54. (Previously presented) The method of claim 42 wherein the antibody is administered as a 4mg/kg dose and then weekly administration of 2mg/kg.

Remarks/Arguments

Claims 34-39, 42-22 and 47-54 are pending in this application. Claims 50 and 51 are withdrawn from consideration, and claims 34-39, 42-44, 47-49 and 52-54 are rejected on various grounds. Claim 52 has been amended. The amendment is of formal nature and does not introduce new matter.

Claim Rejections Withdrawn

Applicants note the withdrawal of the rejection of claim 43 under 35 U.S.C. 112, second paragraph; the rejection of claims 20, 25, 28, 29, 32, and 46 under 35 U.S.C. 112, first paragraph for alleged lack of written description; the rejection of claims 38-40 and 43-49 under 35 USC 112, first paragraph for alleged lack of enablement; and the rejection of claims 20, 25-28, 32, 34-37, 48 and 49 under 35 USC 102(b).

Claim Rejections Maintained and New Grounds of Rejection

Claim Rejections – 35 USC § 112

Claims 52 and 53 have been rejected under 35 U.S.C. 112, second paragraph, as being indefinite for lacking antecedent basis for the phrase "the third therapeutic agent." The present amendment of claim 52 is believed to obviate this rejection.

Claim Rejections – 35 USC § 103

(1) Claims 20, 25-28, 32, 34-49 were rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Baselga-1996 (Baselga et al., Journal of Clinical Oncology, 14(3):737-744, 1996) in view of Perez (Perez et al., Seminars in Oncology, 34(5, suppl 11):41-45, 1996) and further in view of Baselga-1994a (Baselga et al., Proceedings of the American Association for Cancer Research, 35:380, Abstract #2262, 1994).

Baselga-1996 is cited for its teaching of methods of treating breast cancer by administration of a recombinant humanized anti-HER2 antibody that binds to the 4D5 epitope (trastuzumab). The Examiner acknowledges that Baselga-1996 fails to teach the further administration of a chemotherapeutic agent, e.g. a taxoid and growth inhibitory agents, or the administration of trastuzumab in combination with a taxoid and further with carboplatin.

Perez is cited for its alleged teaching that the combination of paclitaxel and carboplatin may be used in the treatment with advanced breast cancer, and that both agents have significant single-agent activity.

Baselga-1994a (erroneously referred to as "Baselga-1994") is cited for allegedly teaching a method of treating nude mice bearing human breast tumor xenografts overexpressing ErbB2 (HER2) by administration of murine 4D5 in combination with paclitaxel. According to the rejection, Baselga-1994a "teaches that the combination of paclitaxel and the 4D5 antibody was synergistic in the effect of the combination on tumor growth inhibition." (Office Action, page 6)

The Examiner concludes that in view of the cited combination of references it would have been *prima facie* obvious to treat cancer patients overexpressing ErbB2 with a combination of an antibody that binds the 4D5 epitope and a taxoid, "because both agents where [sic] taught in the prior art to be useful in treating breast cancer, and because the prior art suggested their combination, and because the prior art demonstrated that in nude mouse xenograft studies the combination of the two agents was more efficacious in inhibiting tumor cell growth than either agents used alone and more efficacious than sum of the effects of either agent used alone." (Office Action, sentence bridging pages 7-6) The Examiner cites In re Kerkhoven, 626 F.2d 846, 850, 205 USCP 1069, 1072 (CCPA 1980)) as allegedly holding it is obvious to combine two compositions, in order to form a third composition, when each of the two compositions is taught by the prior art to the useful for the same purpose.

For there to be obviousness of the invention as claimed it would have to be shown that on an objective analysis of the prior art as a whole the claimed invention could have been achieved with a reasonable expectation of success. Such showing has not been made in the present case.

The Invention

The present invention is based on a rare quality and extent of data actually included in the application, in particular including in the application of actual human clinical trials involving hundreds of patients. The trials, involving tests of anti-ErbB2 antibody with various chemotherapeutic agents, in particular taxoids, demonstrated unexpected results. Specifically, the application contains the first and unexpected experimental demonstration that anti-ErbB2

antibody plus chemotherapy with a taxoid has the advantage of increased time-to-diseases progression, correlating with increased clinical benefit, in human patients, avoiding the overall adverse side effects associated with other cancer drug combinations, such as an ani-ErbB2 antibody-anthracycline derivative combination. As demonstrated by the data presented in paragraph 6 of the Declaration of Susan D. Hellmann dated December 20, 2004 (the Second Hellman Declaration) the extension of time to disease progression was synergistic in that the combination of trastuzumab and paclitaxel extended time to disease progression to a greater extent than the additive effect of the two anti-cancer agents administered as single agents.

There is no basis in the cited prior art for any reasonable expectation of being able successfully to achieve these clinically significant results.

The cited combination of references

The Examiner acknowledges that Baselga-1996 only shows the anti-cancer effect of a humanized anti-ErbB2 antibody that binds to the 4D5 epitope (trastuzumab) as a single agent in human patients.

Perez teaches that paclitaxel and carboplatin, as single agents or in combination, are effective in the treatment of breast cancer in human patients.

In contrast to Baselga-1996 and Perez, the results of Baselga-1994a about the efficacy of the combination of a murine anti-ErbB2 antibody (4D5) in combination with paclitaxel were generated in a murine xenograft model. The studies mentioned in Baselga-1996 did not involve actual therapy of a human patient. As explained in the Second Hellmann Declaration, at the priority date of this application, preclinical data, concerning the combined administration of anti-ErbB2 antibodies and taxoids, obtained in various models were inconsistent and controversial (see, Second Hellmann Declaration, paragraph 7). This is true, whether the Examiner accepts Applicants' interpretation of the results of Pegram et al. (Oncogene 18:2241-2251, 1999). Whether Pegram et al. "teaches away" from the invention claimed in the present application (as Applicants maintain), or just fails to support the Baselga-1994a xenograft data, the results are inconsistent, and there is no good reason for accepting one over the other when making decisions about designing a treatment approach for human patients with breast cancer overexpressing the ErbB2 receptor. Based on the inconsistent pre-clinical data, the expected clinical outcome of

administering an anti-ErbB2 antibody with paclitaxel to human patients was unpredictable, especially since the mouse xenograft data provide absolutely no information on side effects that might occur in human patients, such as those observed in the human clinical trial disclosed in the present application for a combination of an an-ErbB2 antibody and anthracycline derivatives.

Doubts surrounding the predictive value of the preclinical models are also supported by the attached paper of Vosloglou-Nomikos et al. (Clinical Cancer research 4227-4239 (9):4227-4239 (2003)), which is a meta analysis of the predictive value of three preclinical cancer models, including human xenograft models, based on a retrospective, literature-based study. Four solid tumor types were selected: breast, NSCLC, ovary and colon. Page 4228 reports that a recent NCI examination found that, with the exception of NSCLC, preclinical activity in human xenografts of a particular tumor type did not correlate significantly with Phase II activity in the same type of tumor. Thus, on page 4236 the authors report that the human xenograft model "failed to adequately predict clinical performance both in te disease and compound-oriented setting for breast and colon tumors.

Therefore, the xenograft work described in Baselge-1994a, when combined with Baselga et al. 1996 and Perez does not provide a reasonable expectation for the extension (especially a synergistic extension) of time to disease progression, as disclosed in the present application.

With regard to the cited case law, Applicants submit that In re Kerkhoven does that warrant the finding of *prima facie* obviousness in the present case. In Kerkhoven, the court held that claims directed to a process of preparing a spray-dried detergent by mixing together two conventional spray-dried detergents were *prima facie* obvious. As the Examiner is aware, an important element of obviousness inquiry is the degree of predictability or unpredictability in the pertinent art. In contrast to the art of detergents, the art of anti-cancer drugs, and cancer treatment in general, is highly unpredictable. Therefore, from the fact that two or three anti-cancer agents are efficacious individually, one of ordinary skill could not reasonably predict the behavior of the same drugs when administered in various combinations, especially when they act by different mechanisms of action. In addition, even if the mere fact that the claims include administration of a combination of anti-cancer agents were sufficient to reach the conclusion of a

prima facie case of obviousness, this finding has been effectively rebutted by Applicants' arguments and the evidence of record, establishing that the methods of the present invention result in unexpectedly superior results.

Accordingly, the Examiner is respectfully requested to reconsider and withdraw the present rejection.

Claims 38 and 43 were rejected under 35 USC 103(a) as allegedly being unpatentable over Baselga-1996 in view of Perez, in view of Baselga-1994a, and further in view of can Oosterom (van Oosterom, et al., Anti-Cancer Drugs, 6(3):356-368, 1995). The first three documents were cited as in the previous rejection. Oosterom was cited for its teaching that docetaxel is a taxoid that has activity in the treatment of breast cancer.

In response to the previous rejection, Applicants have shown that the combination of an anti-ErbB2 antibody which binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid is not rendered obvious by a combination of Baselga-1996, Perez and Baselga-1994a. The use of a particular taxoid, such as docetaxel, is not obvious for the same reasons. Therefore, the Examiner is respectfully requested to reconsider and withdraw the present rejection.

Double Patenting

(1) Claims 34-49, 42-44, 47-49, and 52-54 were rejected on the ground of obviousness-type double patenting as allegedly being unpatentable over claims 1, 4, and 8-29 of US 5,720,954 in vie view of Baselga-1996, I view of Perez and further in view of Baselga-1994a.

US 5,720,954 was cited as claiming methods of treating a patient having a carcinoma that overexpresses HER2 receptor comprising administering to the patient a cytotoxic factor and an antibody that binds to the HER2 receptor in amounts to eliminate the patient's tumor burden, where the antibody may be a 4D5 anti-HER2 antibody and where the cytotoxic factor may be a chemotherapeutic agent. The claims do not specifically recite that the chemotherapeutic agent may be a taxoid. The other documents were cited as in the previous rejections.

The rejection is respectfully traversed.

In response to the previous rejections under 35 USC 103, Applicants have shown that the claimed treatment comprising the administration of an ErbB2 antibody binding to epitope 4D4 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent provides unexpected results in a human clinical setting, over the combination of Baselga-1996, Perez and Baselga-1994a. The addition of US 5,720,954, which has no disclosure or suggestion that the treatment claimed in the present application would result in the extension of time to disease progression without increasing the incidence of side-effects. Accordingly, the invention claimed in the rejected claims is not obvious over the invention claimed in claims 1, 4 and 8-29 of US 5,720,954, and the present rejection should be withdrawn.

(2) Claims 34-39, 42-44, 47-49, and 52-54 were rejected on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 14, 23, 27-33 and 36 of US 5,770,195, in view of Beselga-1996, in view of Perez and further in view of Baselga-1994a.

The rejection is respectfully traversed.

Claims 14, 23, 27-33 and 36 of US 5,770,195 are drawn to method of inhibiting the growth of tumor cells that overexpress HER2 receptor of EGF receptor comprising administering to a patient a cytotoxic factor and an antibody that binds to the HER2 receptor in amounts to eliminate the patient's tumor burden, where the antibody may be a 4D5 anti-HER2 antibody and the cytotoxic factor may be a chemotherapeutic agent. The claims do not recite that the chemotherapeutic agent may be a taxoid, or that it be in combination with a further growth inhibitory agent.

In response to the previous rejections under 35 USC 103, Applicants have shown that the claimed treatment comprising the administration of an ErbB2 antibody binding to epitope 4D4 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent provides unexpected results in a human clinical setting, over the combination of Baselga-1996, Perez and Baselga-1994a. The addition of US 5,770,195, which has no disclosure or suggestion

that the treatment claimed in the present application would result in the extension of time to disease progression without increasing the incidence of side-effects. Accordingly, the invention claimed in the rejected claims is not obvious over the invention claimed in claims 14, 23, 27-33 and 36 of US 5,770,195, and the present rejection should be withdrawn.

(3) Claims 34-39, 42-44, 47-49, and 52-54 were rejected on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1-7 and 9-13 of US 6,387,371 in view of Baselga-1996, in view of Perez, and further in view of Baselga-1994a.

Claims 1-7 and 9-13 of US 6,387,371 are drawn to methods of treating cancer that overexpressed HER2 receptor comprising administering to the patient a factor which suppresses cell growth, and an antibody that binds to an extracellular domain of the HER2 receptor. The claims do not specifically recite that the chemotherapeutic agent may be a taxoid, or that the antibody is a 4D5 anti-HER2 antibody.

In response to the previous rejections under 35 USC 103, Applicants have shown that the claimed treatment comprising the administration of an ErbB2 antibody binding to epitope 4D4 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent provides unexpected results in a human clinical setting, over the combination of Baselga-1996, Perez and Baselga-1994a. The addition of US 6,387,371, which has no disclosure or suggestion that the treatment claimed in the present application would result in the extension of time to disease progression without increasing the incidence of side-effects. Accordingly, the invention claimed in the rejected claims is not obvious over the invention claimed in claims 1-7 and 9-23 of US 6,387,371 and the present rejection should be withdrawn.

In conclusion, all claims pending in this application are believed to be in *prima facie* condition for allowance, and an early action to that effect is respectfully solicited.

The Commissioner is authorized to charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account 50-4634 (Attorney Docket No. GNE-0329R1D1).

Respectfully submitted,

(Panpan Gao 43,626) on behalf of

Date: December 29, 2008

Ginger R. Dreger, Esq.
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LIBC/3476467.1 GD3 123851-184962
12/29/2008 12:25 PM

Electronic Patent Application Fee Transmittal

Application Number:	10356824
Filing Date:	03-Feb-2003
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Filer:	Ginger R. Dreger/cheryl rogers
Attorney Docket Number:	P1256R1D1

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1110	1110

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**1110**

Electronic Acknowledgement Receipt

EFS ID:	4531662
Application Number:	10356824
International Application Number:	
Confirmation Number:	4326
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Customer Number:	35489
Filer:	Ginger R. Dreger/cheryl rogers
Filer Authorized By:	Ginger R. Dreger
Attorney Docket Number:	P1256R1D1
Receipt Date:	29-DEC-2008
Filing Date:	03-FEB-2003
Time Stamp:	18:39:46
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1110
RAM confirmation Number	5426
Deposit Account	504634
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		amendment.pdf	931374 0d39a9bcdc1a6d6f32957aa850babf5a0ed8a85cf	yes	27

Multipart Description/PDF files in .zip description

Document Description	Start	End
Miscellaneous Incoming Letter	1	1
Extension of Time	2	2
Amendment/Req. Reconsideration-After Non-Final Reject	3	3
Claims	4	6
Applicant Arguments/Remarks Made in an Amendment	7	14
NPL Documents	15	27

Warnings:

Information:

2	Fee Worksheet (PTO-06)	fee-info.pdf	29769 46f1b2610c59c0985e928d6fb9e982e9d803885	no	2

Warnings:

Information:

Total Files Size (in bytes):	961143

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/21 (11-07)
Approved for use through 11/30/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

TRANSMITTAL FOR

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	10/356,824
Filing Date	February 3, 2003
First Named Inventor	Paton, et al.
Art Unit	1643
Examiner Name	Holleran, Anne L.
Confirmation No.	4326
Attorney Docket Number	GNE-0329 R1D1

ENCLOSURES *(Check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☒ **Amendment/Reply**
  - ☐ **After Final**
  - ☐ Affidavits/declaration(s)
- ☒ **Extension of Time Request**
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ **Appeal Communication to TC (Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ **Other Enclosure(s) (please Identify below): Copy of article "Clinical Predictive Value of the *in Vitro* Cell Line, Human Xenograft, and Mouse Allograft Preclinical Caner Models"**

Remarks

AUTHORIZATION TO CHARGE DEPOSIT ACCOUNT 50-4634 FOR ANY FEES DUE IN CONNECTION WITH THIS PAPER, REFERENCING ATTORNEY'S DOCKET NO. 123851-184962 (GNE-0329 R1D1).

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	GOODWIN PROCTER LLP		
Signature			
Printed name	Panpan Gao, Esq.		
Date	December 29, 2008	Reg. No.	43,626

FILED VIA EFS — December 29, 2008

PTO/SB/22 (04-07)
Approved for use through 09/30/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARMENT OF COMMERCE

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2006 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) GNE-0329 R1D1
Application Number 10/356,824	Filed February 3, 2003
For TREATMENT WITH ANTI-ERBB2 ANTIBODIES	
Art Unit 1643	Examiner Holleran, Anne L.

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$
☐	Two months (37 CFR 1.17(a)(2))	$490	$245	$
☑	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 1,110
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☑ The Director has already been authorized to charge fees in this application to a Deposit Account.

☑ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 50-4634 (Ref. Atty. No. 123851-184962)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed (Form PTO/SB/96).

☑ attorney or agent of record. Registration Number 33,055

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34

(Panpan Gao 43,626) on behalf of
Signature

December 29, 2008
Date

GINGER R. DREGER, ESQ.
Typed or printed name

650/752-3100
Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☑ Total of 1 forms are submitted.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
10/356,824	02/03/2003	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
FOR	NUMBER FILED	NUMBER EXTRA					
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 12/29/2008	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 17	Minus	** 20	= 0	X $ =		OR	X $52=	0
Independent (37 CFR 1.16(h))	* 3	Minus	***3	= 0	X $ =		OR	X $220=	0
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/MARSHA R. RICHARDS/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/03/2003	Virginia E. Paton	P1256R1D1	4326

35489 7590 03/20/2009

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EXAMINER	
HOLLERAN, ANNE L	

ART UNIT	PAPER NUMBER
1643	

MAIL DATE	DELIVERY MODE
03/20/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/356,824	PATON ET AL.	
	Examiner	Art Unit	
	ANNE L. HOLLERAN	1643	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *29 December 2008*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *34-39,42-44 and 47-54* is/are pending in the application.

4a) Of the above claim(s) *50 and 51* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *34-39, 42-44, 47-49 and 52-54* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

The amendment filed 12/29/2008 is acknowledged. Claims 34-39, 42-44, 47-54 are pending

Claims 50 and 51, drawn to inventions comprising the use of third therapeutic agents, which are not carboplatin (another ErbB2 antibody, claim 50; a vascular endothelial growth factor antibody, claim 51) are **withdrawn from consideration** in view of the election of species requirement for the third therapeutic agent set forth in the Office action mailed out 2/16/2006.

With respect to claims 52 and 53, which recite a third therapeutic agent is a growth inhibitory agent or a DNA alkylating agent, these claims are considered generic to the elected species of third agent, carboplatin, because carboplatin is a growth inhibitory agent and a DNA alkylating agent. Claims 52 and 53 are examined to the extent that they read on methods using a third agent that is carboplatin.

Claims 34-39, 42-44, 47-49 and 52-54 are examined on the merits.

Claim Rejections Withdrawn:

The rejection of claims 52 and 53 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is withdrawn in view of the amendment to claim 52.

Claim Rejections Maintained and New Grounds of Rejection:

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 34-39, 42-44, 47-49, 52 and 53 remain/are rejected under 35 U.S.C. 103(a) as being unpatentable over Baselga-1996 (Baselga, et al., Journal of Clinical Oncology, 14(3): 737-

744, 1996, March; cited in the IDS) in view of Perez (Perez et al., Seminars in Oncology, 23(5, suppl 11): 41-45, 1996, October) and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994).

This rejection is applied to claims 52 and 53, the limitations of which were discussed in the rejection of the previous Office action.

Applicants' arguments have been carefully considered, but fail to persuade. Applicants argue that the Baselga-1994a reference concerns mouse xenograft data, whereas the data of Baselga-1996 and Perez are not generated in mice; and that the studies mentioned in Baselga-1996 do not actually involve therapy of human patient; and that, as outlined in the second Helmann declaration, there were two different mouse models showing different results for the combination of an anti-ErbB2 antibody and a taxoid; and that there is no good reason for accepting one over the other in making decisions about designing a treatment approach. With respect to the second mouse model, which results are demonstrated in Pegram (Oncogene, 18: 2241-2251, 1999) this data was available to the public after the filing date of the instant application, whereas the data of Baselga-1994a was available before the filing date. In formulating a rejection under 35 USC 103(a), the Office states what would have been obvious to one of ordinary skill in the art "at the time the invention was made". At the time the invention was made the information available to one of ordinary skill in the art is the teachings of the references of record, which would have motivated one to try to administer the combination of an anti-Her-2 antibody and a taxoid and a further growth inhibitory agent, as argued in the rejection of record. Applicants also cite Vosloglou-Nomikos (Clincial Cancer Research 9: 4227-4239, 2003) to state that human xenograft model failed to adequately predict clinical performance in

Phase II trials. However, the claims are not limited to activity in Phase II trials, and furthermore, the elements of the claimed invention were available to one of ordinary skill in the art because the therapeutic agents involved are agents that were known and established in the treatment of breast cancer and there was evidence that the agents might also be synergistic when used to together.

Applicants submit that In re Kerkoven does not warrant a finding of prima facie obviousness in the present case, because In re Kerkoven is directed to claims of a process of preparing a spray-dried detergent by mixing together two conventional spray-dried detergents, which does not have the same degree of unpredictability as does the art of anticancer therapy. While the examiner agrees that in general anticancer therapy is a more unpredictable field than is the field of formulating spray-dried detergent, this is off-set by the fact that the agents involved in the present claims were already well known in the art for the purpose of treating breast cancer, and the concept of combining anti-ErbB2 antibodies with chemotherapeutic agents was also known in the art. Therefore, this situation appears to be one where the separate elements were suggested as was their combination and the result of combining them resulted in an anticipate success.

Claims 38 and 43 remain rejected under 35 U.S.C. 103(a) as being unpatentable over Baselga-1996 (Baselga, et al., Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in the IDS) in view of Perez (Perez et al., Seminars in Oncology, 23(5, suppl 11): 41-45, 1996, October) in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994; cited in the IDS), and further in view of van

Oosterom (van Oosterom, et al. Anti-Cancer Drugs, 6(3): 356-368, 1995, June, Abstract only; cited in the IDS) for the reasons of record.

Claims 38 and 43 include within their scope methods that comprise the administration of docetaxel.

Applicants' arguments for the previous rejection are applied for this rejection. As they were not found persuasive for the above rejection, they are not found persuasive for this rejection.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 34-39, 42-44, 47-49, and 52-54 remain rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4, and 8-29 of U.S. Patent No. 5,720,954 (cited in the IDS) in view of Baslega-1996 (Baselga, J. et al. Journal of

Clinical Oncology, 14(3): 737-744, 1996, March; cited in IDS), in view of Perez (Perez et al., Seminars in Oncology, 23(5, suppl 11): 41-45, 1996, October; cited above) and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994) for the reasons of record.

Claims 34-39, 42-44, 47-49, and 52-54 remain rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 14, 23, 27-33 and 36 of U.S. Patent No. 5,770,195 (cited in IDS) in view of Baslega-1996 (Baselga, J. et al. Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in IDS), in view of Perez; cited previously) and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994) for the reasons of record.

Claims 34-39, 42-44, 47-49, and 52-54 remain rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-7 and 9-13 of U.S. Patent No. 6,387,371 in view of Baslega-1996 (Baselga, J. et al. Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in IDS), in view of Perez; cited previously), and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994) for the reasons of record.

Applicants' arguments for the previous rejection under 5 USC 103(a) are applied for the obviousness-type double patent rejections above. As they were not found persuasive for the

rejections under 35 USC 103(a), they are not found persuasive for the rejections on the grounds of nonstatutory obviousness-type double patenting.

Conclusion

No claim is allowed.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anne Holleran, whose telephone number is (571) 272-0833. The examiner can normally be reached on Monday through Friday from 9:30 am to 5:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Larry Helms, can be reached on (571) 272-0832. Any inquiry of a general nature or relating to the

status of this application or proceeding should be directed to the Group receptionist whose telephone number is (571) 272-1600.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. The faxing of such papers must conform to the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The Official Fax number for Group 1600 is (571) 273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll free).

Anne L. Holleran
Patent Examiner
March 16, 2009
/Alana M. Harris, Ph.D./
Primary Examiner, Art Unit 1643

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10356824	PATON ET AL.
	Examiner	Art Unit
	ANNE L HOLLERAN	1643

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
updated search of US Patents, EAST, history in file	6/23/2008	alh
updated search of US Patents, EAST, history in file	3/16/2009	alh

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	2916	424/130.1,133.1,138.1,141.1,143.1,155.1,174.1.ccls.	USPAT	OR	OFF	2009/03/16 16:15
L4	259	(her2 or erbb2 or cerbb2 or erbb2her2 or cerbb2her2 or p185 or p185her2 or p185erbb2 or p185cerbb2 or p185her2cerbb2 or p185her2erbb2 or p185erbb2her2).clm.	USPAT	OR	OFF	2009/03/16 16:16
L5	47	1 and 4	USPAT	OR	OFF	2009/03/16 16:16

3/16/2009 4:16:52 PM
C:\Documents and Settings\aholleran\My Documents\EAST\Workspaces\10356824.wsp

PTO/SB/31 (06-09)
Approved for use through 07/31/2009. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

NOTICE OF APPEAL FROM THE EXAMINER TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

Docket Number (Optional): GNE-0329-R1D1

FILED VIA EFS – JULY 16, 2009

on _____

Signature _____

Typed or printed name _____

In re Application of: SUSAN D. HELLMANN et al.

Application Number	Filed
10/356,824	February 3, 2003

For TREATMENT WITH ANTI-ErbB2 ANTIBODIES

Art Unit	Examiner
1643	Holleran, Anne L.

Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20 (b)(1)) $540.00

☐ Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by half, and the resulting fee is: $ _____

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 50-4634.

☐ A petition for an extension of time under 37 CFR 1.136(a) (PTO/SB/22) is enclosed.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

☒ attorney or agent of record. Registration number 33,055

☐ attorney or agent acting under 37 CFR 1.34. Registration number if acting under 37 CFR 1.34. _____

/GINGER R. DREGER/
Signature

GINGER R. DREGER
Typed or printed name

650-752-3100
Telephone number

JULY 16, 2009
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☒ *Total of 1 forms are submitted.

This collection of information is required by 37 CFR 41.31. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11, 1.14 and 41.6. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Patent Application Fee Transmittal

Application Number:	10356824
Filing Date:	03-Feb-2003
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Filer:	Ginger R. Dreger/Sherrie Dufault
Attorney Docket Number:	GNE-0329-D1

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Notice of appeal	1401	1	540	540
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	130	130
Miscellaneous:				
		Total in USD ($)		**670**

Electronic Acknowledgement Receipt	
EFS ID:	5716821
Application Number:	10356824
International Application Number:	
Confirmation Number:	4326
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Customer Number:	35489
Filer:	Ginger R. Dreger/Sherrie Dufault
Filer Authorized By:	Ginger R. Dreger
Attorney Docket Number:	GNE-0329-D1
Receipt Date:	16-JUL-2009
Filing Date:	03-FEB-2003
Time Stamp:	16:06:23
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$670
RAM confirmation Number	2387
Deposit Account	504634
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Notice of Appeal Filed	07-16-09NOA.pdf	38694 91972ba3205ff8345449826cd1d022d3191 8b69	no	1
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	32034 842a7b6685105ca9606d1efb1acfd8a2b36 0be6	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	70728		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/03/2003	Virginia E. Paton	GNE-0329-D1	4326

35489 7590 08/25/2009

GOODWIN PROCTER LLP
135 COMMONWEALTH DRIVE
MENLO PARK, CA 94025

EXAMINER	
HOLLERAN, ANNE L	

ART UNIT	PAPER NUMBER
1643	

MAIL DATE	DELIVERY MODE
08/25/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Interview Summary	Application No.	Applicant(s)	
	10/356,824	PATON ET AL.	
	Examiner	Art Unit	
	ANNE L. HOLLERAN	1643	

All participants (applicant, applicant's representative, PTO personnel):

(1) *ANNE L. HOLLERAN*. (3)*Atylya Agarwal*.

(2) *Ginger Dreger*. (4)_____.

Date of Interview: *23 July 2009*.

Type: a)☐ Telephonic b)☐ Video Conference
c)☒ Personal [copy given to: 1)☐ applicant 2)☒ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☐ No.
If Yes, brief description: _____.

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Agreement with respect to the claims f)☐ was reached. g)☒ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Disussed this case with reference to 09/208,649 because this is a divisional of 09/208,649 and because the claimed subject matter overlaps. Therefore, arguments and evidence having to do with unexpected results and level of unpredictability in the art that are presented for 09/208,649 will likely be presented for this case as well.*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/Anne L Holleran/ Examiner, Art Unit 1643	

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Virginia E. Paton	Attorney Docket #:	GNE-0329R1D1
Serial No.	10/356,824	Group Art Unit	1643
Filing Date	02/03/2003	Examiner:	Holleran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
Title:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS – SEPTEMBER 17, 2009

Mail Stop – Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPLICANT'S STATEMENT OF THE SUBSTANCE OF INTERVIEW IN ACCORDANCE WITH 37 C.F.R. § 1.133(b)

Sir:

Applicants express their thanks to the Examiner for the opportunity to discuss the rejections raised in the final Office Action mailed on March 20, 2009 during a personal interview on July 23, 2009. This application was discussed with reference to application Serial No. 09/209,649 due to the related subject matter. Thus, the rejections under 103(a), including the Baselga-1994 reference, were discussed. Applicants indicated that they will provide evidence of unpredictability in their response to the pending Office Action.

Respectfully submitted,

Date: September 17, 2009

By Electronic Signature: /GINGER R. DREGER/
Ginger R. Dreger, Esq.
Reg. No. 33,055

GOODWIN PROCTER LLP
135 Commonwealth Drive
Menlo Park, CA 94025
Tel: (650) 752-3100
Fax: (650) 853-1038

Electronic Acknowledgement Receipt

EFS ID:	6092521
Application Number:	10356824
International Application Number:	
Confirmation Number:	4326
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Customer Number:	35489
Filer:	Ginger R. Dreger/Sherrie Dufault
Filer Authorized By:	Ginger R. Dreger
Attorney Docket Number:	GNE-0329-D1
Receipt Date:	17-SEP-2009
Filing Date:	03-FEB-2003
Time Stamp:	14:51:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant summary of interview with examiner	09-17-09-RespInterviewSummary.pdf	19574 8294a3238bd6b1ea58a5ab1f90bfb38e6ad9f3a7e	no	1

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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New International Application Filed with the USPTO as a Receiving Office

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PTO/SB/30 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
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Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	10/356,824
Filing Date	February 3, 2003
First Named Inventor	VIRGINIA E. PATON et al.
Art Unit	1643
Examiner Name	Holleran, Anne L.
Attorney Docket Number	GNE-0329-R1D1

This is a Request for Continued Examination (RCE) under 37 CFR 1 .114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. ☐ Other _____

   b. ☒ Enclosed

      i. ☒ Amendment/Reply

      ii. ☒ Affidavit(s)/ Declaration(s)

      iii. ☐ Information Disclosure Statement (IDS)

      iv. ☒ Other Exhibits A-H to Declaration of Mark X. Sliwkowski, Ph.D.

2. Miscellaneous

   Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a

   a. ☐ period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   The Director is hereby authorized to charge the following fees any underpayment of fees or credit any overpayments to

   a. ☒ Deposit Account No. 50-4634

      i. ☒ RCE fee required under 37 CFR 1.17(e)

      ii ☒ Extension of time fee (37 CFR 1.136 and 1.17)

      iii ☐ Other _____

   b. ☐ Check in the amount of $ _____ enclosed

   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/GINGER R. DREGER/	Date	October 15, 2009
Name (Print/Type)	Ginger R. Dreger	Registration No.	33,055

FILED VIA EFS – OCTOBER 15, 2009

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Virginia E. Paton	Attorney Docket #:	GNE-0329R1D1
Serial No.	10/356,824	Group Art Unit	1643
Filing Date	02/03/2003	Examiner:	Holleran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
Title:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS – OCTOBER 15, 2009

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Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO FINAL OFFICE ACTION

Sir:

This is in response to the final Office Action mailed on March 20, 2009 in connection with the above-identified patent application. A Notice of Appeal was filed on July 16, 2009, setting the time for filing an Appeal Brief to September 16, 2009. The present Amendment and Response is accompanied by a Request for a One-Month Extension of Time and is filed along with a Request for Continued Examination (RCE). Also accompanying the present Amendment and Response is a Declaration of Mark X. Sliwkowski, Ph.D., the entry and consideration of which is respectfully requested.

Amendments to the Claims begin on page **2** of this paper.

Remarks/Arguments begin on page **5** of this paper.

Listing of the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

1-33. (Canceled)

34. (Previously presented) A method for the treatment of a human patient with breast cancer that overexpresses ErbB2 receptor, comprising administering a combination of an antibody that binds ErbB2, a taxoid, and a further growth inhibitory agent to the human patient in an amount effective to extend the time to disease progression in the human patient, wherein the antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

35. (Currently amended) The method of claim 34 wherein the antibody ~~if~~ is a humanized 4D5 anti-ErbB2 antibody.

36. (Previously presented) The method of claim 34 wherein the antibody cross-blocks binding of 4D5 to the ErbB2 extracellular domain sequence.

37. (Previously presented) The method of claim 34 wherein the antibody binds to amino acid residues in the region from about residue 529 to about residue 625 of the ErbB2 extracellular domain sequence.

38. (Previously presented) A method for the treatment o f a human patient with breast cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of a combination of an anti-ErbB2 antibody which binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid, and a further therapeutic agent, to the human patient.

39. (Previously presented) The method of claim 38 wherein the breast cancer is metastatic breast carcinoma.

40-41. (Canceled)

42. (Previously presented) The method of claim 38 wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

43. (Previously presented) The method of claim 38 wherein the taxoid is paclitaxel or docetaxel.

44. (Previously presented) The method of claim 38 wherein efficacy is measured by determining the time to disease progression or the response rate.

45-46. (Canceled)

47. (Previously presented) The method of claim 38, wherein the further therapeutic agent is selected from the group consisting of: another ErbB2 antibody, EGFR antibody, ErbB3 antibody, ErbB4 antibody, vascular endothelial growth factor (VEGF) antibody, cytokine, and growth inhibitory agent.

48. (Previously presented) A method for the treatment of a human patient with ErbB2 overexpressing breast cancer, comprising administering a combination of an antibody that binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in the human patient.

49. (Previously presented) The method of claim 48 wherein the breast cancer is metastatic breast carcinoma.

50. (Withdrawn) The method of claim 38 wherein the third therapeutic agent is another ErbB2 antibody.

51. (Withdrawn) The method of claim 38 wherein the third therapeutic agent is a vascular endothelial growth factor (VEGF) antibody.

52. (Previously presented) The method of claim 38 wherein the further therapeutic agent is a growth inhibitory agent.

53. (Previously presented) The method of claim 52 wherein the growth inhibitory agent is a DNA alkylating agent.

54. (Previously presented) The method of claim 42 wherein the antibody is administered as a 4mg/kg dose and then weekly administration of 2mg/kg.

Remarks/Arguments

Claims 34-39, 42-22 and 47-54 are pending in this application. Claims 50 and 51 are withdrawn from consideration, and claims 34-39, 42-44, 47-49 and 52-54 are rejected on various grounds. Claim 35 has been amended to correct an obvious typographical error.

Claim Rejections Withdrawn

Applicants note the withdrawal of the rejection of claims 52 and 53 under 35 U.S.C. 112, second paragraph as "indefinite" in view of the amendment to claim 52.

Claim Rejections Maintained and New Grounds of Rejection

Claim Rejections – 35 USC § 103

(1) Claims 34-39, 42-44, 47-49, 52 and 53 are/remain rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Baselga-1996 (Baselga et al., Journal of Clinical Oncology, 14(3):737-744, 1996) in view of Perez (Perez et al., Seminars in Oncology, 34(5, suppl 11):41-45, 1996) and further in view of Baselga-1994a (Baselga et al., Proceedings of the American Association for Cancer Research, 35:380, Abstract #2262, 1994).

In addressing Applicants' arguments filed in response to a similar rejection in the Office Action mailed June 26, 2008, the Examiner makes the following comments:

(i) The Examiner found Applicants' argument that the mouse models of Baselga-1994a and Pegram (1999) showed different results, and therefore there was no good reason to accept one over the other in making treatment decisions, not convincing, because obviousness determination under 35 U.S.C 103(a) is based on the assessment of information that was known in the art "at the time the invention was made." Since Baselga-1994a was known in the art before the effective filing date of this application, while Pegram was not, the Examiner maintains that at the time the invention was made Baselga-1994a would have motivated one of ordinary skill in the art to try to administer the combination of an anti-HER-2 (anti-ErbB2) antibody and a taxoid and a further growth inhibitory agent.

(ii) The Examiner reads Vosloglou-Nomikos (Clinical Cancer Research 9:4227-4239, 2003), submitted by Applicants to illustrate doubts surrounding the predictive value of data obtained in xenograft models, for the narrower teaching that a human xenograft model failed to adequately predict clinical performance in Phase II clinical trials. The Examiner notes that the claims are not limited to activity in Phase II trials, and further that "the elements of the claimed invention were available to one of ordinary skill in the art because the therapeutic agents involved are agents that were known and established in the treatment of breast cancer and there was evidence that the agents might also be synergistic when used . . .together." (Office Action, page 3, lines 1-5)

(iii) In regard of the applicability of In re Kerkoven, the Examiner agrees with Applicants that in general anti-cancer therapy is a more unpredictable field than is the field of formulating spray-dried detergent, but asserts that "this is off-set by the fact that the agents involved in the present claims were already well known in the art for the purpose of treating breast cancer, and the concept of combining anti-ErbB2 antibodies with chemotherapeutic agents was also known in the art." (Office Action, page 5, first full paragraph). The Examiner asserts that this situation "appears to be one where the separate elements were suggested as was their combination and the result of combining them resulted in an anticipate[d] success." (Office Action, page 5, first full paragraph)

Applicants continue to disagree, and respectfully traverse the rejection.

To further support Applicants' earlier arguments, enclosed is a Declaration of Mark X. Sliwkowski, Ph.D. Dr. Sliwkowski has extensive experience in the field of drug development, and in particular, in developing various therapeutic approaches targeting members of the ErbB receptor family, including ErbB2. Dr. Sliwkowski has participated in the development of trastuzumab (Herceptin®), a humanized anti-ErbB2 antibody that binds to epitope 4D5 within the ErbB2 extracellular domain sequence. Dr. Sliwkowski is also involved in investigating combination therapeutic approaches for the treatment of cancer, including breast cancer.

At the priority date of the present application one of ordinary skill would have had no motivation to treat human breast cancer patients with an anti-ErbB2 antibody that binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid

As Dr. Sliwkowski explains in section 7 of his Declaration, drugs in the treatment of cancer are known to exert their effects at different points within the cell cycle. Dr. Sliwkowski cites Woods et al., Mol. Med. 1:506-526, 1995 (Exhibit B) to illustrate that before the priority date of the present application it was known that treatment of cancer cells with paclitaxel (a taxoid) disrupts the formation of normal splindles at metaphase, leading to arrest of the cells in the G2/M phase of the cell cycle, and ultimately to apoptotic cell death. As Dr. Sliwkowski explains, citing Huzdiak et al., Molecular and Cellular Biology 9(3):1165-1172, 1989 (Exhibit C) and Lewis et al., Cancer Research 56:1457-1465, 1996 (Exhibit D), it was also known before the priority date of the present application that the antibody 4D5 acts in the S phase of cell cycle, which precedes the G2 phase. Thus, at the priority date of the present application one of ordinary skill in the art would have anticipated that paclitaxel would provide little to no additional benefits to treatment with trastuzumab alone, since trastuzumab arrests the cell cycle before paclitaxel would be able to act. The same is true for treatment with a combination of other antibodies binding to epitope 4D5 within the ErbB2 extracellular domain and other taxoids for the same reasons.

In section 8 of his Declaration, Dr. Sliwkowski cites the well known example of tamoxifen and anthracycline chemotherapeutics, where the addition of tamoxifen to standard chemotherapy, involving anthracycline derivatives, provided little to no clinical benefit, since tamoxifen arrested the cell cycle in the G0-G1 phase, before anthracyclines could exert their anticancer activity. In the possession of this knowledge, and knowing that, just like tamoxifen, antibody 4D5 acts in the S phase of cell cycle, one of ordinary skill at the time the present invention was made would not have been motivated to combine an anti-ErbB2 antibody that binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid to treat breast cancer, since the expectation would have been that the addition of a taxoid would carry no additional clinical benefit.

This is true despite the data disclosed in the Baselga-1994a abstract, due to the low predictive value of data generated in xenograft models of human cancer, which is discussed below and in the rest of the Declaration of Dr. Sliwkowski.

Even if a motivation had existed to try combination treatment with an anti-ErbB2 antibody that binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, the outcome of such treatment would have been highly unpredictable

Even if one assumes *arguendo* that Baselga et al., 1994a provided one of ordinary skill in the art at the time the present invention was made with sufficient motivation to try treating human breast cancer patients with an anti-ErbB2 antibody that binds to epitope 4D5 within the ErbB2 extracellular domain sequence and a taxoid, there would have been no reasonable expectation of success for such treatment. First of all, as Dr. Sliwkowski explains in section 8 of his Declaration, in view of prior experience with the combined administration of tamoxifen and anthracyclines, an antagonistic interaction between the antibody and taxoid would have been a reasonable expectation.

Furthermore, as explained in section 9 of the Sliwkowski Declaration, even in the absence of knowledge about the seemingly incompatible mechanisms of action of taxoids and anti-ErbB2 antibodies that binds to epitope 4D5 within the ErbB2 extracellular domain sequence, such as trastuzumab, the outcome of such treatment would have been highly unpredictable. As objective evidence of such unpredictability, Dr. Sliwkowski refers to a publication by National Cancer Institute (NCI) scientists reporting that after a decade of screening potential anticancer agents, their xenograft models had only moderate predictive value (see Johnson, JI., et al, British Journal of Cancer (2001) 84(1), 1424-1431; Exhibit H of the Declaration). According to the authors, *"[f]or 39 agents with both xenograft data and Phase II clinical trial results available, in vivo activity in a particular histology in a tumour model did not closely correlate with activity in the same human cancer histology, casting doubt on the correspondence of the pre-clinical models to clinical results."* (Abstract) Indeed, while breast cancer xenografts are described as most useful for predicting clinical response against *any* disease, correlation with clinical activity was found against NSCL, melanoma, and ovarian cancer, but *not with clinical breast cancer*! (page 1426, 2nd column) Dr. Sliwkowski explicitly states that, based on his many years of experience and

familiarity with the development of anticancer drugs, individual xenograft models of human tumors, such as the breast cancer xenograft model used by Baselga et al. – 1994a, do not mimic closely enough the biological characteristics and do not capture the inherent variability of human tumors, which greatly limits their value in predicting patient response in a human clinical situation. This variability, and consequently the high degree of unpredictability, is clearly evidenced by the different results reported by Pegram et al. (Oncogene, 18:2241-2251, 1999) of record, which shows hat animals treated with rhuMAb HER2 alone had smaller tumor volumes at day 20 than those treated with the combination of a taxoid (taxol) plus rhuMAb HER2. As the authors state at page 2245, paragraph 2, column 2, "the difference between rhuMAb HER2 alone and rhuMAb HER2 did not reach statistical significance. Thus, Pegram et al. is further evidence of the high degree of unpredictability in regard to this particular combination.

Vosloglou-Nomikos (2003), submitted with Applicants' earlier response is further evidence of the serious limitations of xenograft models in predicting human clinical performance. In this regard, the Examiner's comments that the claims are not limited to activity in Phase II clinical trials are believed to be misplaced. The claims are directed to the treatment of human patients, and thus require activity in human patients. Voslogluo-Nomikos (2003), just as Pegram (1999) and Johnson et al. (2001) are clear evidence of the unpredictability of anti-cancer activity in human patients, based on data generated in xenograft models of human cancer. This, coupled with the additional unpredictability based on the conflicting and seemingly incompatible mechanisms of action of the anti-ErbB2 antibodies and taxoids used in the claimed treatment methods, one of ordinary skill in the art would not have had a reasonable expectation that the claimed treatment method would provide clinical benefits to human patients. This conclusion is not changed by the rest of the references, Baselga-1996, which only shows the anti-cancer effect of a humanized anti-ErbB2 antibody that binds to the 4D5 epitope (trastuzumab) as a single agent in human patients, and Perez, which teaches that paclitaxel and carboplatin, as single agents or in combination, are effective in the treatment of breast cancer in human patients.

Evidence of record demonstrates that the claimed treatment method provides unexpected clinical benefits

Clinical trials, conducted on hundreds of breast cancer patients, demonstrated unexpected clinical benefits of the combined administration of anti-ErbB2 antibodies binding to the 4D5 epitope of ErbB2 and taxoids. Specifically, the application contains the first and unexpected experimental demonstration that anti-ErbB2 antibody plus chemotherapy with a taxoid has the advantage of increased time-to-diseases progression, correlating with increased clinical benefit, in human patients, avoiding the overall adverse side effects associated with other cancer drug combinations, such as an ani-ErbB2 antibody-anthracycline derivative combination. As demonstrated by the data presented in paragraph 6 of the Declaration of Susan D. Hellmann dated December 20, 2004 (the Second Hellman Declaration) the extension of time to disease progression was synergistic in that the combination of trastuzumab and paclitaxel extended time to disease progression to a greater extent than the additive effect of the two anti-cancer agents administered as single agents.

Based on the foregoing arguments and the evidence of record, including the Declaration of Dr. Sliwkowski submitted with the present Response, the Examiner is respectfully requested to reconsider and withdraw the present rejection.

(2) Claims 38 and 43 remain rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Baselga-1996 (Baselga et al., Journal of Clinical Oncology, 14(3):737-744, 1996) in view of Perez (Perez et al., Seminars in Oncology, 34(5, suppl 11):41-45, 1996) and further in view of Baselga-1994a (Baselga et al., Proceedings of the American Association for Cancer Research, 35:380, Abstract #2262, 1994), and further in view of van Oosterom (Van Oosterom et al., Anti-Cancer Drugs, 6(3):356-368 (1995) – Abstract only).

According to the rejection, claims 38 and 44 "include within their scope methods that comprise the administration of docetaxel," and since Applicants' arhuments for the previous rejection were not found to be convincing, "they are not found persuasive for this rejection."

Applicants submit that in view of the foregoing arguments and the evidence of record the previous rejection under 35 U.S.C. 103(a) should be withdrawn, and the present rejection should be withdrawn for the same reasons.

Double Patenting

(1) Claims 34-49, 42-44, 47-49, and 52-54 were rejected on the ground of obviousness-type double patenting as allegedly being unpatentable over claims 1, 4, and 8-29 of US 5,720,954 in vie view of Baselga-1996, I view of Perez and further in view of Baselga-1994a.

(2) Claims 34-39, 42-44, 47-49, and 52-54 were rejected on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 14, 23, 27-33 and 36 of US 5,770,195, in view of Beselga-1996, in view of Perez and further in view of Baselga-1994a.

(3) Claims 34-39, 42-44, 47-49, and 52-54 were rejected on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1-7 and 9-13 of US 6,387,371 in view of Baselga-1996, in view of Perez, and further in view of Baselga-1994a.

According to the Office Action, "Applicants' arguments for the previous rejection under 35 U.S.C. 103(a) are applied for the obviousness-type double patenting rejections above. As they were not found persuasive for the rejections under 35 U.S.C. 103(a), they are not found persuasive for the rejections on the grounds of nonstatutory obviousness-type double patenting." (Passage bridging pages 7 and 8)

Applicants submit that the arguments and evidence of record should overcome the previous rejections under 35 U.S.C. 103(a) and, for the same reasons, also the present obviousness-type double patenting rejections.

In conclusion, all claims pending in this application are believed to be in *prima facie* condition for allowance, and an early action to that effect is respectfully solicited.

The Commissioner is authorized to charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account **50-4634** (Attorney Docket No. **GNE-0329R1D1 (123851-184962)**).

Respectfully submitted,

Date: October 15, 2009

By Electronic Signature: /GINGER R. DREGER/
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THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Virginia E. Paton	Attorney Docket #:	GNE-0329R1D1
Serial No.	10/356,824	Group Art Unit	1643
Filing Date	02/03/2003	Examiner:	Holleran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
Title:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS – OCTOBER 15, 2009

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF MARK X. SLIWKOWSKI, Ph.D.

I, MARK X. SLIWKOWSKI, Ph.D. declare and say as follows: -

1. I obtained a B.S. in Animal Science and Agricultural Biochemistry from the University of Delaware, a Ph.D. in Biochemistry with minor in Physical Chemistry from North Carolina State University, and completed postdoctoral training at the National Institutes of Health, National Heart, Lung and Blood Institute, Laboratory of Biochemistry.

2. After six years of research experience at Triton Biosciences, Inc. (Berlex Biosciences, Inc.), I joined Genentech, Inc. in 1991 as a senior scientist, where my current title is Staff Scientist, Research Oncology.

3. During my employment at Genentech, I have worked on a number of programs involving drugs directed against the human epidermal growth factor receptor family (also known as the HER or ErbB family). The HER or ErbB family, is frequently activated in a number of proliferative diseases including cancer. Our laboratory focuses on studying various aspects of this receptor activation process including the biochemical nature of the receptor complexes, the activation of signal transduction pathways, and the resultant biological outcomes. These

investigations have contributed to the development of a number of therapeutic approaches that target the receptor family such as Herceptin® (trastuzumab) and Tarceva® (erlotinib), which have received U.S. Food and Drug Administration (FDA) approval. Working closely with the Molecular Diagnostics and Pathology Departments, our current effort deals primarily with identifying a molecular profile that may aid in selecting patients who benefit from treatment with the dimerization inhibitor anti-HER2 antibody, pertuzumab. We are also actively investigating combination therapeutic approaches for the treatment of cancer.

4. My Scientific Curriculum Vitae, including my list of publications, patents, pending patent applications, and awards, is enclosed as **Exhibit A** and forms part of this Declaration.

5. I am familiar with and understand the disclosure of the above-identified patent application, including the claims currently pending. I am also familiar with and understand the Office Action mailed on March 20, 2009 in connection with the above-identified patent application, and the prior art references cited in that Office Action. The pending claims are directed to the treatment of human patients with breast cancer that overexpresses ErbB2 by administration of a combination of an anti-ErbB2 antibody, a taxoid, and a further therapeutic agent, such as a growth inhibitory agent, where the anti-ErbB2 antibody binds epitope 4D5 within the ErbB2 extracellular domain sequence. A prototype of antibodies that binds epitope 4D5 within the ErbB2 extracellular domain sequence is Herceptin® (trastuzumab), and representative taxoids are paclitaxel and docetaxel.

6. According to the Office Action, the invention claimed would have been obvious to one of ordinary skill in the art at the time the invention was made. One of the references cited in support of this conclusion is an Abstract by Baselga, J., et al (Proceedings of the American Association for Cancer Research, 35:380, Abstract #2262, 1994), which reports on the antitumor activity of paclitaxel and docetaxel in combination with the murine anti-HER2 antibody 4D5 in the treatment of human breast cancer xenografts in nude mice. According to the authors, their results suggest synergy between paclitaxel and 4D5.

7. Drugs used in the treatment of cancer are known to exert their effects at different points within the cell cycle. For example, mitotic inhibitors, such as taxoids, stabilize microtubules in their polymerized state and lead to a G2/M block in the cell cycle. In contrast, so called "targeted therapies" such as tamoxifen and trastuzumab, arrest cells in the G0-G1/S transition of the cell cycle. Before the 1997 priority date of this application, it was known that treatment of cancer cells with paclitaxel (Taxol®) disrupts the formation of normal spindles at metaphase, leading to arrest of the cells in the G2/M phase of the cell cycle and ultimately to apoptotic cell death (see, e.g. Woods et al., Mol. Med. 1:506-526, 1995 – **Exhibit B**). It was further known that antibody 4D5 has cytostatic effect, i.e. inhibits cell growth (Huzdiak et al., Molecular and Cellular Biology 9(3):1165-1172, 1989 – **Exhibit C**). It was also reported that treatment with 4D5 antibody reduces the percentage of tumor cells in the S phase of cell cycle, which precedes the G2 phase (see, e.g. Lewis et al., Cancer Research 56:1457-1465, 1996 – **Exhibit D**). These initial findings were subsequently confirmed and refined by Lane, Heidi A., et al., (Molecular and Cellular Biology, 20(9):3210-3223, 2000 – **Exhibit E**) and others. Based on the pre-1997 reports, at the priority date of this application a skilled scientist would have anticipated that paclitaxel would provide little or no additional benefit to treatment with trastuzumab alone, since trastuzumab would arrest the cell cycle before paclitaxel would be able to act.

8. An antagonistic interaction between trastuzumab and paclitaxel would have been viewed as a reasonable possibility based on prior knowledge, such as prior experience with the combined administration of tamoxifen and anthracyclines. Tamoxifen is used in women with breast cancer whose tumors express the estrogen receptor. Binding of tamoxifen inhibits estrogen from binding to estrogen receptor and prevents its ability to promote cell cycle progression. Women, who are first diagnosed with breast cancer, are now treated with multiple therapeutic modalities that frequently include surgery, radiation, chemotherapy, endocrine therapy, and monoclonal antibody therapy. Two decades ago it was recognized that the addition of the anti-estrogen, tamoxifen, to standard chemotherapy regimens resulted in little or no benefit with either advanced breast cancer or in the adjuvant setting. (Osborne et al., Journal Clinical Oncology, 7(6):710-717 (1989) – **Exhibit F**) A mechanistic basis for this apparent antagonism that is observed in the clinic was provided by Woods et al. (Biochem Pharmacol 47(8):1449-

1452, 1994 – **Exhibit G**). In this report, experimental data demonstrated that the antagonism with anthracyline and tamoxifen is likely related to the capacity of tamoxifen to decrease the growth of estrogen-receptor positive tumors and to arrest these cells in the G0-G1 phase of the cell cycle, so that the cell cycle is arrested before the anthracyclines could exert their activity. Based on the antagonistic interaction between tamoxifen and anthracyclines, despite the 1994 Baselga et al. abstract, one of ordinary skill at the priority date of the present application would have had reasons to expect that trastuzumab, or other anti-HER2 antibodies acting by inducing cell cycle arrest in the G1 phase, would antagonize the effect of taxoids, such as paclitaxel, since they arrest cell cycle before it reaches the G2/M phase, where taxoids exert their apoptotic antitumor activity.

9. In addition, even in the absence of knowledge about the seemingly incompatible mechanisms of action of taxoids and trastuzumab, based on the data reported in the Baselga et al. 1994 abstract one of ordinary skill at the priority date of this application would not have had a reasonable expectation of successfully using a combination of a taxoid, and an anti-ErbB2 antibody that binds epitope 4D5 within the ErbB2 extracellular domain sequence, along with an additional growth inhibitory agent in the treatment of human patients with ErbB2 overexpressing cancer. Although human tumor xenografts have been extensively used for rapid screening of the efficacy of anticancer drugs, significant controversy exists about the usefulness of these preclinical models in predicting response of human patients to therapy, because many agents which show high activity in xenograft models prove to be inactive, or show disappointingly low or different activity, in the clinical setting. Thus, for example, after a decade of screening in various preclinical models, scientists of the National Cancer Institute reported that their xenograft models had only moderate predictive value. (Johnson, JI., et al, British Journal of Cancer (2001) 84(1), 1424-1431 – **Exhibit H**) According to the authors, "*[f]or 39 agents with both xenograft data and Phase II clinical trial results available, in vivo activity in a particular histology in a tumour model did not closely correlate with activity in the same human cancer histology, casting doubt on the correspondence of the pre-clinical models to clinical results.*" (Abstract) Indeed, while breast cancer xenografts are described as most useful for predicting clinical response against *any* disease, correlation with clinical activity was found against NSCL, melanoma, and ovarian cancer, but *not with clinical breast cancer*! (page 1426, 2nd column)

Based on my many years of personal experience and familiarity with the development of anticancer drugs, individual xenograft models of human tumors, such as the breast cancer xenograft model used by Baselga et al., do not mimic closely enough the biological characteristics and do not capture the inherent variability of human tumors, which greatly limits their value in predicting patient response in a human clinical situation. This unpredictability applies not only to the testing of single agents, but also to the combinations of two or more anticancer agents, essentially for the same reasons.

10. On the basis of the explanation set forth in paragraphs 7-9 of this Declaration, and the enclosed evidence, it is my considered scientific opinion that the xenograft data reported in the 1994 Baselga Abstract would not have motivated one of ordinary skill in the art at the priority date of the present application to treat a human breast cancer patient whose cancer overexpresses ErbB2 with combined administration of an antibody that binds to epitope 4D5 within the ErbB2 extracellular domain and a taxoid. It is further my considered scientific opinion that, while at the 1997 priority date of this patent application, the clinical efficacy of 4D5 and paclitaxel, as single agents, in the treatment of human breast cancer was demonstrated, one of ordinary skill at that time would not have had a reasonable expectation that a combination of an anti-ErbB2 antibody binding to the 4D5 epitope and a taxoid, such as paclitaxel could be successfully used to treat human breast cancer patients, or that the reported synergistic interaction between 4D5 and paclitaxel would also hold true to human breast cancer patients.

11. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further, that these statements are made with the knowledge that willful statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent granted thereon.

Dated: 15 October 2009

MARK X. SLIWKOWSKI, Ph.D.

EXHIBIT A

MARK X. SLIWKOWSKI

Genentech, Inc.
Research Oncology
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650/225-1247 Ph.
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EDUCATION

National Institutes of Health (1982-1985)
National Heart, Lung and Blood Institute, Laboratory of Biochemistry
Staff Fellow with Dr. Thressa C. Stadtman

North Carolina State University (1978-1981)
Ph.D. in Biochemistry with minor in Physical Chemistry
Advisor: Dr. Harold E. Swaisgood

University of Delaware (1972-1976)
B.S. in Animal Science and Agricultural Biochemistry (Pre-Vet)

EXPERIENCE

GENENTECH, INC.

Staff Scientist 2002-present

HER3-EGFR Dual Antibody Project (DAF) (2006-present) Late Stage Research Team Leader responsible for moving project into Early Clinical Development (ECD). Current member of ECD core team.

Armed Antibody Program (2003-present) Lead multi-disciplinary research and development team efforts to assess arming monoclonal with cytotoxic agents. Served as the major liaison in coordinating collaboration with outside companies.

Trastuzumab-DM1 Program (2004) Research team leader and early development team leader. Considered prototype for armed antibody platform. Current member of T-DM1 core team. Participate in development program.

Director 2003-2008

Formed Translational Oncology Department. Managed expansive growth from 2004-2007, including small molecule expertise. Managed Director of Assay & Automation Technology from 2005-2007.

Senior Scientist 1991-2001

Heregulin (1991-1996) Participated in the initial characterization of heregulin. Helped define a role for HER2/ErbB2 as a co-receptor with HER3, HER4 and EGFR.

HERCEPTIN® (1993-present) Member of the core project team throughout Phase II and Phase III clinical development. Responsibilities included studies on the development of an *in vitro* diagnostic assay, mechanism of action, mechanism of cardiotoxicity, coordinating biological and biochemical assays, obtaining data to support new clinical indications and designing studies to test novel chemotherapeutic combinations. Also responsible for managing all extramural research activities.

Pertuzumab (1997-present) As part of our heregulin studies, recognized the potential of blocking ligand-activated HER2 as an anti-cancer therapy. Led research and developmental research teams. Participated on developmental assessment team that resulted in rhuMAb 2C4/pertuzumab being moved into development in August 2000. Helped facilitate Roche decision to co-develop rhuMAb 2C4. Currently lead research effort and serve on pertuzumab core team.

Tarceva®(2000-2007) One of several Genentech employees that encouraged Business Development to pursue in-licensing OSI-774. Participated in due diligence team. Led research effort on Tarceva and serve on Tarceva core team.

Triton Biosciences, Inc. (Berlex Biosciences, Inc.)

Staff Scientist 1990 – 1991
Initiated a program for the identification and isolation of ligands for receptor tyrosine kinases. Participated in project to establish structure-activity relationship for TGFα. Supervised protein and peptide chemistry laboratories consisting of 2 scientists and 5 research associates.

Senior Research Scientist 1987 – 1990
Project Leader for development stage of HTLV-1 program. (Interdisciplinary team consisting of 3 scientists and 8 research associates.) Developed folding procedure for TGFα. Collaborated extensively with Immunology group on projects involving differentiation and cytotoxicity.

Research Scientist 1985 – 1987
One of the first bench scientists hired. Established protein chemistry laboratory purified recombinant retroviral proteins for development of diagnostic viral immunoassay.

NIH Postdoctoral Position 1982 – 1985
Studied mechanisms by which selenium is incorporated into bacterial proteins. Purified several selenium-containing proteins and gained extensive experience in peptide mapping.

AWARDS

Genentech Inc. Outstanding Commercial Collaborator Award, 2008
North Carolina State University Outstanding Alumni Award, 2008
Genentech Inc. Most Commercially Significant Patent Award, 2007 (Patent No. 7,097,840)
Genentech Inc. Most Commercially Significant Patent Award, 2006 (Patent No. 6,949,245)
Industry Scientist of the Year, Pharmaceutical Achievement Award, 2005
Triton R&D Award, 1989
Industrial Initiative for Science and Math Education Award, 1989
American Society of Biological Chemistry Travel Grant, 1985
Phi Lambda Upsilon Chemistry Honor Society, 1981
Gamma Delta Sigma Agricultural Honor Society, 1981

Outstanding Graduate Student Teaching Award , 1979

PROFESSIONAL AFFILIATIONS

American Society of Clinical Oncology
American Association for Cancer Research
American Society for Biochemistry and Molecular Biology

INVITED PRESENTATIONS (SINCE 2006)

Van Andel Research Institute, Grand Rapids, June 2009
Keystone Symposium, Whistler, BC, March 2009
European Antibody Congress, Geneva, December 2008
Istituto Nazionale Tumori of Milan, April 2008
Oncology Leaders' Forum Boston, November 2007
ASTRO Los Angeles, October 2007
FASEB Symposium, Tucson, August 2007
ESMO Congress, Istanbul, Turkey October 2006
Congress of the International Assoc. for Breast Cancer Research, Montreal September 2006
SPORE Breast Cancer Workshop, Baltimore, July 2006
Stanford University May 2006
Chair of New Biological Agents on the Horizon AACR Annual Meeting, Washington DC, April 2006
Conference on Obstacles to Translational Medicine, San Francisco, March 2006
Vanderbilt University, January 2006

PEER REVIEW ACTIVITIES

Department of Defense Breast Cancer Program
Nature ad hoc
Oncogene ad hoc
Cancer Research ad hoc
Clinical Cancer Research ad hoc
Cancer Cell ad hoc

PUBLICATIONS

1. Yao E, Zhou W, Lee-Hoeflich ST, Truong T, Haverty PM, Eastham-Anderson J, Lewin-Koh N, Gunter B, Belvin M, Murray LJ, Friedman LS, Sliwkowski MX, Hoeflich KP. Suppression of HER2/HER3-mediated growth of breast cancer cells with combinations of GDC-0941 PI3K inhibitor, trastuzumab, and pertuzumab. Clin Cancer Res 2009;15:4147-56.

2. Polson AG, Sliwkowski MX. Toward an effective targeted chemotherapy for multiple myeloma. Clin Cancer Res 2009;15:3906-7.

3. Makhija S, Amler LC, Glenn D, Ueland FR, Gold M, Dizon DS, Paton V, Lin C-Y, Januario T, Ng K, Strauss A, Kelsey SM, Sliwkowski MX, Matulonis U. Clinical activity of gemcitabine plus pertuzumab in platinum-resistant ovarian cancer, fallopian tube, or primary peritoneal cancer: low mRNA expression of the HER2-coreceptor HER3 may be predictive of pertuzumab activity. J Clin Oncol 2009;in press.

4. Krop IE, Beeram M, Modi S, Holden SN, Yu W, Girish S, Tibbitts J, Yi J-H, Sliwkowski MX, Jacobson FS, Lutzker SG, Burris HA. A Phase I Study of Trastuzumab-DM1, HER2 Antibody-Drug Conjugate, Given Every 3 Weeks to Patients with HER2+ Metastatic Breast Cancer J Clin Oncol 2009; to be submitted.

5. Junttila TT, Parsons K, Olsson C, Lu Y, Xin Y, Theriault J, Meng G, Totpal K, Kelley RF, Sliwkowski MX. Superior in vivo efficacy of afucosylated trastuzumab in the treatment of HER2 amplified breast cancer. to be submitted 2009.

6. Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, Sampath D, Sliwkowski MX. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. Cancer Cell 2009;15:429-40.

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EXHIBIT B

Taxol-Induced Mitotic Block Triggers Rapid Onset of a p53-Independent Apoptotic Pathway

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ABSTRACT

Background: At therapeutic concentrations, the antineoplastic agent taxol selectively perturbs mitotic spindle microtubules. Taxol has recently been shown to induce apoptosis, similar to the mechanism of cell death induced by other antineoplastic agents. However, taxol has shown efficacy against drug-refractory cancers, raising the possibility that this pharmacological agent may trigger an alternative apoptotic pathway.

Materials and Methods: The kinetics and IC_{50} of mitotic (M) block, aberrant mitosis, and cytotoxicity following taxol treatment were analyzed in human cell lines as well as normal mouse embryo fibroblasts (MEFs) and MEFs derived from p53-null mice. Apoptosis was followed by DNA gel electrophoresis and by in situ DNA end-labeling (TUNEL).

Results: Taxol induced two forms of cell cycle arrest: either directly in early M at prophase or, for those cells progressing through aberrant mitosis, arrest in G_1 as multimininucleated cells. TUNEL labeling revealed that DNA nicking occurred within 30 min of the arrest in prophase. In contrast, G_1-arrested, multimininucleated cells became TUNEL positive only after several days. In the subset of cells that became blocked directly in prophase, both wt p53-expressing and p53-null MEFs responded similarly to taxol, showing rapid onset of DNA nicking and apoptosis. However, p53-null MEFs progressing through aberrant mitosis failed to arrest in the subsequent G_1 phase or to become TUNEL positive, and remained viable.

Conclusions: Taxol induces two forms of cell cycle arrest, which in turn induce two independent apoptotic pathways. Arrest in prophase induces rapid onset of a p53-independent pathway, whereas G_1-block and the resulting slow (3–5 days) apoptotic pathway are p53 dependent.

INTRODUCTION

Taxol is a complex plant alkaloid with a unique taxane ring structure which appears to target microtubules (MTs) specifically both in vitro and in vivo (1–6). Taxol binds to the polymeric microtubule form of tubulin in a 1:1 stoichiometry with $\alpha\beta$-tubulin heterodimer subunits (2,4,7). Binding markedly shifts the dynamic instability equilibrium of MT polymers, stabilizing against dissociation and hence augmenting polymerization. Bound taxol overrides the need for GTP hydrolysis in tubulin heterodimer polymerization, and consequently it also serves to lower the critical concentration for tubulin polymerization (2,7); in vivo this is manifested by the ability of taxol to override the centrosomal MT nucleating activity in interphase cells, resulting in the appearance of non-centrosomally linked MT bundles throughout the cytoplasm (1,5,6,8). Currently, there has been considerable interest in taxol as a cancer therapeutic agent, in particular because it has shown activity against leukemias, late stage ovarian, and metastatic breast cancers refractory to standard chemotherapeutic treatments (9–12). Phase I and II trials have also shown some efficacy against advanced small cell lung cancer (13), melanoma (14,15) and head and neck cancers (16).

Microtubules constitute a major class of

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structural filaments in the eukaryotic cell and play a key role in a wide repertoire of cellular processes: endocytosis, vesicular movement, cell morphology and motility, and mitosis to name but a few (17). Somewhat surprisingly, however, taxol concentrations within the therapeutic range are not necessarily sufficient to cause gross rearrangements of interphase MT arrays and leave a functional MT scaffold intact. Therefore, it is not immediately apparent why taxol should be cytotoxic rather than merely cytostatic. In cultured human cell lines, taxol has been shown to induce a block in the cell cycle at the G_2/M transition (1,18–23). At the G_2/M transition, cellular MTs undergo a dramatic reorganization associated with a sudden change in the centrosomal MT-nucleating activity (5,6,23,24). Interphase MTs dissociate (even in the presence of taxol) as the centrosomes concurrently elaborate the mitotic spindle MTs (5,6,23,24). Interestingly, this sudden change in centrosomal activity correlates with association of $p34^{cdc2}$/cyclin B complex with the centrosome (23,25). Taxol induces aberrant multipolar spindle formation that causes mitotic arrest (5,6,26). Recent studies with human lines have provided strong evidence that it is this arrest in M that correlates with cytotoxicity (18,20–22,27,28). For HeLa cells, the EC_{50} for arrest at the G_2/M transition is similar for the EC_{50} for taxol-induced cytotoxicity and 1 to 2 orders of magnitude lower than the doses required to induce gross changes in interphase MT arrays (18). Rodent cells are also sensitive to taxol in ranges that perturb mitosis but appear to be more tolerant of arrest in mitosis; they eventually assume a multimininucleated phenotype resulting from aberrant mitosis and lack of cytokinesis. Doses effecting these changes are also cytotoxic (5,6,19). Species differences between human and rodent lines have been attributed to differences in $p34^{cdc2}$ regulation (19). Taken together, these studies predict that taxol should be therapeutically active only against cells that are rapidly cycling. In balance, this appears to be the case, and the side effects of neutropoenia and leukopoenia as well as gastrointestinal-related side effects have been shown to be consistent with the hypothesis that taxol effects are due to mitotic arrest (29,30). However, exceptions exist where tumor regression is found despite the lack of actively cycling tumor cells and studies with leukemic lines have indicated that sensitivity to taxol correlates with the induction of MT bundles rather than multipolar aster formation (8,31). Therefore, the nature of taxol's therapeutic activity remains to be fully elucidated.

Cell death can be induced by either necrosis or an active process known as apoptosis (32–36). The latter appears to be closely linked to the programmed cell death that occurs in development (32–35). Typically, high levels of insult induce necrosis, whereas lower levels induce apoptosis (33,35,36). In several cases of cellular differentiation, growth factors serve not only as proliferative and differentiating signals but also serve to rescue precursor proliferating cells from apoptosis (33–35). Thus certain cell types, most dramatically cells of the hematopoetic lineages, are particularly poised for apoptosis (low threshold for apoptosis defined in Refs. 33 and 35). It has now been well established that DNA-damaging cancer therapeutics and γ-irradiation exert their cytotoxic effects by inducing the programmed apoptotic pathway following arrest in G_1 or G_2 as opposed to necrosis (36–42). The tumor suppressor, p53, has been shown to play a key role in this process. The process of DNA nicking in and of itself induces elevated p53 levels (43,44) which in turn induce G_1 arrest by up-regulation of the cdk inhibitor WAF1/Cip1/p21 (45–48) to prevent progression past the G_1/S checkpoint of cells with damaged DNA. Extensive DNA damage leads to prolonged G_1 arrest and apoptosis (43). Similarly, commonly used antineoplastic agents that target DNA, which induce arrest in G_1 or G_2, trigger the same p53 response (42–44,49–55). Interestingly, many advanced stage tumor types which have poor prognosis with established antineoplastic therapies express a high percentage of p53 mutations (55–59). For many tumor types, a late stage event in the progression of the cancer is the clonal expansion of cells expressing mutant p53 alleles (55–59). Mutation of the normal cellular p53 gene has been shown to be sufficient to induce cellular immortalization (55,59,60) and can enhance tumorigenicity (61–64). p53 normally functions as a homo-oligomer; hence, a single mutant p53 allele can lead to a dominant negative phenotype (63,65). The discovery that Li Fraumeni patients, genetically predisposed to an abnormally high incidence of cancer, fail to mount a normal p53-mediated response, together with studies indicating that transgenic mice carrying germ line knock-out of p53 alleles are more prone to develop spontaneous cancers and fail to mount an appropriate apoptotic response to γ-irradiation or DNA-damaging antineoplastic agents, provides strong support for the

hypothesis that p53 plays an important role in the apoptotic pathways underlying elimination of aberrant tumor cells (42–44,49,50,52–55,61,66,67). Alternative non-p53-dependent apoptotic pathways also exist. These operate in normal development; for example apoptosis resulting from growth-factor deprivation appears to occur via a p53-independent pathway (33,35,52,54,68). Since taxol induces predominantly arrest in early M rather than G_1 or G_2 arrest in human cells, it is of interest to know whether the apoptotic pathway triggered by taxol (69–71) is dependent on p53.

Here we describe detailed comparative analyses of taxol effects on several human carcinoma lines as well as a normal human line. Kinetic analysis reveals that the majority of human cells become blocked at the G_2/M transition in prophase, with only a small percentage progressing through a protracted aberrant mitosis, manifested by a multimininucleated phenotype, to become arrested in the subsequent G_1 stage. The lag between mitotic block and cell death is 20–30 hr, with similar EC_{50} values for both processes. G_1-arrested multimininucleated cells are also destined to die but with much slower kinetics (several days). We further demonstrate that both forms of taxol-induced cell cycle arrest lead to apoptosis. However, apoptosis triggered by arrest in M is initiated very rapidly, within 30 min of arrest at prophase, and appears to operate by a p53-independent pathway. In contrast, both the G_1 arrest and subsequent apoptotic death of cells progressing through an aberrant mitosis are p53 dependent.

MATERIALS AND METHODS

Cells

Hela, Hs578T, Hs578Bst, MCF7, and Rat1 cells were all obtained from ATCC and each grown in the medium specified by ATCC at 37°C in a humid atmosphere containing 5% CO_2. wt p53 Mouse embryo fibroblasts (MEFs) were isolated by trypsinizing the muscle mass of 13-day mouse embryoes, filtering through 70-μm filters and plating at 0.3×10^6 cells per cm^2 in DMEM (Gibco, Gaithersburg, MD, U.S.A.) containing 10% FBS (ICN) and 1 × penicillin/streptomycin (Gibco). After 1 h nonadherent cells were removed. MEFs were passaged twice before being used for experiments. MEFs lacking p53 (p53−/− MEFs) were obtained from T. Jacks (MIT). p53 status was verified by Western blot analyses. For comparative nuclear and viability/growth analyses, cells were seeded both in 48-well plates at 2.5×10^4 cells per well (for viability and cell counting) and onto #1 glass coverslips in 6-well plates at 5×10^5 cells per well (for mitotic scoring). Twenty-four hours later, the medium was replaced with medium containing taxol at the specified concentrations. Taxol was always added directly from a DMSO stock to prewarmed medium immediately prior to treating the cells; final DMSO concentration was always less than 0.1%. DMSO up to 1% had little effect on cell growth. At specified times, nonadherent cells were collected and the adherent cells trypsinized. After pelleting briefly at 2,000 rpm for 2 min in an Eppendorf centrifuge, the cell pellet was resuspended in a final volume of 50–100 μl 2% Trypan blue in PBS. Concurrently, coverslips were fixed in Omnifix for 10 min then stained with 2 μg/ml Hoechst 33342 (Boehringer-Mannheim, Indianapolis, IL) in PBS. To assay cells in suspension, 0.5 ml medium was removed after very gentle swirling, cells pelleted and stained in 2 μg/ml Hoechst in 30% ethanol. Stained cells were viewed under epifluorescence with a Zeiss Axiophot microscope. Two sets of 250 cells, located by random walk of the stage control, were scored as prophase, mitotic, multimininucleated or normal interphase for each time point. The ratio of attached to nonadherent cells derived from the total cell counts was used to convert scoring of Hoechst-stained adherent and nonadherent cells to overall percentages in the total population.

To obtain cells synchronized in the cell cycle, mitotic cells were collected by selective shake-off from 70 to 80% confluent (higher densities helped minimize detachment of interphase cells) HeLa cells with two successive 7-min incubations at 37°C in Ca^{2+} and Mg^{2+}-free Hanks' buffered salt solution (CMF-HBSS; 37°C). Harvested cells were cooled to 15°C to arrest cells in mitosis without depolymerizing the MTs, and collected by centrifugation at 15°C for 5 min at 1000 × g. A small aliquot of cells was fixed and Hoechst-stained to assess percent mitotic cells and the remainder plated onto coverslips or incubated as required. Only preparations exceeding 50% mitotic cells were used for experiments.

End Labeling of Cellular DNA and Agarose Gel Electrophoresis

Cellular DNA was isolated from cells at different times following taxol treatment by proteinase K and RNAaseA digestion of cell lysates followed

by phenol chloroform extraction and ethanol precipitation following standard protocols. DNA was end labeled with ^{32}P-γ ATP using terminal transferase (Boehringer Mannheim), electrophoresed on a 1.4% agarose gel and transferred to nitrocellulose. The dried blot was exposed to Kodak X-OMAT film for 15–30 hr at −80°C.

TUNEL Labeling and Immunofluorescence

For tubulin immunofluorescence (IF) cells were fixed in −20°C methanol and processed for IF using anti-β tubulin mAbs (Amersham, Arlington Heights, IL). For labeling DNA ends in situ, the TUNEL method described by Gavrieli et al. (72) was modified as follows. Cells were fixed for 5 min in 2% formaldehyde in TBS (10 mM Tris HCl (pH 8.0), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA) and permeabilized in 0.5% Triton X-100 in TBS for 15 min. After successive washes in TBS and TTD buffer (100 mM Na cacodylate (pH 6.6), 5 mM $CoCl_2$, 0.26 mg/ml BSA) cells were end-labeled with terminal transferase using digitonin-coupled dUTP (Boehringer Mannheim) followed by counter labeling with FITC-conjugated mouse anti-digitonin mAbs as described (Boehringer Mannheim). For this application of terminal transferase activity, we found considerable variation in enzyme batches. To ensure consistent results the following control was included. Fixed cells were treated with 50 μl/ml micrococcal nuclease (Boehringer Mannheim) in 10 mM Tris HCl (pH 8.5), 1 mM $MgCl_2$, 1 mM $CaCl_2$ for 20 min and then processed for TUNEL labeling as described above. Only enzyme batches that yielded clean nuclear staining following this treatment were used for these experiments. In addition, etoposide treated cells were also used as controls for a positive reaction. To assess the kinetics of DNA nicking following taxol-induced arrest at the G_2/M transition, mitotic cells harvested by selective shake-off were plated onto coverslips and incubated for 10 hr at 37°C. One hundred nanomolar taxol was then added and after an additional 3–4 hr, coverslips were collected at 30-min intervals, fixed, processed for TUNEL staining, and counterstained with Hoechst 33342. Nonadherent cells were also processed. Two sets of 250 cells per coverslip were scored. Control untreated cells typically exhibited a peak of mitosis between 16–17 hr after the shake-off.

RESULTS

Kinetics of Mitotic Block and Cytotoxicity Following Taxol Treatment

To establish the correlation between mitotic block and cell death and the kinetics between these two events, different human cell lines, including HeLa (human cervical carcinoma), Hs578T and MCF-7 (human breast adenocarcinoma), and Hs578Bst (derived from normal breast tissue) were treated with 10^{-9} to 10^{-6} M taxol. Care was taken to score both attached and detached cells for cell number and mitotic state since prolonged mitotic block (>3–4 hr) caused cells to detach irreversibly from the substratum since even if these detached prophase cells were transfered to another well, they failed to attach. The results obtained for HeLa cells are shown in Fig. 1. A lag of three–four hr elapsed after taxol addition before a statistically significant increase in the percent of cells in mitosis compared with the controls (~3%) was observed. The estimated time to complete mitosis is 20–30 min (24). HeLa cells have a doubling time of around 16 hr, therefore, at any one time the number of cells predicted to be in M should be 2.1–3.1%. This predicted value corresponded exactly to the observed value scored for the untreated cells of 3.1

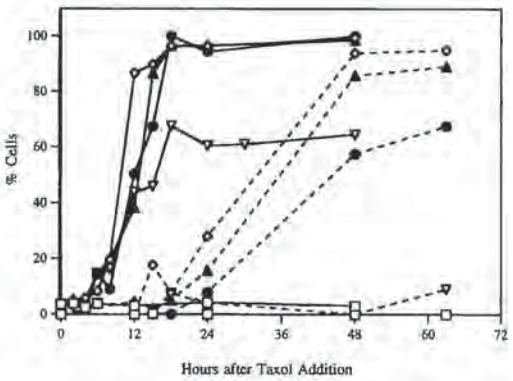


FIG. 1. Kinetics of taxol-induced block in mitosis and cytotoxicity in HeLa cells

Cells were treated with 1 μM (◇), 100 nM (▲), 25 nM (●), 10 nM (▽), or 0 (□) taxol and scored at intervals for arrest in prophase by Hoechst staining (solid lines) or cell death by Trypan blue staining (dashed lines). Both attached and detached cells were scored, since cells arrested in prophase soon detach, and summed to give the total cell number. The ratio of cell numbers attached:detached was used to calculate overall percentage of cells in M.

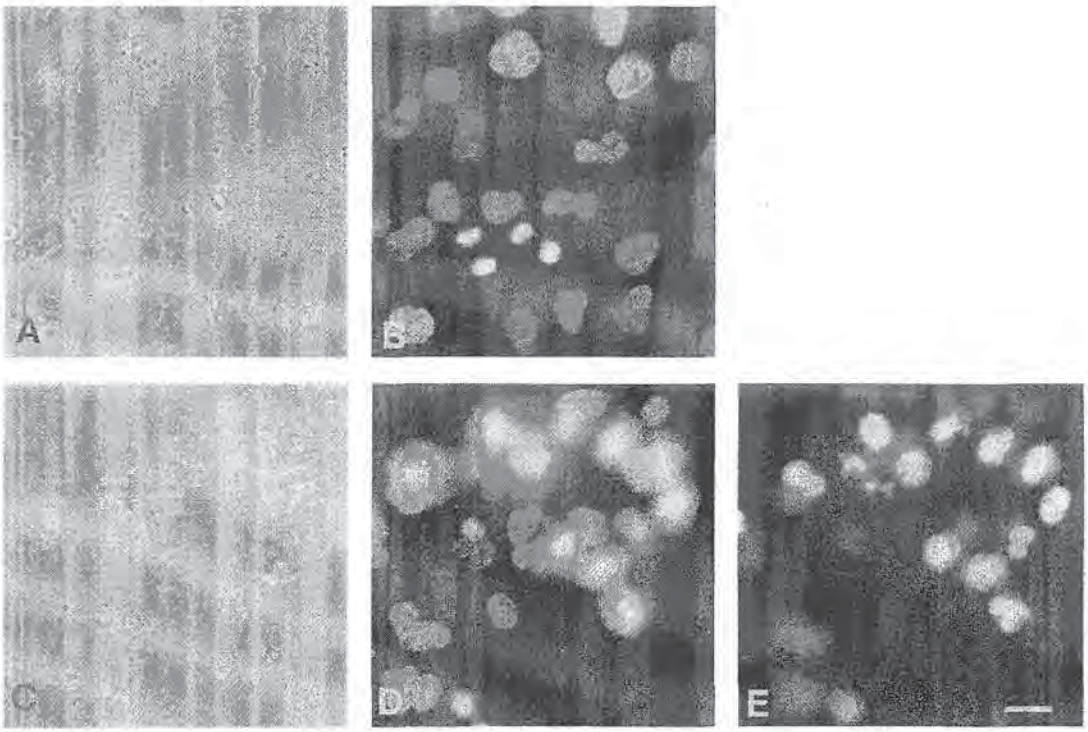


FIG. 2. Taxol induces either arrest in prophase or multimininucleation

HeLa cells were grown on #1 coverslips, and fed regular medium (Control; A and B) or treated with 100 nM taxol for 24 hr (C–E). Cells were stained with Hoechst 33342 (B, D, and E) and viewed with epifluorescence. The corresponding phase is shown in the left-most panel. Panel E shows prophase-blocked cells in focus; Panel D shows the same field but with the attached interphase cells in focus. Bar in Panel E represents 10 μm; magnification is the same for all panels.

± 0.3%. Should taxol immediately effect mitotic block, the rate of accumulation in M for the whole population should reflect these kinetics. Therefore the lag between taxol addition and onset of mitotic block indicated either that a minimum time was required to accumulate sufficient intracellular taxol to reach an adequate MT-taxol equilibrium to induce this effect or that time was required for some downstream effects to be manifested. Short, 1-hr taxol treatments revealed that a similar pattern of lag followed by accumulation in M could be observed for the first 8–15 hr (depending on taxol concentration) indicating that the lag was due to some downstream effect (data not shown).

From 4 hr onwards, progressively more cells became blocked in prophase (i.e., at the G_2/M transition), at a rate consistent with the doubling time exhibited by the untreated cells. For Hela cells treated with ≥100 nM, more than 95% of the cells became blocked in prophase within 15–18 hr, equivalent to the doubling time of the cells (Figs. 1 and 2E). Cell death commenced 24–36 hr later, as evidenced by Trypan blue exclusion, with the slope of taxol-induced cytotoxicity ressembling that of mitotic block at taxol concentrations at or above 25 nM (Fig. 1). At concentrations around the IC_{50} and below, this relationship is obscured when the data is expressed on a percentage basis since from 2 to 3 days onwards, the cells that passage through M normally continue to rapidly proliferate (see below). A small percentage of the residual attached cells was seen to be multimininucleated (Fig. 2E). These cells were observed at taxol concentrations of 10 to 25 nM and amounted to less than 5% of the total cell number. At these concentrations a few mitotic figures were observed that had proceeded past the block at prophase to metaphase, but they displayed grossly misaligned

TABLE 1. Comparison of EC_{50} values for mitotic arrest and cytotoxicity induced by taxol in various human cell lines

Cell Type	Mitotic Arrest[a] (nM)	Aberrant Mitosis[a] (nM)	Cytotoxicity[b] (nM)
Hela	8.4 ± 1.5	8.4 ± 1.5	25.6 ± 5.6
Hs578T	16.6 ± 7.6	10.0 ± 5.3	14.8 ± 4.9
Hs578Bst	18.5 ± 6.5	12.5 ± 0.5	10.0
MCF-7	6.7 ± 1.7	6.7 ± 0.5	47.0 ± 7.2

All individual curve fits gave R^2 values of 0.98 to 1.0. Cells were treated with taxol concentrations ranging from 1 to 0 μM and scored after "24 hr for mitotic effects as described in Materials and Methods.

[b]Cytotoxicity estimations based on # Trypan blue positive cells after 72 hr. Data was analyzed by Inplot or Prism (Graphpad, CA, U.S.A.) to obtain EC_{50} values. Experiments were carried out in triplicate.

chromosome arrays (Fig. 3I). Detailed microscopy observations indicated that multimininucleation was the consequence of passage through aberrant mitosis, with the individual mininuclei apparently resulting from lamin repolymerization and nuclear envelope reformation occurring around each misaligned clump of chromosomes. Noticeably, multimininucleated cells were not observed until at least 18 hr in the presence of taxol. These cells became arrested in the subsequent G_1 stage, as evidenced by the constant amount of Hoechst-stained chromatin seen immediately after their first appearance and at all subsequent time points. Multimininucleated cells also died but with much slower kinetics of 3–6 days following G_1 arrest.

Similar correlations between taxol-induced cell cycle arrest and cytotoxicity were also seen with the Hs578T, MCF7, and Hs578Bst lines (doubling time of 2, 3, and 7 days, respectively). With the slower growing MCF7 and Hs578Bst cultures, it was clear that taxol only remained effective for the first 24–48 hr following addition of taxol-containing medium. Cells entering M around 48 hr appeared to be only transiently blocked in this phase of the cell cycle, passaging through aberrant M to arrested in the cell cycle as multimininucleated cells. Thereafter, cells entering M progressed through normal mitosis. Addition of fresh taxol to the medium at this point was required to block the next cohort of cells entering M, confirming that taxol remained effective only for a limited time in slower growing cell lines (data not shown). However, within the time period taxol remained fully effective, it exerted a similar effect on all the human lines we analyzed, namely, selective perturbation of mitosis, which in turn induced arrest at one of two stages of the cell cycle. The majority of cells were blocked directly in M (mitotic arrest in Table 1) and died 24–48 hr later. The remaining cells that did progress through M (aberrant mitosis in Table 1 represents the sum of M-blocked plus cells that pass through abberant M to become multimininucleated) appeared to become arrested in G_1, based on the constant chromatin content contained by these multimininucleated cells immediately following their appearance (i.e., bona fide G_1) and at all subsequent time points. This G_1 arrest was confirmed by FACS analysis for MCF7 and Hs578T cells (these cell lines exhibited a greater fraction of multimininucleated cells than did HeLa cells) since no progression past the 4N DNA compartment was observed in the adherent multimininucleated cell population (data not shown). These G_1-arrested multimininucleated cells died after 3–6 days.

Anti-tubulin IF revealed that 2–4 hr of exposure to taxol above 10 nM induced abnormal multipolar spindles in the prophase-blocked cells (Fig. 3 C and D) compared with the normal bipolar spindle arrays of untreated cells (Fig. 3B). In contrast, gross rearrangements of MT arrays in interphase cells required concentrations in the 1–10 μM range (5,6,73). As shown in Table 1, the EC_{50} values for taxol-induced cytotoxicity at 72 hr correlated with the EC_{50} for arrest in early M (7–18.5 nM) and were two orders of magnitude lower than the 1–10 μM concentrations required for gross perturbations of interphase MT arrays (73). The EC_{50} values for M arrest and cytotoxicity were similar for all human lines examined. No difference was noted between the

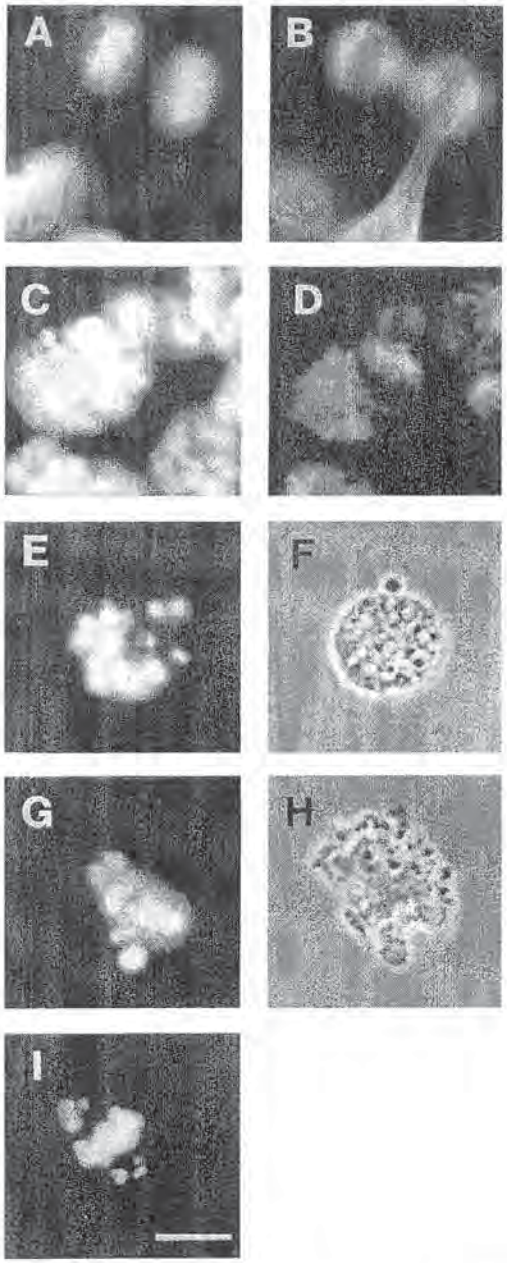


FIG. 3. Sequence of changes induced by taxol in HeLa cells

Untreated (A and B) and taxol-treated (4 hr; C and D) cells processed for anti-tubulin IF (B and D) and counterstained with Hoechst 33342 nuclear dye (A and C) to reveal multipolar spindles induced by taxol. A few cells undergo aberrant mitosis exhibiting irregular chromosome arrays at metaphase (I). After 24-hr taxol treatment, Hoechst staining reveals DNA becomes globular (E and G Hoechst staining; F and H corresponding phase) especially when cells have extensive membrane blebbing (H). Exposure times for Panels E and G were shorter than for Panels A, C, and I. Bar in Panel I represents 10 μm; magnification is the same for all panels.

tumor-derived lines and the Hs578Bst line derived from normal breast tissue.

Taxol-Induced Cytotoxicity Occurs by an Apoptotic Pathway

Microscopic observations of Hoechst-stained cells, indicated that initially cells blocked by taxol in prophase displayed defined chromatin structure (Fig. 3C; note that this 3D structure cannot be illustrated well by a single plane of focus, given the tightly compacted nature of the chromatin, but the structure was clearly evident with fine focussing up and down through several optical planes). However, by 24 hr following addition of taxol, the chromatin of many cells began to appear globular and more brightly stained (Fig. 3 E and corresponding phase image F; note, the exposure times for Panels E and G were reduced compared with Panels A, C, and I). The chromatin became progressively more globular as the cells exhibited extensive membrane blebbing and appeared phase-dense (Fig. 3 G and H). This highly globular and intensely staining DNA pattern was reminiscent of the late sequence of events seen in etoposide-treated cells used as a positive control for apoptosis. However, etoposide-treated cells become arrested in interphase at G_1 and exhibit a well-characterized sequence of apoptotic changes in chromatin morphology, initially developing punctate chromatin staining under the nuclear membrane but becoming progressively more globular and brightly Hoechst-stained as the nuclear structure breaks down (see Fig. 5 G and H). Therefore, etoposide-treated cells are not directly comparable to taxol-treated cells arrested in M. We therefore carried out gel electrophoresis with ^{32}P-end labeled DNA extracted from taxol-treated cultures to detect DNA ladders, the commonly used hallmark of apoptosis (33,34,36). We employed end labeling to visualize fragments on a molar basis and increase sensitivity to pinpoint the earliest time apoptosis could be monitored by gross DNA fragmentation. Figure 4 shows the results obtained with HeLa cells although identical results were also obtained with Hs578T and MCF-7 cells (data not

FIG. 4. Taxol induces the DNA nucleosomal ladders that are indicative of apoptosis

DNA isolated from cells treated with 0 (Lane 1), 1 μM (Lane 2), or 100 nM taxol (Lane 3) for 6, 24, or 72 hr as indicated above the lanes. Arrowheads indicate positions of 1 kb ladder markers (Gibco-BRL). Panels underneath represent Ethidium bromide staining of the same gel prior to transfer.

shown). No evidence for DNA fragmentation was observed after 6 hr taxol treatment and DNA fragmentation was only evident after 24 hr at either 100 nM or μM taxol. Pronounced DNA ladders were only evident after 72 hr, which corresponded to the time the cells became Trypan blue positive.

Bulk DNA analysis does not reveal whether apoptosis is selectively occuring in those cells that are blocked in M at prophase. To address this issue we performed in situ digitonin-dUTP end-labeling (TUNEL, 72). Since enzyme batches were highly variable in this assay, and the procedure was found to be very sensitive to permeabilization and fixation conditions, we routinely used as a control, normal cells that had been briefly treated for 20 min with 50 μl/ml micrococcal nuclease after fixation before being processed for TUNEL labeling (Fig. 5 I and J). Only batches of terminal transferase that gave uniformly bright-staining nuclei after in situ nuclease digestion were used. (It should be noted that poor permeabilization and enzyme penetration resulted in only peripheral labeling around the perimeter of the nucleus, again underscoring the importance of internal controls for this technique). TUNEL labeling revealed that indeed only those taxol-treated cells blocked in prophase stained positively (Fig. 5 D–F). To ensure that the preferential staining of blocked prophase cells was not simply due to more efficient enzyme penetration in mitotic cells due to dissolution of the nuclear envelope and because the chromatin is condensed, we rigorously analyzed many fields of control cells and found all untreated mitotic cells at all stages of mitosis to be TUNEL negative (Fig. 5 A–C, indicated by m). Only the occasional dead cell stained positively in untreated populations (Fig. 5 A–C, indicated by arrowhead). It was also readily apparent that all M-blocked cells appeared to be positive even though 8 hr of treatment was well before the time cell death was apparent by Trypan blue staining or DNA ladders were evident by gel electrophoresis. TUNEL labeling and staining of the multimininucleated cells confirmed morphological observations of Hoechst-stained cells which indicated that these cells also progressed to an apoptotic pathway of cell death. However, multimininucleated cells only began to exhibit positive TUNEL staining from 3 days onwards after G_1 arrest in a much more staggered fashion (Fig. 5 K and L; Hs578T cells are shown since multimininucleation was more common in these cells than in HeLa cells which have a rapid doubling time).

The remarkable concordance between the number of M-blocked and TUNEL-positive cells implied that DNA nicking was induced rapidly after block in prophase. To establish the exact timing for onset of DNA nicking after arrest in M, cells were synchronized in the cell cycle by se-

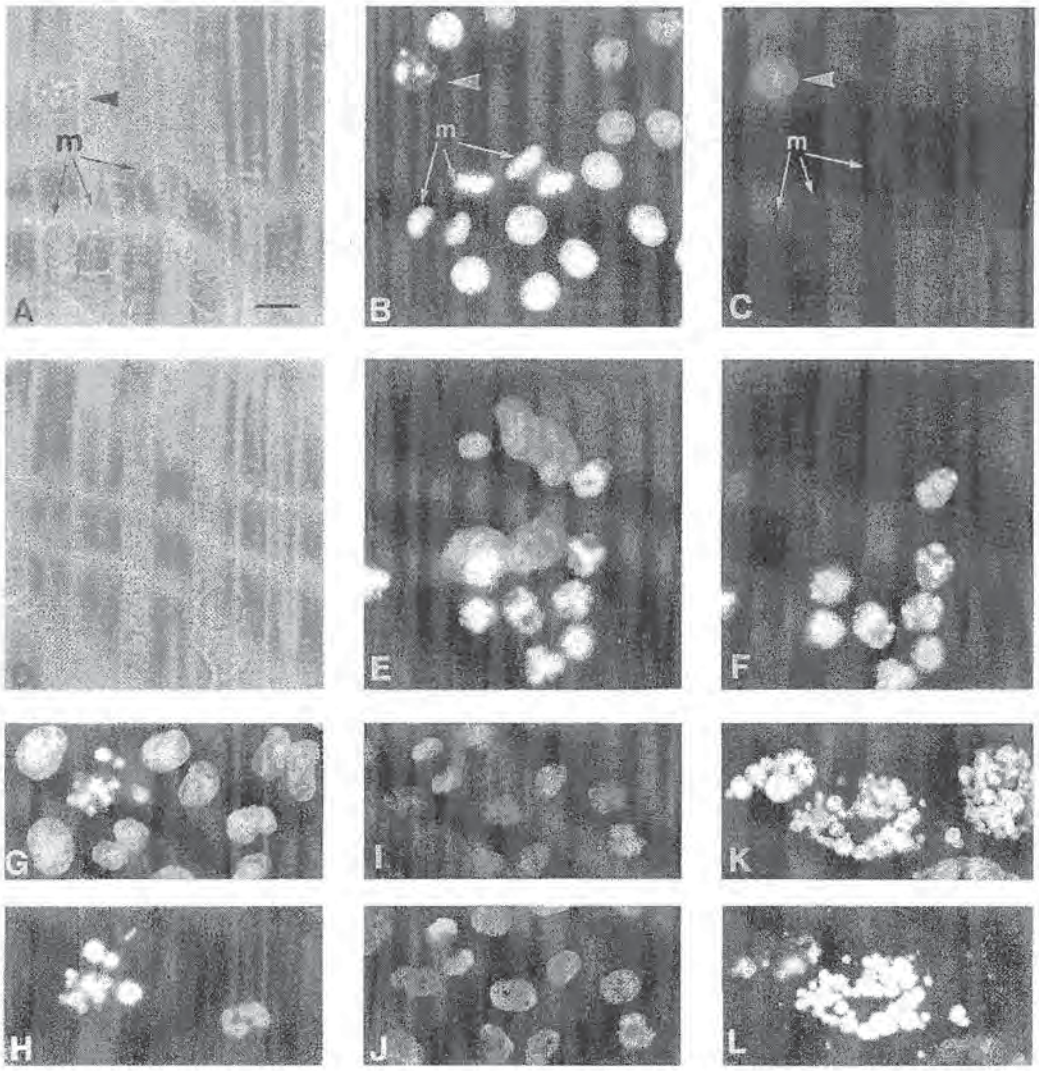


FIG. 5. TUNEL staining for in situ identification of apoptotic cells reveals that DNA nicking is detected first in taxol-treated cells blocked in prophase.

HeLa cells grown on coverslips in normal medium (A–C, I and J) or treated with 100 nM taxol for 8 hr (D–F) or 100 nM etoposide for 3 days (G and H), as well as multimininucleated G_1-arrested Hs578T cells remaining attached after 4 days following 25 nM taxol treatment (K and L) were processed for TUNEL labeling of nicked DNA (C, F, H, J, and L) and counterstained with Hoechst 33342 (B, E, G, I, and K). Cells in Panels I and J were treated with micrococcal nuclease for 20 min after fixation and prior to TUNEL labeling. Bar in Panel A represents 10 μm; magnification is the same for all panels.

lective shake-off of mitotic cells. Although the yield is low using this method, it was imperative not to use any artificially induced block that in itself could trigger or prime the cells for apoptosis. The cells were then plated onto coverslips and incubated for 10 hr before being treated with 100 nM taxol. This timing was chosen to permit the cells obtained by selective shake-off maximal time for normal culture before entry into M, yet compensate for the lag effect (4 hr, see above) for taxol to effect M block. After a further 3 hr, coverslips were collected at 30-min intervals. Control cells entered mitosis around 16 hr after the shake-off. Using this approach more than

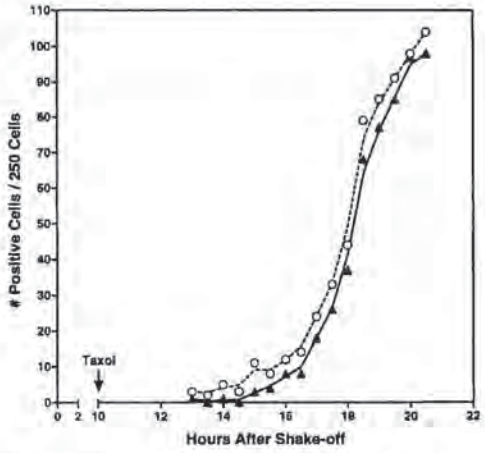


FIG. 6. Time course for onset of block in prophase (o) and appearance of TUNEL positive cells (▲) in cell-cycle synchronized cell cultures.

TUNEL staining occurred exclusively in cells blocked in prophase. Cells were plated onto coverslips immediately after shake-off (60% cells in mitosis by Hoechst staining). After 12 hr, 200 nM taxol was added and coverslips collected at the indicated time points, fixed, processed for TUNEL, and counterstained with DAPI. Two sets of 250 mitotic cells were scored concurrently for arrest in prophase and TUNEL staining.

50% of the cells entered M as a cohort over a 2–5 hr period enabling the lag between arrest in prophase and appearance of TUNEL-positive cells to be accurately measured. As shown in Fig. 6, the lag between block in M and appearance of TUNEL-positive cells was remarkably short. The delay between the two curves is only ~30 mins.

p53 Plays a Role in G_1 Arrest Following Aberrant Mitosis but Does Not Affect Taxol-Induced Cell Death Following Arrest in M

p53 plays a major role in the transducing sequence leading to apoptosis following DNA damage (39–42,50,52,62). To determine whether p53 is involved in taxol-mediated apoptosis, we compared the effects of taxol on arrest in early M, aberrant mitosis (scored by multimininucleation) and loss of viable cells in wt p53 MEFs to those in p53-null MEFs derived from p53−/− transgenic mice (39,53). Not surprisingly, the status of p53 had no effect on taxol's primary biological mode of action, namely perturbation of mitotic MTs. Hence, block in prophase (solid

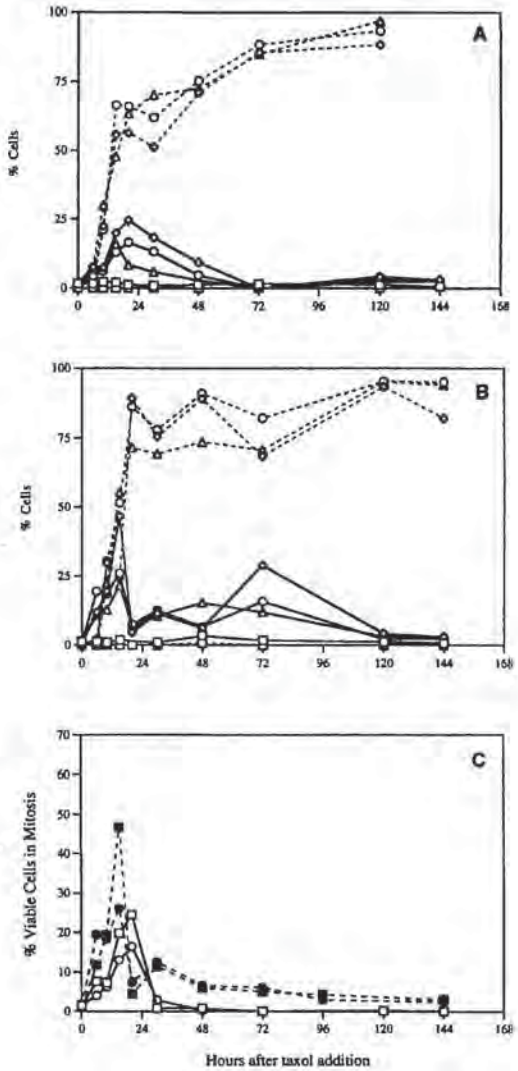


FIG. 7. Comparison of wt p53-expressing MEFs with p53-null MEFs response to taxol.

(A) wt p53 and (B) p53-null MEFs treated with 1 μM (◇), 250 nM (○), or 100 nM (△) taxol and scored at the indicated times for percentage of cells in prophase (solid lines) and multimininucleation (dotted lines). (C) a comparison of viable cells in M (adherent cells) wt p53 (solid lines) or p53-null MEFs (dotted lines). Taxol concentrations 1 μM (□) and 250 nM (○) are shown.

lines) and the appearance of multimininucleated cells (dashed lines) were similar in both cell lines (Fig. 7 A and B). However, it was readily apparent that, while normal wt p53-expressing MEFs became blocked in the cell cycle as multimini-

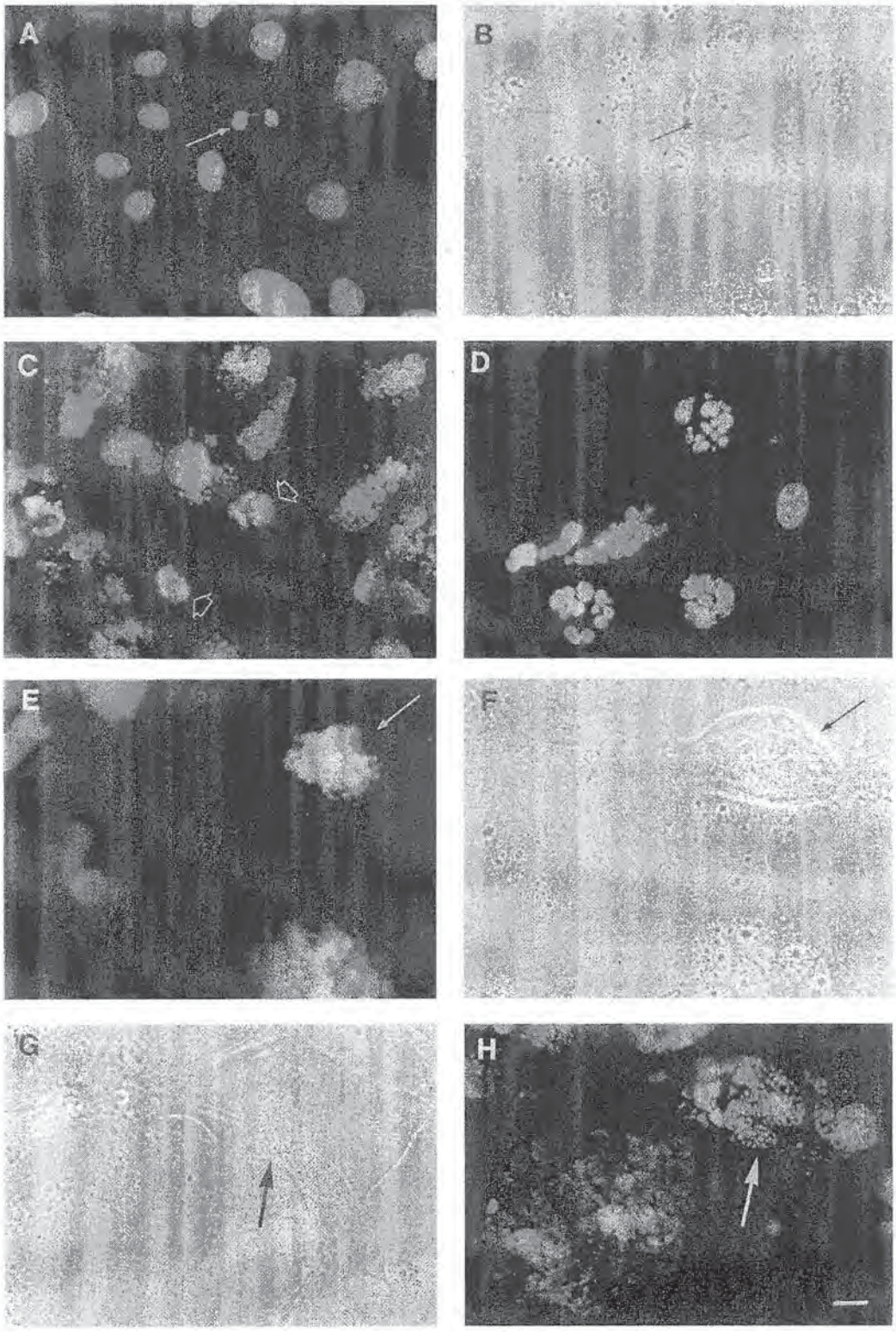
nucleated cells immediately following passage through aberrant mitosis (i.e., in G_1), multimininucleated p53−/− MEFs continued through several rounds of mitosis in the absence of cytokinesis. Figure 7C represents a comparison of viable cells in mitosis, assayed as number of mitotic cells attached to the coverslip (since those M-blocked cells destined to die detach), following addition of taxol to the cultures. This graph illustrates how wt p53 MEFs (solid lines) cease mitosis after the first wave of mitosis following taxol addition to the cultures, as evidenced by a mitotic index of 0 within 48 hr. In particular, all mitotic cells observed in M between 24 and 48 hr were cells with normal chromatin content: multimininucleated wt p53 cells were never seen to enter mitosis. In contrast, p53-null MEFs (dashed lines) continue to cycle through M throughout the subsequent 6-day period. Furthermore, multimininucleated p53-null MEFs were observed in mitosis throughout the 6-day period. The micrographs in Fig. 8 visualize these differences. The reference size of untreated, control cells in mitosis (indicated by the arrow) and interphase cells is shown in Panels A (Hoechst-stained nuclei) and B (corresponding phase). Those wt p53-expressing MEFs cells in prophase during the first round of mitosis following addition of taxol at 18 hr (indicated by arrowheads in Fig. 8C) compare in size with untreated mitotic cells (indicated by arrow in Fig. 8A above). p53-null MEFs in prophase between 8 and 18 hr and those multimininucleated cells at 18 hr were of identical size to those shown in Panels C and D (data not shown in this figure, but see Fig. 11).

Multimininucleated wt p53 MEFs were never seen in mitosis throughout the detailed observations made during the subsequent 1- to 6-day period. Furthermore, those multimininucleated cells remaining attached at 6 days (Fig. 8D) were a similar size with similar chromatin content to multimininucleated cells fixed after 18 hr of taxol treatment (Fig. 8C) indicating arrest in G_1. In contrast, multimininucleated p53−/− MEFs continued to enter mitosis but as a result of continued aberrant mitosis and lack of cytokinesis, they become progressively larger in size with a progressively greater complement of mini-nuclei (i.e., greater polyploidy; Fig. 8 E–H). Compare the size of the mitotic cell after 6 days taxol treatment in Panels E and F with the size of cells in mitosis in Panels A, B (control), and C (wt p53 after 18 hr). The cell indicated by the arrow in Panels G and H is just at the onset of chromosome condensation and so clearly reveals the condensation of all the individual mininuclei within the same cell, confirming the entry of multimininucleated cells into M 6 days after taxol treatment. In addition compare the considerably larger size of the multimininucleated cell to the lower left of the cell entering M indicated in Panels G and H, seen after 6 days, to the size of the multimininucleated wt p53 cells seen after both 18 hr and 6 days shown in Panels C and D, respectively. FACS analysis was carried out with propidium iodide stained cells that had been fixed after 0 hr, 24 hr, and 72 hr of taxol treatment. This confirmed that only the p53-null MEF population proceeded past the 4N DNA compartment after 24 hr and 3 days of taxol treatment. By 3 days, 70% of the p53-null MEFs were seen to be in the 8N and greater DNA compartment compared with 10% of untreated cells. In contrast, wt p53-expressing MEFs showed no such trends (data not shown; similar to trends shown by Cross et al. in Ref. 75 for MT destabilizing drugs).

Hoechst-staining revealed that multimininucleated MEFs expressing wt p53 developed all the morphological characteristics of apoptosis between 4 and 5 days following G_1 arrest: initially, the DNA began to develop more intense punctate staining under the nuclear membrane and subsequently the DNA became highly globular. Little evidence of such changes was observed in p53-null MEFs. TUNEL staining of the two lines

FIG. 8. P53-null MEFs fail to arrest in the G_1 following the first aberrant mitosis following exposure to taxol.

wt p53-expressing (A–D) and p53-null (E–H) were treated with 250 nM taxol for 0 (A and B), 18 hr (C), or 6 days (D–H; note that cells were refed taxol-containing medium after 3 days to maintain biologically active levels of taxol) and fixed and stained with Hoechst 33342 (A, C, D, E, and H). The open arrowheads in Panel C indicate the size of prophase nuclei and compare this with the size of the mitotic nucleus indicated in the field of the control cells shown in Panel A (corresponding phase Panel B). This size contrasts markedly with the size of the Hoechst stained p53-null prophase nucleus seen after 6 days of taxol treatment indicated by the arrow in Panel E (corresponding phase Panel F to compare with Panel B). The arrow in Panels G and H indicates a cell just beginning chromatin condensation. Bar in Panel H represents 10 μm; magnification is the same for all panels.



confirmed this difference dramatically. As illustrated in Fig. 9, the multimininuclei in wt p53-expressing MEFs all stain very brightly by TUNEL labeling 3–6 days following taxol-induced G_1 arrest (Fig. 9 A–C represents cells after 6 days taxol treatment). In contrast, the multimininuclei in p53-null MEFs remain negative after 6 days (Fig. 9 D–F).

The above results might predict that taxol would only have a cytostatic effect on MEFs lacking p53 protein. However, both wt p53 and p53−/− MEFs showed a similar decline in viable cell numbers at higher taxol concentrations (above 100 nM; Fig. 10). Although the majority of taxol-treated cells for both MEF lines succeeded in passaging through an aberrant mitosis, a subset did appear to be completely arrested in prophase at taxol concentrations ≥100 nM. These detached but rapidly disappeared. Since the numbers of detached, prophase-blocked cells exactly equaled the subsequent (within 2 hr) drop in cell numbers we concluded they must have been cleared by phagocytotic activity of the adherent interphase fibroblasts. Furthermore, Hoechst staining indicated some vesicular Hoechst staining consistent with an endosomal pattern within the interphase cells at the time points coinciding with the loss of cells. (We observed similar results in Rat1 fibroblasts; data not shown.) By comparing the EC_{50} values for block in M, overall aberrant mitosis and net loss of viable cells with respect to starting numbers, it was immediately apparent that the EC_{50} for taxol-induced cytotoxicity for both wt p53 and p53−/− MEFs correlate with arrest in early M rather than overall perturbation of mitosis (Table 2). Since the appearance of prophase blocked cells is only transient and compounded by the phagocytotic activity of these fibroblasts this correlation is easily missed if detailed scoring is not carried out throughout the critical period of 8–18 hr following addition of taxol.

As mentioned above, those MEFs blocked directly in prophase detached and became nonadherent. Although detachment and Hoechst staining were consistent with these cells being destined for apoptosis, they were too transitory to obtain clearcut morphological evidence of apoptosis. Therefore, we sought to obtain convincing evidence that taxol-induced, early-prophase blocked cells in both MEF lines had initiated apoptosis using TUNEL staining. Cells were fixed after 8–10 hr of exposure to 1 μM taxol, while the cells were still adherent (peak detachment of M-blocked cells usually occurred between 10 and 15 hr) for ease of processing for TUNEL. As shown in Fig. 11, prophase-blocked cells are seen to be TUNEL positive in both wt p53 (Fig. 11, C and D) and p53-null (Fig. 11 E and F) lines and compare with the negative staining of the untreated mitotic cells (Fig. 11, A and B). Also note the multimininucleated cell at the bottom of Panels C and D which is TUNEL negative at this time point.

DISCUSSION

The data presented above clearly indicates that taxol-induced cytotoxicity correlates with perturbation of mitosis and cell cycle arrest for both the carcinoma lines and the normal HS578Bst line we studied. Our data supports the proposals first made by Kung et al. (Ref. 20, see also Refs. 19 and 23) and Jordan et al. (18) that taxol-induced cytotoxicity correlates with selective perturbation of mitosis rather than gross changes in interphase arrays as proposed by Rowinsky et al. (8) based on their studies of taxol effects on leukemic lines. We detected two forms of taxol-induced cell cycle arrest: arrest in prophase at the G_2/M transition and, for those cells that succeeded in passing through this block and underwent aberrant mitosis; arrest in the subsequent G_1 stage. For all the human cell lines that we examined, the major fraction of the population entering M during the time period taxol remained effective became arrested in prophase, with only a minor fraction becoming arrested in G_1 as multimininucleated cells. As noted previously by others, we found rodent cells to be more tolerant of mitotic arrest per se than human lines (5,6,18,23). Rather the majority of the population proceeded through a dysfunctional mitosis to become arrested in G_1 as multimininucleated cells (the restitution nuclei described by DeBrabander et al. in their studies on rodent PtK2 cells [5,6]). However, this species difference is a relative difference rather than an absolute one since we demonstrate here that rodent cells also displayed taxol-induced arrest in early M but at higher taxol concentrations than the EC_{50} for aberrant M and subsequent G_1 arrest. As we have shown, this can be readily missed due to the transitory nature of the detached prophase-blocked cells due to phagocytosis by the adherent interphase cells. Both forms of cell cycle arrest led to cell death which by all morphological and DNA gel criteria occurred by apoptosis irrespective of the species cell type studied.

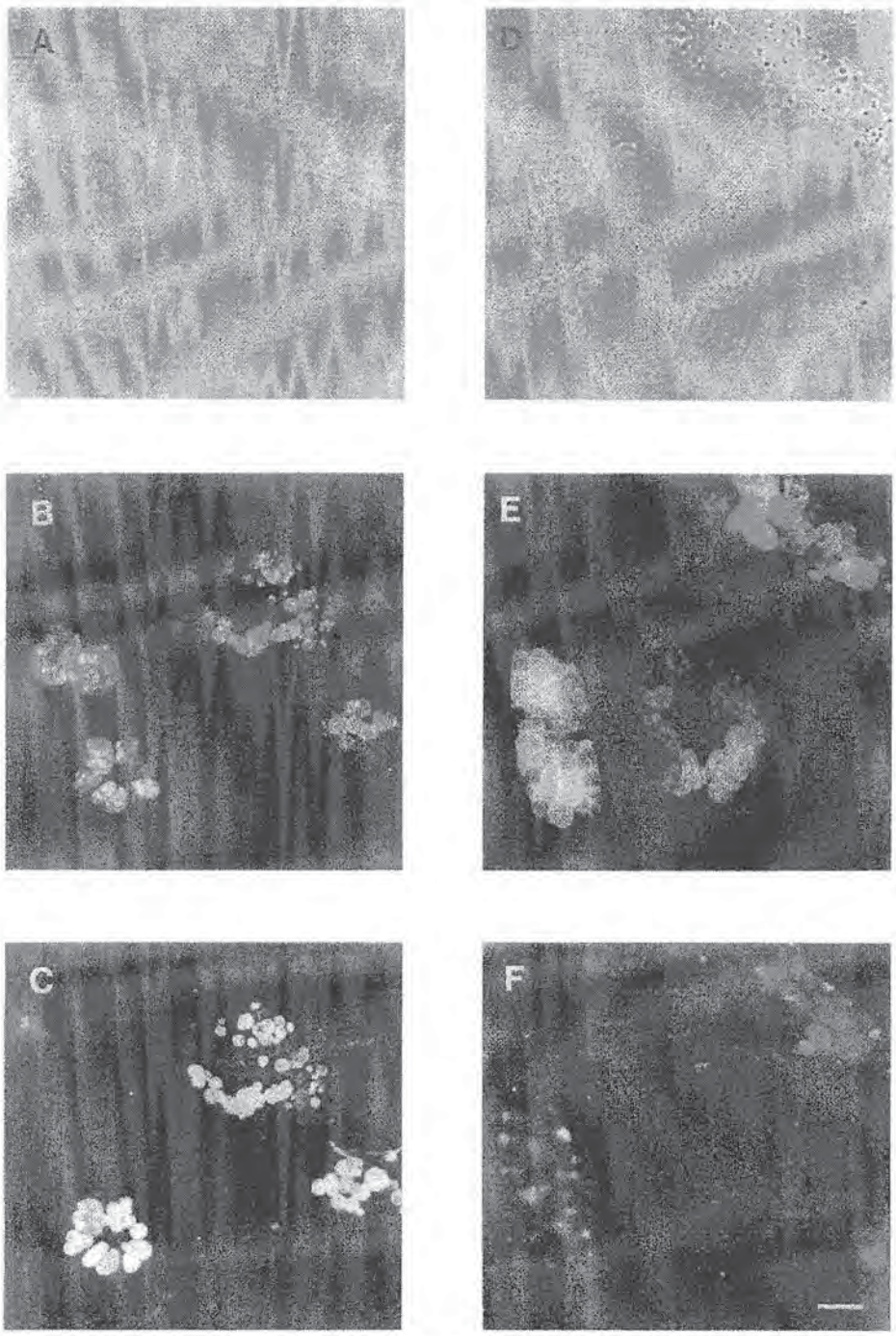


FIG. 9. Multimininucleated p53-null MEFs fail to become TUNEL positive

wt p53-expressing (A–C) or p53-null (D–F) MEFs were treated for 6 days with 250 nM taxol, then processed for TUNEL (C and F) and counterstained with DAPI (B and E). Phase images of the same fields are shown in Panels A and D. Bar in Panel F represents 10 μm; magnification is the same for all panels.

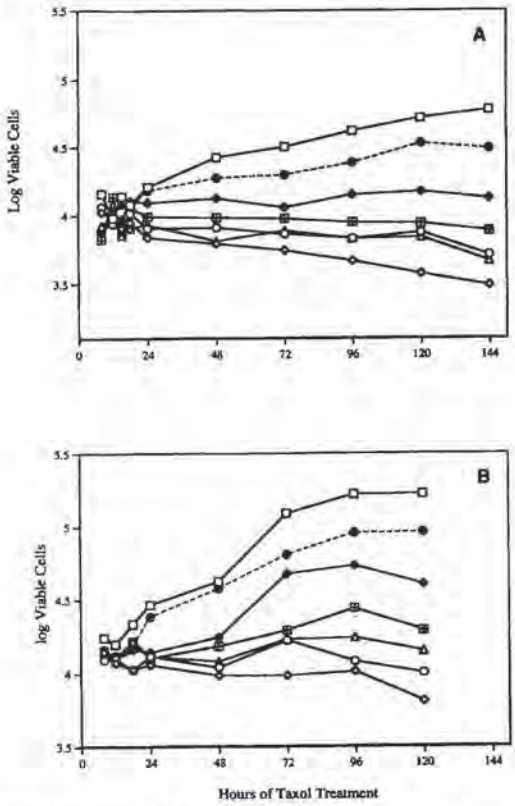


FIG. 10. At μM taxol concentrations, both p53-expressing and -null MEFs exhibit a similar taxol-induced decline in cell number

Normal p53-expressing (A) and p53-null (B) MEFs grown in 48-well plates were treated with 1 μM (◇), 250 nM (○), 150 nM (△) 100 nM (⊞), 50 nM (◆), 25 nM (-●-), or 0 (□) taxol, and both adherent and detached cells in triplicate wells were counted at the indicated time intervals. Data is plotted as log cell number.

Our morphological and kinetic studies indicated that cell death occurred more rapidly in those cells arrested in M. Multimininucleated cells died with much slower kinetics over 3–7 days following taxol treatment. Those interphase cells that did not passage M during the time period taxol remained effective (within 48 hr), appeared to survive and subsequently proliferate normally. We employed the TUNEL method for in situ DNA end labeling to accurately determine the time course for induction of apoptosis following both types of cell cycle arrest, since classical DNA ladders were only detectable after 24 hr. TUNEL labeling revealed not only that the onset of DNA nicking occured first specifically in those cells arrested in prophase but also that this occurred surprisingly rapidly; within 30 min following taxol-induced arrest in prophase. In contrast, those cells that progressed through aberrant mitosis and were arrested in the subsequent G_1 stage, became TUNEL positive only 2–4 days following G_1 arrest. This was similar for all the human cell types examined, as well as Rat1 (data not shown) and wt p53-expressing MEFs.

The majority of commonly used cancer therapeutic agents, including γ-irradiation, cause DNA damage and have been shown to exert their cytotoxic effects by triggering apoptosis (35–43). DNA nicking has been shown to directly elevate p53 protein expression which in turn induces arrest in the subsequent G_1 or G_2 stage of the cell cycle and then activates apoptosis by an as yet uncharacterized pathway (43,44). Elevated p53 levels directly up-regulate expression of the broad specificity cyclin-cdk kinase inhibitor, WAF1/Cip1/p21 (45–48,54,74) which appears to mediate the p53-dependent inhibition of cyclin-dependent kinase activities in G_1 following

TABLE 2. Comparison of EC_{50} values for taxol-induced mitotic effects and cytotoxicity between ±p53 MEF phenotypes

Cell Type	Mitotic Arrest[a] (nM)	Aberrant Mitosis[a] (nM)	Cytotoxicity[b] (nM)
wt p53 MEF	135.6 ± 9.6	46.2 ± 5.3	95.4 ± 32
p53 −/− MEF	120.0 ± 6.1	47.2 ± 0.1	110.7 ± 17.4

[a]Scoring after 24 hr.

[b]Determinations based on absolute decline in total cell number after 6 days with respect to starting numbers at time of taxol addition.

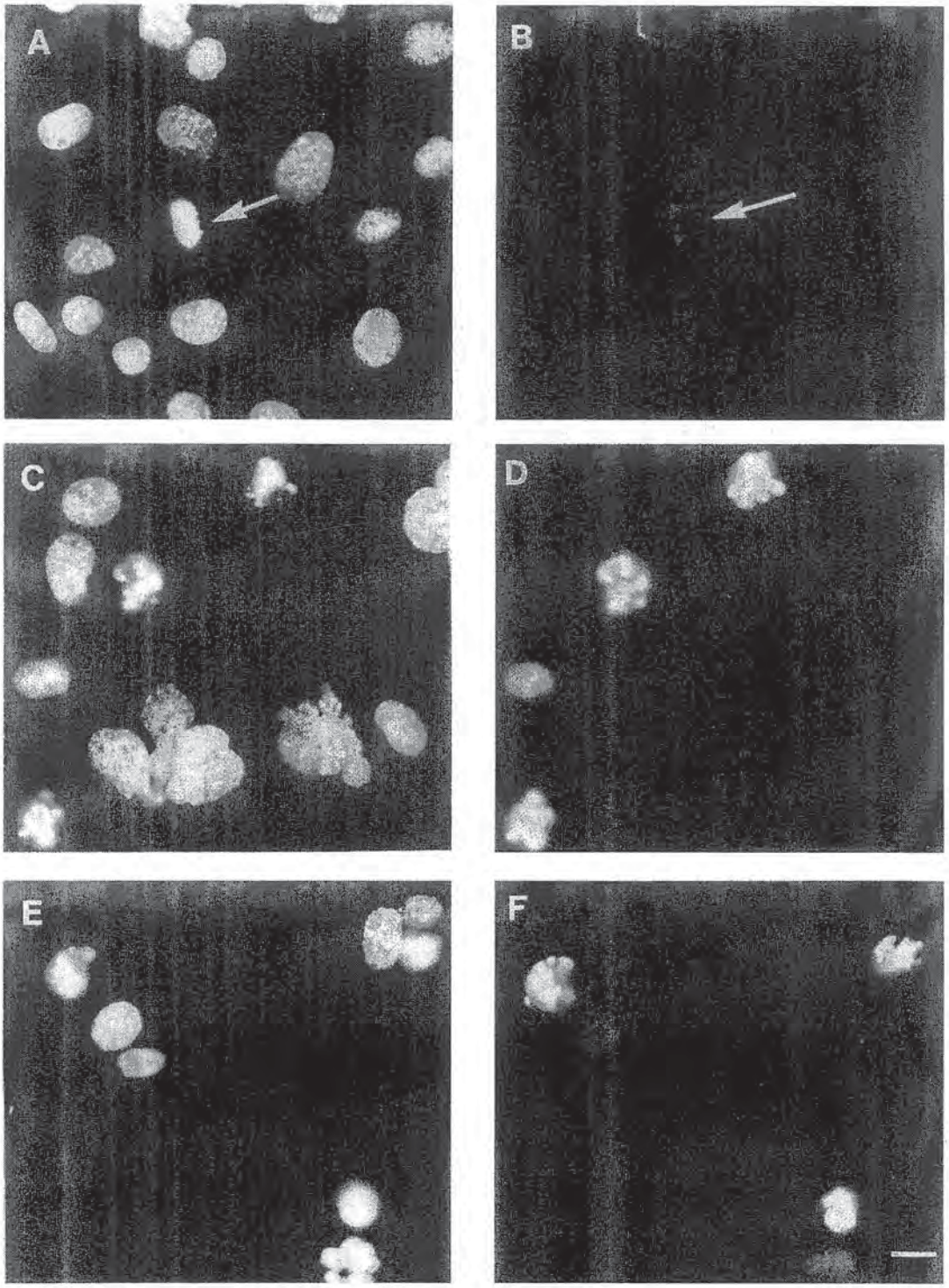


FIG. 11. Taxol-induced prophase-blocked wt p53 and p53-null MEFs both exhibit signs of apoptosis
wt-expressing MEFs (A, B, C, and D) and p53-null MEFs (E and F) were treated with 0 (A and B) or 1 μM taxol (C–F) for 8 hr prior to fixation and processing for TUNEL (B, D, and F) and counterstained with DAPI (A, C, and E). Arrow in Panels A and B indicates a control cell in metaphase. Bar in Panel F represents 10 μm; magnification is the same for all panels.

γ-irradiation (45,47,54,62). The G_2 block induced by many DNA-damaging antineoplastic agents entails a similar p53-mediated up-regulation of WAF1/Cip1/p21 (41,54).

Since taxol exhibits a completely different mechanism of action to the other antineoplastic agents, binding specifically to and thereby stabilizing intracellular MTs (1–4), which in turn induces predominantly arrest in M (1,3,26), we examined the role of p53 in taxol-induced apoptosis. For this we chose to compare MEFs expressing wt p53 with MEFs with a null p53 phenotype for an unambiguous answer. As expected, p53 status did not affect the primary consequences of taxol treatment. Taxol induced the same aberrant spindle formation in both wt p53 and p53-null MEFs. This led to a subset (~1/3) of cells exhibiting true arrest in prophase at relatively high taxol concentrations (EC_{50} ~ 130 nM) and the remainder proceeding through aberrant mitosis to become multimininucleated cells (EC_{50} ~ 46 nM). However, whereas the wt p53-expressing MEFs became arrested in this first G_1, the p53-null MEFs failed to arrest. They continued to progress through several rounds of the cell cycle in the absence of cytokinesis, becoming progressively larger with a progressively larger complement of mininuclei. Detailed morphological studies by Hoechst-chromatin staining of treated cell populations strongly suggested the wt p53-expressing multimininucleated cells became immediately arrested in G_1 since the chromatin content remained identical from the time these aberrant cells first appeared (i.e., bona fide G_1) to all subsequent time points. This was confirmed by FACS analysis. Studies on similar p53+/+ and p53−/− cell lines by Cross et al. (75), but using the MT-destabilizing agents nocodazole and colcemid, gave FACS DNA content data that was identical to the data we obtained with taxol-treated MEFs. Both by morphological criteria and by TUNEL labeling it was evident that multimininucleated wt p53-expressing MEFs developed all the hallmarks of apoptotic cell death. In contrast, the multimininucleated p53-null MEFs failed to exhibit signs of apoptotic death throughout the following 7-day period. Despite this, higher taxol concentrations induced a similar decline in total viable cell number in both lines rather than exerting solely a cytostatic effect in the p53 null line as might be predicted from the above observations. Detailed morphological characterization throughout the time course of the first cell cycle (~15–18 hr) revealed that in fact this decline in viable cells correlated with the EC_{50} for arrest in prophase which was similar for both MEF lines. TUNEL staining of the two MEF lines confirmed that a subset of cells seen in prophase after 10 hr of taxol treatment did initiate DNA nicking, indicative of the induction of apoptosis.

Taken together, our results indicate that the antineoplastic mechanism of action of taxol evokes two distinct apoptotic pathways. The first is triggered by arrest in prophase at the G_2M transition which leads to the rapid activation (within 30 min) of a p53-independent apoptotic pathway. The second, manifested by those cells that succeed in progressing through an aberrant mitosis, is a p53-dependent arrest in G_1 which leads to a slower p53-dependent apoptotic pathway. We observed G_1 arrest at taxol concentrations around the EC_{50} values for mitotic effects in all the human lines, albeit for a very small percent for the faster growing HeLa and Hs578T cells. Yet, Hs578T cells have been shown to harbor mutant p53 carrying a point mutation (67). Since mutant forms of p53 have been shown to have potential "gain of function" activity (63–64), it is clear that p53 protein carrying a point mutation is not synonymous with a null phenotype. Recently, it has been shown that antineoplastic agents may still be effective at inducing up-regulated expression of WAF1/Cip1/p21, the downstream effector of p53-mediated G_1 or G_2 arrest, in cell lines carrying point mutations in p53 (77).

The study by Cross et al. (75), which appeared during the final preparation of this manuscript, investigated the effects of the MT-destabilizing drugs, nocodazole and colcemid, which also perturb mitosis, on p53+/+ and p53-null MEFs. Although the data convincingly showed that wt p53 MEFs became arrested with 4N DNA content, but p53-null MEFs failed to arrest and accumulated progressively increasing amounts of DNA, the authors concluded that this data indicated that p53 was essential for a spindle checkpoint in M. However, the 4N DNA compartment was not subdivided into normal G_2, cells in M and multimininucleated cells. Therefore, the data could indicate equally well that only the arrest in G_1 following aberrant mitosis is compromised by the absence of p53. The detailed microscopic analyses we present here, clearly show that for taxol concentrations ≥100 nM, nearly all cells remaining after 24 hr are multimininucleated cells, so represent cells that have passaged aberrant mitosis to become aberrant 4N DNA-containing cells in G_1. Since MT-destabilizing drugs effect a similar M and G_1 block to taxol

(18–20,22), it is clear the accumulation of cells in the 4N DNA compartment observed by Cross et al. (75) actually represent multimininucleated cells after 24 hr. By accounting for the fate of all the cells, which revealed the loss of M-blocked cells by phagocytosis, and titrating a taxol concentration that can effect a block in M at prophase in these murine cells, the data presented here in this report indicates that in fact the mitotic checkpoint is independent of p53 status. Our preliminary observations indicate that cyclin B levels are higher in taxol-blocked prophase cells than in normal cells in prophase through metaphase (data not shown). Further experimentation is required to determine whether this is indicative of heightened $p34^{cdc2}$/cyclin B kinase activity. However, these observations are interesting in light of the following observations: (1) prolonged $p34^{cdc2}$/cyclin B kinase activity might underly colcemid cytotoxicity (45,46); (2) an increase in $p34^{cdc2}$/cyclin B kinase activity is observed just prior to cell death after cisplatin-induced G_2 block (i.e., downstream from the initial p53-WAF1/Cip1/p21 response effecting the G_2 arrest [39,41]); (3) initiation of apoptosis involves $p34^{cdc2}$ kinase activation (78) and up-regulation of cyclin A (79,80); and (4) deletion of the wee^1 kinase responsible for maintaining the $p34^{cdc2}$ kinase in a phosphorylated inactive form (81) induces a "mitotic catastrophe" state that bears all the morphological features of taxol-blocked apoptotic cells in prophase (82). It remains to be shown whether, indeed, arrest in M directly activates a $p34^{cdc2}$ kinase apoptotic response, in comparison with the p53-WAF1/Cip1/p21-dependent response that effects G_1 block by inhibition of cyclin/cdk kinase activities well in advance of the active process of apoptosis.

Both p53-dependent and -independent pathways of apoptosis have been shown exist. While the former clearly operate in response to DNA damage resulting from γ-irradiation and DNA damaging agents (35–43), apoptotic pathways operating in normal development resulting from growth factor deprivation function through p53-independent pathways (52,54,68). Our data suggest that direct block in prophase also bypasses a p53-WAF1/Cip1/p21-dependent pathway. This could provide some explanation as to why taxol, uniquely among all the chemotherapeutic agents utilized to date, has shown efficacy against advanced stage, drug refractory leukemia, ovarian, and metastatic breast cancers (9–12). A high proportion of many types of advanced solid tumor types express mutant p53 (55–59,63,64). Thus, the commonly used cancer therapeutic agents that induce DNA damage and thereby directly activate the p53-dependent apoptotic pathway would be predicted to be ineffective against late-stage tumors consisting of clonally expanded, p53 mutant-expressing cells. By inducing a rapidly activating, M-block-dependent apoptotic pathway that is p53-independent (and presumably WAF1/Cip1/p21-independent), taxol may therefore bypass at least this one common pathway of aquired resistance to antineoplastic agents. The MT destabilizing drugs (e.g., vinblastine, vincristine, colchicine, podophyllotoxin) also appear to induce cell cycle arrest specifically in M, but to date their use in the clinic has been restricted by a poor therapeutic index. Although taxol appears to have an improved therapeutic index and to be efficacious for a broader spectrum of cancer types, its use is still limited by its poor solubility and side effect profile. Therefore, the discovery of additional structural classes of antineoplastic agents that exhibit a mechanism of action similar to that of taxol and specifically induce arrest in M-phase (73), but have the potential for an improved side effect and solubility profile, might prove highly useful in the fight against cancer.

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EXHIBIT C

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p185^{HER2} Monoclonal Antibody Has Antiproliferative Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor

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The *HER2*/c-*erbB*-2 gene encodes the epidermal growth factor receptorlike human homolog of the rat *neu* oncogene. Amplification of this gene in primary breast carcinomas has been shown to correlate with poor clinical prognosis for certain cancer patients. We show here that a monoclonal antibody directed against the extracellular domain of p185^{HER2} specifically inhibits the growth of breast tumor-derived cell lines overexpressing the *HER2*/c-*erbB*-2 gene product and prevents *HER2*/c-*erbB*-2-transformed NIH 3T3 cells from forming colonies in soft agar. Furthermore, resistance to the cytotoxic effect of tumor necrosis factor alpha, which has been shown to be a consequence of *HER2*/c-*erbB*-2 overexpression, is significantly reduced in the presence of this antibody.

HER2/c-*erbB*-2, the human homolog of the rat protooncogene *neu* (4, 34), encodes a 1,255-amino-acid glycoprotein with extensive homology to the human epidermal growth factor (EGF) receptor (4, 21, 33, 34, 42). The *HER2*/c-*erbB*-2 gene product, p185^{HER2}, has all of the structural features and many of the functional properties of subclass I growth factor receptors (reviewed in references 43 and 44), including cell surface location and an intrinsic tyrosine kinase activity. However, the ligand for this putative growth factor receptor has not yet been identified.

Amplification of the *HER2*/c-*erbB*-2 gene has been found in human salivary gland and gastric tumor-derived cell lines (13, 34), as well as in mammary gland carcinomas (21, 22, 40, 42). Slamon et al. (35) surveyed 189 primary breast adenocarcinomas and determined that the *HER2*/c-*erbB*-2 gene was amplified in about 30% of the cases. Most importantly, *HER2*/c-*erbB*-2 amplification was correlated with a negative prognosis and high probability of relapse. Similar although less frequent amplification of the *HER2*/c-*erbB*-2 gene has been reported for gastric and colon adenocarcinomas (45, 46). Experiments with NIH 3T3 cells also suggest a direct role for the overexpressed, structurally unaltered *HER2*/c-*erbB*-2 gene product p185^{HER2} in neoplastic transformation. High levels of *HER2*/c-*erbB*-2 gene expression attained by coamplification of the introduced gene with dihydrofolate reductase by methotrexate selection (18) or by using a strong promoter (6) was shown to transform NIH 3T3 fibroblasts. Only cells with high levels of p185^{HER2} are transformed, i.e., have an altered morphology, are anchorage independent, and will form tumors in athymic mice.

Overexpression of p185^{HER2} may, furthermore, contribute to malignant tumor development by allowing tumor cells to evade one component of the antitumor defenses of the body, the activated macrophage (17). Macrophages play an important role in immune surveillance against neoplastic growth in vivo (1, 2, 38), and Urban et al. (39) have shown that tumor cells made resistant to macrophages display enhanced tumorigenicity. Tumor necrosis factor alpha (TNF-α) has been shown to play a role in activated macrophage-mediated tumor cell killing in vitro (3, 11, 23, 29, 39). NIH 3T3 cells transformed by a transfected and amplified *HER2*/c-*erbB*-2 cDNA show increased resistance to the cytotoxic effects of activated macrophages or TNF-α in direct correlation with increased levels of p185^{HER2} expression. Furthermore, breast tumor cell lines with high levels of p185^{HER2} exhibit resistance to TNF-α. Resistance to host antitumor defenses could facilitate the escape of cells from a primary tumor to establish metastases at distant sites.

To further investigate the consequences of alteration in *HER2*/c-*erbB*-2 gene expression in mammary gland neoplasia and to facilitate investigation of the normal biological role of the *HER2*/c-*erbB*-2 gene product, we have prepared monoclonal antibodies against the extracellular domain of p185^{HER2}. One monoclonal antibody (4D5) was characterized in more detail and was shown to inhibit in vitro proliferation of human breast tumor cells overexpressing p185^{HER2} and, furthermore, to increase the sensitivity of these cells to the cytotoxic effects of TNF-α.

MATERIALS AND METHODS

Cells and cell culture. Human tumor cell lines were obtained from the American Type Culture Collection. The mouse fibroblast line NIH 3T3/HER2-3$_{400}$, expressing an amplified *HER2*/c-*erbB*-2 cDNA under simian virus 40 early promoter control, and the vector-transfected control cell line NIH 3T3/CVN have been described previously (18).

Cells were cultured in a 1:1 mixture of Dulbecco modified Eagle medium and Ham nutrient mixture F-12 supplemented with 2 mM glutamine, 100 u of penicillin per ml, 100 μg of streptomycin per ml, and 10% serum. Human tumor cell lines were cultured with fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.); NIH 3T3 derivatives were cultured with calf serum (Hyclone Laboratories, Inc., Logan, Utah.).

Immunization. Female BALB/c mice were immunized with NIH 3T3/HER2-3$_{400}$ cells expressing high levels of

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p185^{HER2}. The cells were washed once with phosphate-buffered saline (PBS) and detached from the plate with PBS containing 25 mM EDTA. After low-speed centrifugation, the cells were suspended in cold PBS (2×10^7 cells per ml). Each mouse was injected intraperitoneally with 0.5 ml of this cell suspension on weeks 0, 2, 5, and 7.

On weeks 9 and 13, 100 μl of a Triton X-100 membrane preparation of p185^{HER2}, partially purified by wheat germ agglutinin chromatography (700 μg of protein per ml) (25), was administered intraperitoneally. Three days before fusion, 100 μl of the enriched p185^{HER2} protein was administered intravenously.

Fusion and screening. Mice with high antibody titers as determined by immunoprecipitation of p185^{HER2} were sacrificed, and their splenocytes were fused as described previously (26). Spleen cells were mixed at a 4:1 ratio with the fusion partner, mouse myeloma cell line X63-Ag8.653 (20), in the presence of 50% polyethylene glycol 4000. Fused cells were plated at a density of 2×10^5 cells per well in 96-well microdilution plates. The hypoxanthine-azaserine (12) selection for hybridomas was begun 24 h later. Beginning at day 10 postfusion, supernatants from hybridoma-containing wells were tested for the presence of antibodies specific for p185^{HER2} by an enzyme-linked immunosorbent assay with the wheat germ agglutinin chromatography-purified p185^{HER2} preparation (28). Enzyme-linked immunosorbent assay-positive supernatants were confirmed by immunoprecipitation and cloned twice by limiting dilution.

Large quantities of specific monoclonal antibodies were produced by preparation of ascites fluid; antibodies were then purified on protein A-Sepharose columns (Fermentech, Inc., Edinburgh, Scotland) and stored sterile in PBS at 4°C.

Immunoprecipitations and antibodies. Cells were harvested by trypsinization, counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), and plated 24 h before being harvested for analysis of p185^{HER2} expression. Cells were lysed at 4°C with 0.8 ml of HNEG lysis buffer (18) per 100-mm plate. After 10 min, 1.6 ml of lysis dilution buffer (HNEG buffer with 1% bovine serum albumin and 0.1% Triton X-100) was added to each plate, and the extracts were clarified by centrifugation at 12,000 × *g* for 5 min.

Antibodies were added to the cell extracts and allowed to bind at 4°C for 2 to 4 h. Immune complexes were collected by adsorption to protein A-Sepharose beads for 20 min and washed three times with 1 ml of HNEG buffer–0.1% Triton X-100. Autophosphorylation reactions were carried out for 20 min at 4°C in 50 μl of HNEG wash buffer containing 5 mM $MnCl_2$ and 3 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol, Amersham Corp., Arlington Heights, Ill.). The autophosphorylation reaction conditions have been described previously (18). Proteins were separated on sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gels and analyzed by autoradiography.

The polyclonal antibody, G-H2CT17, recognizing the carboxy-terminal 17 amino acids of p185^{HER2}, has been described previously (18). The anti-EGF receptor monoclonal antibody 108 (16) was provided by Joseph Schlessinger, Rorer Biotechnology, Inc.

Fluorescence-activated cell sorting. SK-BR-3 human breast tumor cells overexpressing the *HER2*/c-*erbB*-2 gene (17, 22) or A431 human squamous carcinoma cells overexpressing the EGF receptor gene (14) were grown in T175 flasks. They were detached from the flasks by treatment with 25 mM EDTA–0.15 M NaCl, collected by low-speed centrifugation, and suspended at 1×10^6 cells per ml in PBS–1% fetal bovine serum. One milliliter of each cell line was incubated with 10 μg of either anti-*HER2*/c-*erbB*-2 monoclonal antibody (4D5) or a control antibody (40.1.H1) recognizing the hepatitis B surface antigen. The cells were washed twice and suspended on ice for 30 min in 1 ml of PBS–1% fetal bovine serum containing 10 μg of goat anti-mouse immunoglobulin G $F(ab')_2$ fragments conjugated with fluorescein isothiocyanate dye (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Unbound fluorescein dye was removed by two further washes. The cells were suspended at 2×10^6 per ml in PBS–1% fetal bovine serum and analyzed with an EPICS 753 (Coulter) fluorescence-activated cell sorter. Fluorescein was excited by 300 mW of 488-nm argon laser light, and the emitted light was collected with a 525-nm band-pass filter with a 10-nm band width.

Down-regulation assay. SK-BR-3 cells were plated at 1.5×10^5 cells per 35-mm culture dish in normal medium. After a 6-h period to allow attachment, the medium was replaced by 1.5 ml of methionine-free labeling medium containing 150 μCi of [^{35}S]methionine per ml and 2% dialyzed fetal bovine serum. The cells were metabolically labeled for 14 h and then chased with medium containing 2% dialyzed serum and unlabeled methionine. Either a control monoclonal antibody (40.1.H1) or anti-p185^{HER2} (4D5) was added to a final concentration of 2.5 μg/ml. At 0, 5, and 11 h, extracts were prepared with 0.3 ml of lysis solution and 0.6 ml of dilution buffer. The p185^{HER2} was immunoprecipitated with 2.5 μl of polyclonal antibody G-H2CT17. The washed immune complexes were dissolved in sample buffer, electrophoresed on a SDS-7.5% polyacrylamide gel, and analyzed by autoradiography. Each time point determination was performed in duplicate. Autoradiograph band intensities were quantitated by using a scanner (Ambis Systems).

Cell proliferation assays. The anti-p185^{HER2} monoclonal antibodies were characterized by using the breast tumor cell line SK-BR-3. Cells were detached by using 0.25% (vol/vol) trypsin and suspended in complete medium at a density of 4

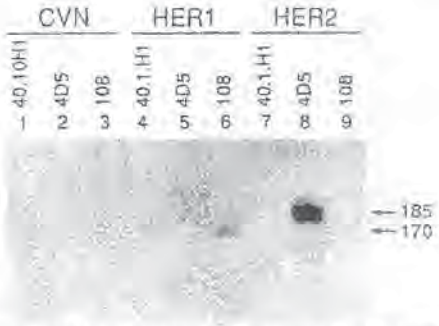

FIG. 1. Specificity of monoclonal antibody 4D5. Three cell lines, NIH 3T3/CVN, NIH 3T3/HER1-EGF receptor, and NIH 3T3/HER2-3_{400}, were plated out at 2.0×10^6 in 100-mm culture dishes. At 24 h, Triton X-100 lysates were prepared and divided into three portions. Either an irrelevant monoclonal antibody (6 μg of anti-hepatitis B virus surface antigen, 40.1.H1; lanes 1, 4, and 7), anti-p185^{HER} monoclonal antibody 4D5 (6 μg; lanes 2, 5, and 8), or anti-EGF receptor monoclonal antibody 108 (6 μg; lanes 3, 6, and 9) was added and allowed to bind at 4°C for 4 h. The immune complexes were collected with 30 μl of protein A-Sepharose. Rabbit anti-mouse immunoglobulin (7 μg) was added to each 4D5 immunoprecipitation to improve the binding of this monoclonal antibody to the protein A-coated beads. Proteins were labeled by autophosphorylation and separated on an SDS–7.5% polyacrylamide gel. The gel was exposed to film at −70°C for 4 h with an intensifying screen. The arrows show the positions of proteins of M_r 185,000 and 170,000.

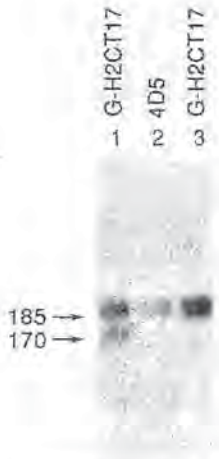

FIG. 2. Binding of monoclonal antibody 4D5 to unglycosylated receptor. NIH 3T3/HER2-3$_{400}$ cells were plated into two 100-mm plates at 2×10^6 cells per plate. After 14 h, the antibiotic tunicamycin was added to one plate at 3 μg/ml. After a further 5.5 h of incubation, Triton X-100 lysates were then prepared from each plate. Immunoprecipitations, the autophosphorylation reaction, and SDS-polyacrylamide gel electrophoresis were performed as described in the legend to Fig. 1. Lanes: 1, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with 2.5 μl of a polyclonal antibody directed against the C terminus of p185^{HER2}; 2, tunicamycin-treated cell lysate (one-third of a plate) immunoprecipitated with 6 μg of 4D5; 3, untreated control lysate (one-third of a plate) immunoprecipitated with the polyclonal antibody. The arrows show the locations of proteins of M_r 185,000 and 170,000.

$\times 10^5$ cells per ml. Aliquots of 100 μl (4×10^4 cells) were plated into 96-well microdilution plates, the cells were allowed to adhere, and 100 μl of media alone or media containing monoclonal antibody (final concentration, 5 μg/ml) was then added. After 72 h, plates were washed twice with PBS (pH 7.5), stained with crystal violet (0.5% in methanol), and analyzed for relative cell proliferation as described previously (36).

For assays in which monoclonal antibodies were combined with recombinant human TNF-α (5.0×10^7 U/mg; Genentech, Inc.), cells were plated and allowed to adhere as described above. Following cell adherence, control medium alone or medium containing monoclonal antibodies was added to a final concentration of 5 μg/ml. Cultures were incubated for another 4 h, and then increasing concentrations of TNF-α were added to a final volume of 200 μl. Following 72 h of incubation, the relative cell number was determined by crystal violet staining. Some samples were analyzed by crystal violet staining following cell adherence for determination of the initial cell number.

RESULTS

Specificity of monoclonal antibody 4D5. Monoclonal antibodies directed against the extracellular domain of p185^{HER2} were prepared by immunizing mice with NIH 3T3 cells transfected with a *HER2*/c-*erbB*-2 cDNA (HER2-3$_{400}$) (17, 18) and overexpressing the corresponding gene product, p185^{HER2}. One antibody exhibited several interesting biological properties and was chosen for further characterization. Antibody 4D5 specifically immunoprecipitated a single ^{32}P-labeled protein of M_r 185,000 from NIH 3T3 cells expressing p185^{HER2} (Fig. 1, lane 8). This antibody did not cross-react with the human EGF receptor (HER1; Fig. 1, lane 5), even when overexpressed in a mouse NIH 3T3 background (Fig. 1, lane 6). Furthermore, it did not immunoprecipitate any proteins from NIH 3T3 cells transfected with a control plasmid (pCVN) which expresses the neomycin resistance and dihydrofolate reductase genes only (Fig. 1, lane 2).

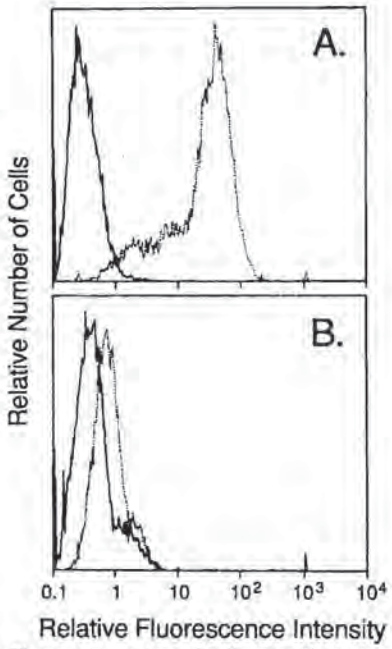

FIG. 3. Fluorescence-activated cell sorter histograms of human tumor cells binding anti-p185 monoclonal antibody 4D5. ——, Binding by the control antibody, 40.1.H1, directed against the hepatitis B surface antigen; ·····, binding by the anti-*HER2*/c-*erbB*-2 antibody, 4D5. The antibodies were first allowed to react with the cell surface. After a wash step, bound antibody was labeled by addition of fluorescein-conjugated F(ab')$_2$ fragment of goat anti-mouse immunoglobulin G. (A) Binding of the antibodies to the human breast tumor line SK-BR-3, which contains an amplification of the *HER2*/c-*erbB*-2 gene and expresses high levels of the *HER2*/c-*erbB*-2 gene product p185^{HER2}. (B) Binding of the same antibodies to the human squamous epithelial cell line A431. This cell line expresses low levels of mRNA for *HER2*/c-*erbB*-2 and high levels (2×10^6 receptors per cell) of the EGF receptor.

To determine the nature of the epitope recognized by 4D5, NIH 3T3/HER2-3$_{400}$ cells were treated with tunicamycin, which prevents addition of N-linked oligosaccharides to proteins (15, 41). Cells treated with this antibiotic for 5.5 h contained two proteins which were immunoprecipitated by a polyclonal antibody against the carboxy-terminal peptide of p185^{HER2} (Fig. 2, lane 1). The polypeptide of 170,000 M_r represents unglycosylated p185^{HER2}. The upper band of ca. 185,000 M_r comigrated with glycosylated p185^{HER2} from untreated cells (Fig. 2, lane 3). Monoclonal antibody 4D5 efficiently immunoprecipitated only the glycosylated form of p185^{HER2} (Fig. 2, lane 2). This experiment suggests either that the epitope recognized by 4D5 consists partly of carbohydrate, or, alternatively, that the antibody recognizes a conformation of the protein achieved only when it is glycosylated.

TABLE 1. Inhibition of SK-BR-3 proliferation by anti-p185^{HER2} monoclonal antibodies[a]

Monoclonal antibody	Relative cell proliferation[b]
7C2	79.3 ± 2.2
2C4	79.5 ± 4.4
7D3	83.8 ± 5.9
4D5	44.2 ± 4.4
3E8	66.2 ± 2.4
6E9	98.9 ± 3.6
7F3	62.1 ± 1.4
3H4	66.5 ± 3.9
2H11	92.9 ± 4.8
40.1.H1	105.8 ± 3.8
4F4	94.7 ± 2.8

[a] SK-BR-3 breast tumor cells were plated as described in Materials and Methods. Following adherence, medium containing 5 μg of either anti-p185^{HER2} or control monoclonal antibodies (40.1.H1 and 4F4) per ml were added.

[b] Relative cell proliferation was determined by crystal violet staining of the monolayers after 72 h. Values are expressed as a percentage of results with untreated control cultures (100%).

The binding of monoclonal antibody 4D5 to human tumor cell lines was investigated by fluorescence-activated cell sorting (Fig. 3). This antibody was bound to the surface of cells expressing p185^{HER2}. Figure 3A shows the 160-fold increase in cellular fluorescence observed when 4D5 was added to SK-BR-3 breast adenocarcinoma cells relative to a control monoclonal antibody. This cell line contains an amplified *HER2*/c-*erbB*-2 gene and expresses high levels of p185^{HER2} (17, 22). In contrast, the squamous carcinoma cell line A431, which expresses about 2×10^6 EGF receptors per cell (14) but only low levels of p185^{HER2} (4), exhibited only a twofold increase in fluorescence with 4D5 (Fig. 3B) when compared with a control monoclonal antibody.

The binding of 4D5 correlated with the levels of p185^{HER2} expressed by these two cell lines. SK-BR-3 cells, expressing high levels of p185^{HER2}, showed an 80-fold increase in relative fluorescence intensity compared with A431 cells. This experiment demonstrates that 4D5 specifically recognizes the extracellular domain of p185^{HER2}.

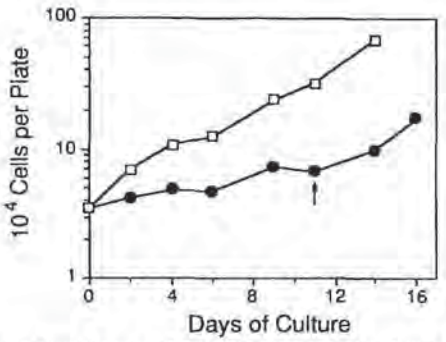

FIG. 4. Growth curve of SK-BR-3 cells treated with anti-*HER2*/c-*erbB*-2 monoclonal antibody 4D5. Cells were plated into 35-mm culture dishes at 20,000 cells per plate in medium containing 2.5 μg of either control antibody (40.1.H1, anti-hepatitis B surface antigen) (□) or anti-p185^{HER2} antibody 4D5 (●) per ml. On the indicated days, cells were trypsinized and counted in a Coulter counter. The determination for each time point and each antibody was done in duplicate, and the counts were averaged. The arrow indicates the day the cells were refed with medium without antibodies.

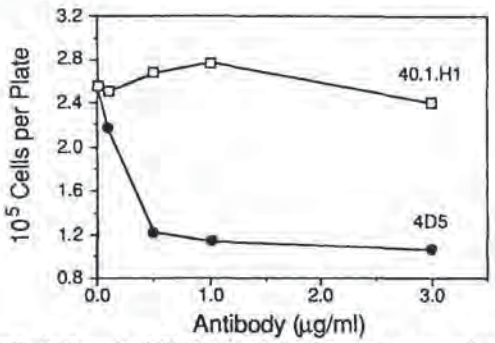

FIG. 5. Growth of SK-BR-3 cells in different concentrations of monoclonal antibody 4D5. The human breast tumor line SK-BR-3 was plated into 35-mm culture dishes at 20,000 cells per dish. Either 0.1, 0.5, 1.0, or 3.0 μg of a control monoclonal antibody (40.1.H1, anti-hepatitis B surface antigen) or monoclonal 4D5 antibody per ml was added at the time of plating. After 8 days of growth, the plates were trypsinized and the cells were counted in a Coulter counter. Each concentration of antibody was plated and counted in duplicate, and the cell numbers were averaged.

Effects on cell proliferation. We used the human mammary gland adenocarcinoma cell line, SK-BR-3, to determine whether monoclonal antibodies directed against the extracellular domain of p185^{HER2} had any effect on the proliferation of cell lines overexpressing this receptorlike protein. SK-BR-3 cells were coincubated with several *HER2*/c-*erbB*-2-specific monoclonal antibodies or with either of two different control monoclonal antibodies (40.1.H1, directed against the hepatitis B surface antigen; 4F4, directed against recombinant human gamma interferon). Most anti-*HER2*/c-*erbB*-2 monoclonal antibodies which recognize the extracellular domain inhibited the growth of SK-BR-3 cells (Table

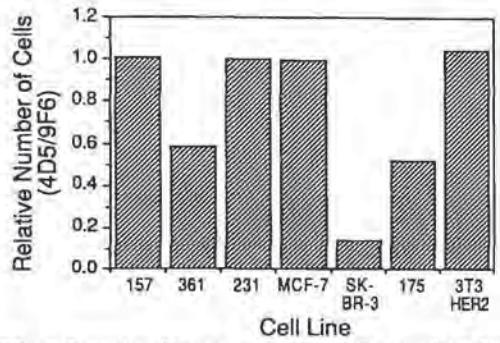

FIG. 6. Screening of breast tumor cell lines for growth inhibition by monoclonal antibody 4D5. Each cell line was plated in 35-mm culture dishes at 20,000 cells per dish. Either a control monoclonal antibody (9F6, anti-human immunodeficiency virus gp120) or the anti-p185^{HER2} monoclonal antibody 4D5 was added on day 0 to 2.5 μg/ml. Because the different cell lines grow at different rates, the cell lines NIH 3T3/HER2-3_{400} and SK-BR-3 were counted after 6 days, cell lines MDA-MB-157, MDA-MB-231, and MCF-7 were counted after 9 days, and cell lines MDA-MB-175VII and MDA-MB-361 were counted after 14 days. The difference in growth between cells treated with 4D5 and 40.1.H1 is expressed as the ratio of cell numbers with 4D5 versus a control monoclonal antibody, 9F6. Each cell line was assayed in duplicate for each antibody, and the counts were averaged.

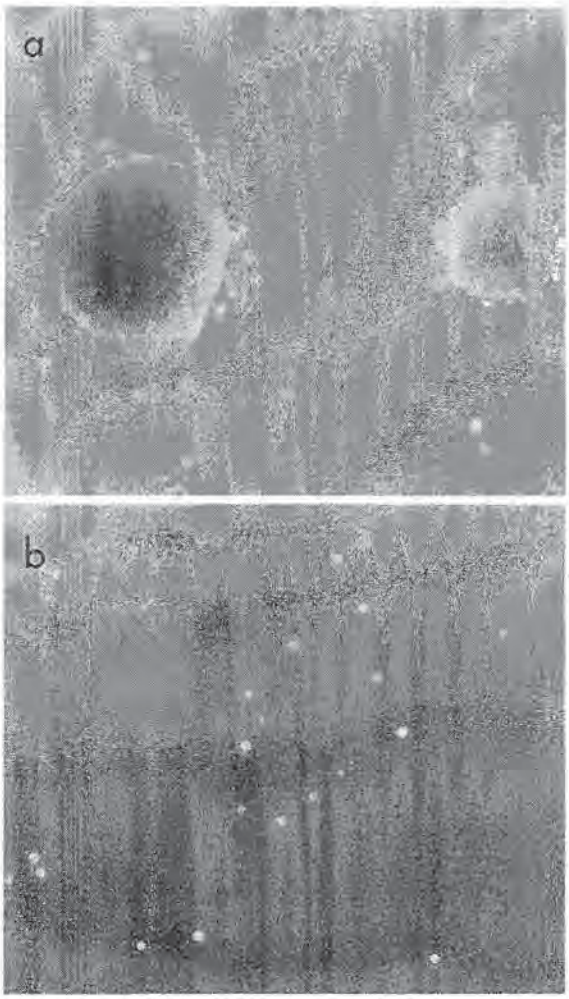

FIG. 7. Inhibition of anchorage-independent growth of NIH 3T3/HER2-3_{400} cells by 4D5. Cells (20,000 per 60-mm plate) were plated in 0.2% soft agar over a 0.4% agar base. After 3 weeks, the plates were photographed at ×100 magnification by using a Nikon microscope with phase-contrast optics. (a) HER2-3_{400} cells plated in agar containing 200 ng of a control antibody (TF-C8) per ml. (b) The same cells plated in agar containing 200 ng of 4D5 per ml.

1). Maximum inhibition was obtained with monoclonal antibody 4D5, which inhibited cellular proliferation by 56%. The control antibodies had no significant effect on cell growth.

Figure 4 compares the growth of SK-BR-3 cells in the presence of either a control antibody, 40.1.H1, or the anti-p185^{HER2} antibody. Proliferation of the cells was inhibited when antibody 4D5 was present. The generation time increased from 3.2 to 12.2 days. To determine whether 4D5 treatment was cytostatic or cytotoxic, antibody was removed by medium change 11 days after treatment. The cells resumed growth at a nearly normal rate, suggesting that the antibody affected cell growth rather than cell viability. The dose-response curve (Fig. 5) showed that a concentration of 200 ng/ml inhibited growth by 50%, whereas maximum

FIG. 8. Effect of antibody binding on p185^{HER2} turnover. SK-BR-3 cells were labeled for 14 h with [^{35}S]methionine. The label was then chased with cold methionine and either an irrelevant monoclonal antibody (40.1.H1, anti-hepatitis B surface antigen) or 4D5 was added to 2.5 μg/ml. The cells on the plates were lysed at 0, 5, and 11 h, and ^{35}S-labeled p185^{HER2} was quantitated by immunoprecipitation with the C-terminal specific polyclonal antibody. The 5- and 11-h time point determinations were performed in duplicate for each of the two antibodies. Proteins were separated by SDS-polyacrylamide gel electrophoresis. The fluor-treated gel was exposed to film for 4 h at room temperature. The arrow indicates the position of a protein of M_r 185,000. Band intensities were quantitated by using an Ambis Systems scanner. Lanes; 1, 0 h; lanes 2 and 3, 40.1.H1 (5 h); lanes 4 and 5, 4D5 (5 h); lanes 6 and 7, 40.1.H1 (11 h); lanes 8 and 9, 4D5 (11 h).

effects were achieved by using a concentration of between 0.5 and 1 μg/ml.

The effect of 4D5 on the proliferation of six additional breast tumor cell lines, as well as mouse NIH 3T3 fibroblasts transformed by p185^{HER2} overexpression (NIH 3T3/HER2-3_{400}), was tested in monolayer growth assays. Cells were plated at low density in medium containing 2.5 μg of either a control antibody or 4D5 per ml. When the cultures approached confluency, cells were removed with trypsin and counted. 4D5 did not have any significant effect on the growth of the MCF-7, MDA-MB-157, MDA-MB-231, or NIH 3T3/HER2-3_{400} cell lines (Fig. 6). It did, however, significantly affect the growth of the cell lines MDA-MB-361 (58% of control) and MDA-MB-175-VII (52% of control), which express high levels of p185^{HER2} (17).

Interestingly, monoclonal antibody 4D5 had no effect on the monolayer growth of the NIH 3T3/HER2-3_{400} cell line. However, it completely prevented colony formation by these cells in soft agar (Fig. 7), a property which had been induced by *HER2*/c-*erbB*-2 amplification (18). In the presence of 200 ng of a control monoclonal antibody (antitissue factor, TC-C8) per ml, 116 (average of two plates) soft-agar colonies were counted, while the same cells plated simultaneously into soft agar containing 200 ng of 4D5 per ml did not yield any colonies.

Monoclonal antibody 4D5 down-regulates p185^{HER2}. To determine whether the antiproliferative effect of 4D5 was due to enhanced degradation of p185^{HER2}, we measured its rate of turnover in the presence or absence of antibody. p185^{HER2} was metabolically labeled by culturing SK-BR-3 cells for 14 h in the presence of [^{35}S]methionine. Cells were then chased for various times, and either a control antibody or 4D5 was added at the beginning of the chase period. At 0, 5, and 11 h, cells were lysed and p185^{HER2} levels were assayed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. p185^{HER2} is degraded more rapidly after exposure of SK-BR-3 cells to 4D5 (Fig. 8). Densitometric evaluation of the data showed that the p185^{HER2} half-life of

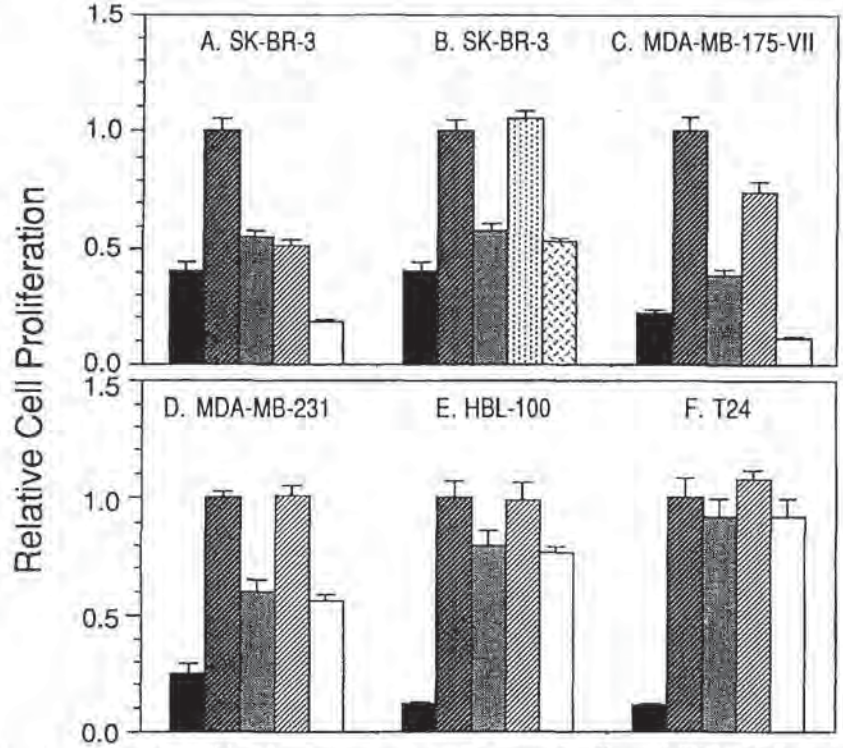

FIG. 9. Monoclonal antibody 4D5 sensitizes breast tumor cells to the cytotoxic effects of TNF-α. Cells were plated in 96-well microdilution plates (4×10^4 cells per well for SK-BR-3, MDA-MB-175-VII, and MDA-MB-231; 10^4 cells per well for HBL-100 and T24) and allowed to adhere for 2 h. Anti-*HER2*/c-*erbB*-2 monoclonal antibody 4D5 (5 μg/ml) or anti-hepatitis B surface antigen monoclonal antibody 40.1.H1 (5 μg/ml) was then added for a 4-h incubation prior to the addition of TNF-α to a final concentration of 10^4 units/ml. After 72 h, the monolayers were washed twice with PBS and stained with crystal violet dye for determination of relative cell proliferation. In addition, some cell monolayers were stained with crystal violet following adherence in order to determine the initial cell density for comparison with cell densities measured after 72 h. The symbols denote initial cell density (■), untreated (control) cells (▨), cells treated with TNF-α (■), 4D5 (▨), TNF-α plus 4D5 (□), 40.1.H1 (⁝⁝⁝); or TNF-α plus 40.1.H1 (⁘).

7 h decreased to 5 h in the presence of antibody (data not shown).

Monoclonal antibody 4D5 enhances TNF-α cytotoxicity. The addition of certain growth factors to tumor cells has been shown to increase their resistance to the cytotoxic effects of TNF-α (37). A prediction based on these findings would be that expression of oncogenes that mimic or replace growth factor receptor function may also increase the resistance of cells to this cytokine. Recently, it was shown that overexpression of the putative growth factor receptor $p185^{HER2}$ in NIH 3T3 cells caused an increase in the resistance of these cells to TNF-α (17). Furthermore, breast tumor cell lines with high levels of $p185^{HER2}$ also exhibited TNF-α resistance.

To further investigate the mechanism by which the 4D5 antibody inhibited cell growth, we investigated the response of three breast tumor cell lines to TNF-α in the presence or absence of this antibody. If the anti-$p185^{HER2}$ monoclonal antibody 4D5 inhibited proliferation of breast tumor cells by interfering with the signalling functions of $p185^{HER2}$, addition of this antibody would be expected to enhance the sensitivity of tumor cells to TNF-α. Both SK-BR-3 (Fig. 9A) and MDA-MB-175-VII (Fig. 9C) were growth inhibited by both the monoclonal antibody 4D5 (5 μg/ml; 50% and 25% inhibition, respectively) and high concentrations of TNF-α (1×10^4 units/ml; 50% and 60% inhibition, respectively). However, the combination of TNF-α and monoclonal antibody 4D5 reduced the SK-BR-3 and MDA-MB-175-VII tumor cell number to a level below that initially plated, indicating the induction of a cytotoxic response. In a separate experiment, SK-BR-3 cell viability was determined directly by using trypan blue dye exclusion, yielding identical results to those described above that were obtained by using crystal violet staining (data not shown). A control monoclonal antibody, 40.1.H1, did not inhibit SK-BR-3 breast tumor cell proliferation, nor did it induce an enhanced sensitivity of this cell line to the cytotoxic effects of TNF-α (Fig. 9B). In addition, the growth of the breast tumor cell line MDA-MB-231, which does not express detectable levels of $p185^{HER2}$ (17), was unaffected by monoclonal antibody 4D5, and the growth inhibition seen with the combination of 4D5 and TNF-α was similar to that observed with TNF-α alone (Fig. 9D). Furthermore, neither HBL-100 (30), a nontransformed but immortalized human breast epithelial cell line (Fig. 9E), nor T24 (27), a human bladder carcinoma cell line (Fig. 9F), expressed high levels of $p185^{HER2}$ (data not shown), and neither demonstrated growth inhibition by 4D5 or an enhanced growth-inhibitory or cytotoxic response to the combination of TNF-α and monoclonal antibody 4D5. These results demonstrate that only tumor cells which

overexpress p185^{HER2} will become sensitized to the cytotoxic effects of TNF-α by antibody 4D5.

DISCUSSION

We have prepared monoclonal antibodies against the extracellular domain of the *HER2*/c-*erbB*-2 gene product, p185^{HER2}, and have found that one of these, 4D5, strongly inhibits the growth of several breast tumor cell lines and furthermore sensitizes p185^{HER2}-overexpressing breast carcinoma cell lines SK-BR-3 and MDA-MB-175-VII to the cytotoxic effects of TNF-α. Monoclonal antibody 4D5 is specific for p185^{HER2} and shows no cross-reactivity with the closely related human EGF receptor expressed in mouse fibroblasts. Of six mammary carcinoma cell lines tested, only the three lines which express high levels of p185^{HER2} (SK-BR3, MBA-MB-175, and MDA-MD-361 [17]) were growth inhibited, and 4D5 did not inhibit the proliferation of a nontransformed human breast epithelial cell line, HBL-100, or the bladder carcinoma cell line T24.

In the presence of the antibody, the inhibition of SK-BR-3 cell growth was nearly complete, but the effect was cytostatic rather than cytotoxic. This property of 4D5 is similar to that described for a subset of monoclonal antibodies to the EGF receptor (19, 31, 32) which inhibit the growth of A431 cells, a human squamous epithelial carcinoma line expressing high levels of the EGF receptor. In this case, these inhibitory antibodies compete with radiolabeled EGF for binding to the receptor, and antibodies which do not block EGF binding have no effect on A431 cell growth. It has been suggested (J. Mendelsohn and H. Masui, Clin. Res. 35:600A, 1987) that these antibodies inhibit cell growth by interfering with an autocrine system involving the EGF receptor and an essential growth factor, transforming growth factor alpha, that is produced by the cells (5). It is therefore intriguing to speculate that antibody 4D5 analogously interferes with ligand binding to the *HER2*/c-*erbB*-2 gene product. Since an appropriate ligand for the putative *HER2*/c-*erbB*-2 receptor has not yet been identified, this possibility cannot yet be tested directly.

The 4D5 antibody had no effect on the growth of NIH 3T3 cells transformed by *HER2*/c-*erbB*-2 overexpression. However, it reversed one property conferred on these cells by amplification of the *HER2*/c-*erbB*-2 cDNA: the formation of colonies in soft agar was prevented by 200 ng of 4D5 antibody per ml. This result is similar to those obtained by Drebin et al. (8) with a monoclonal antibody to the rat *neu* oncogene-encoded p185^{neu}. They also observed that an anti-p185^{neu} monoclonal antibody inhibited colony growth in soft agar and tumor formation by *neu*-transformed NIH 3T3 cells in athymic mice (7–10). This effect was attributed to a lowering p185^{neu} levels by an increase in receptor turnover triggered by antibody binding. The apparent discrepancy between 4D5 effects on proliferation of breast tumor cells versus transfected mouse fibroblast cells is most probably a reflection of the fact that SK-BR-3 cells are authentic cancer cells, in contrast to the NIH 3T3 model system. Whereas SK-BR-3 cells may have evolved to be dependent on *HER2*/c-*erbB*-2-mediated signals for both growth and transformation characteristics, NIH 3T3 cells have acquired a transformed phenotype only as a result of *HER2*/c-*erbB*-2 overexpression, but may proliferate normally in response to other serum growth factors, even in the presence of blocking anti-p185^{HER2} antibody.

Previous work has shown that high-level expression of p185^{HER2} will transform NIH 3T3 cells and has suggested a casual role for amplification of the *HER2*/c-*erbB*-2 gene in mammary gland neoplasia. We have shown here that *HER2*/c-*erbB*-2 gene overexpression in NIH 3T3 cells is associated with increased resistance to the monokine TNF-α and that breast tumor cell lines which overexpress p185^{HER2} are resistant to the cytotoxic effects of TNF-α. The mechanism by which 4D5 inhibits breast tumor cell proliferation and reverses phenotypes associated with high levels of p185^{HER2} expression, such as resistance to TNF-α, is not clear. However, these results suggest that in addition to its ability to transform cells by virtue of overexpression (6, 18), *HER2*/c-*erbB*-2 could play a role in tumor progression by allowing tumor cells overexpressing p185^{HER2} to evade one component of the antitumor immunosurveillance of the host, the activated macrophage (17). These properties of the *HER2*/c-*erbB*-2 gene product may in part explain the aggressive, single-step induction of mammary adenocarcinoma in transgenic mice bearing the *neu* oncogene (24), which encodes the mutated rat homolog of p185^{HER2}.

The experiments presented here demonstrate that a monoclonal antibody which recognizes the extracellular domain of p185^{HER2} inhibits the proliferation of breast tumor cells which overexpress this receptorlike protein. Moreover, treatment with this antibody also sensitizes these tumor cells to the cytotoxic effects of TNF-α. Monoclonal antibodies specific for p185^{HER2} may therefore be useful therapeutic agents for the treatment of human neoplasias, including certain mammary carcinomas, which are characterized by the overexpressing of p185^{HER2}

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EXHIBIT D

[CANCER RESEARCH 56, 1457–1465, March 15, 1996]

Growth Regulation of Human Breast and Ovarian Tumor Cells by Heregulin: Evidence for the Requirement of ErbB2 as a Critical Component in Mediating Heregulin Responsiveness

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ABSTRACT

Alterations in the expression of the epidermal growth factor (EGF) receptor ErbB family are frequently encountered in a number of human cancers. Two of these receptors, ErbB3 and ErbB4, are known to bind a family of related proteins termed heregulins (HRGs) or *neu* differentiation factors. In biologically relevant systems, interaction of HRG with ErbB3 or ErbB4 results in the transactivation of ErbB2. In this report, we show that ErbB2 is a critical component in mediating HRG responsiveness in a panel of human breast and ovarian tumor cell lines. Because HRGs have been reported to elicit diverse biological effects on cultured cells, including growth stimulation, growth inhibition, and induction of differentiation, we systematically examined the effect of rHRGβ1 on tumor cell proliferation. HRG binding studies were performed with a panel of breast and ovarian tumor cell lines expressing a range of levels of ErbB2. The biological responses to HRG were also compared to EGF and to the growth-inhibitory anti-ErbB2 antibody, 4D5. In most cases, HRG stimulation of DNA synthesis correlated with positive effects on cell cycle progression and cell number and with enhancement of colony formation in soft agar. On each cell line tested, the HRG effects were distinguishable from EGF and 4D5. Our findings indicate that HRG induces cell proliferation in a number of tumor cell lines. In addition, we show that methods for measuring cell proliferation, as well as experimental conditions, are critical for determining HRGs effect on tumor cell growth *in vitro*.

INTRODUCTION

Clinical interest in the ErbB family of receptor tyrosine kinases stems from the observations that these receptors are frequently overexpressed in a number of human cancers (1–5). Five different gene products are known to activate the prototypical member of this family, EGFR.[2] In several different tumor types, the coexpression of EGFR with one of its cognate ligands, transforming growth factor α, has been correlated with greater metastatic potential (6, 7). A second group of ligands, which collectively have been termed neuregulins, are known to arise from alternative splicing of a single gene mapped to human chromosome 8p22–p11 (8, 9). This family of proteins which includes HRG (10), NDF (11, 12), gp30 (13, 14), acetylcholine receptor-inducing activity (15), glial growth factor (16), and sensory and motor neuron-derived factor (17) are ligands for ErbB3 and ErbB4 (18). One hallmark of this class of receptors is their propensity for heterodimerization, which upon ligand stimulation can lead to transphosphorylation and ultimately transactivation (19). To date, no ligands have been characterized, to the extent that their cDNA clones have been obtained, that specifically interact with ErbB2. ErbB2 is, however, frequently transactivated by either EGFR ligands or neuregulins, and in several instances, the activation of ErbB2 has been shown to be essential for the generation of an active receptor signaling complex (20–22).

The importance of ErbB2 as a negative prognostic indicator in breast (23) and ovarian (24) cancer is now well established (25). Since HRG (10) and NDF (11) were originally identified based on their ability to activate ErbB2, it is of interest to determine the biological outcome of this activation on the growth of human breast and ovarian tumor cells. To address this question, we have undertaken a systematic study of HRG responsiveness using a panel of human breast and ovarian tumor cell lines with known ErbB2 levels. These cell lines were then characterized with regard to both their affinities and capacities to bind HRG. Antibodies directed against ErbB2 were used to determine if ErbB2 was essential in mediating HRG interactions with ErbB3 or ErbB4. To determine whether HRG treatment of these tumor cell lines resulted in cell proliferation (10) or growth inhibition (12, 26), we performed these assays under a series of well-defined experimental conditions and using a number of different assay formats. In addition, HRG responses were compared to EGF and a cytostatic monoclonal antibody directed against ErbB2, 4D5 (27, 28). Our conclusions from these studies are that ErbB2 plays a critical role in mediating HRG responsiveness over a wide range of ErbB2 expression levels. Cellular responses to exogenous HRG under carefully controlled experimental conditions are cell specific but, in general, result in the proliferation of human breast and ovarian tumor cells. These studies suggest that development of HRG antagonists or compounds that target HRG receptors may find clinical utility in the treatment of a number of important human cancers.

MATERIALS AND METHODS

Cells and Cell Culture. The following cell lines were obtained from the American Type Culture Collection: MCF7, SK-BR-3, MDA-MB-175-VII, MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468, BT-474, HBL-100, T47D, BT-20, BT-483, Caov3, and SK-OV-3. For all experiments, cells were used between passages 5 and 15. All lines were maintained in Ham's F-12:DMEM (50:50) supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine.

HRG Binding Assays. Tumor cells were plated in 24-well plates at 10^5 cells/well in F12/DMEM containing 10% FBS. After 48 h, the cultures were washed two times with F12/DMEM. Cells were placed on ice and briefly incubated with binding buffer (0.1% BSA in F12/DMEM). ^{125}I-labeled rHRGβ1$_{177-244}$ (5 pM to 2 nM) was then added to each well. Binding reactions were performed for 4 h on ice. Kinetic studies showed that equilibrium was obtained at 4 h, and no significant changes in binding constants were observed if the reactions were conducted up to 16 h. Cells were then washed twice with 0.5 ml binding buffer, and bound radioactivity was determined after solubilization with 0.1% SDS in 0.1 N NaOH or with 8 M urea in 3 M acetic acid. Scatchard analysis was performed as described previously (10, 29). The values reported in Table 1 for these binding experiments are the average of three different binding experiments, each of which consisted of triplicate incubations for each concentration of ^{125}I-labeled rHRGβ1$_{177-244}$ tested. Error values for both K_d and sites/cell were on the order of 10% or less for each binding experiment.

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[2] The abbreviations used are: EGFR, epidermal growth factor receptor; HRG, heregulin; NDF, *neu* differentiation factor; FBS, fetal bovine serum; PI3-kinase, phosphatidylinositol 3′-kinase.

Inhibition of HRG binding to tumor cell lines by anti-ErbB2 antibodies were also performed on ice with monolayer cultures in a 24-well-plate format. Anti-ErbB2 monoclonal antibodies were added to each well and incubated for 30 min. ^{125}I-labeled rHRGβ1$_{177-244}$ (25 pM) was added, and the incubation was continued for 4 to 16 h. For several cell lines, incubations were performed for 4 h, since longer incubations at reduced temperatures resulted in cells detaching from the plate.

Tyrosine Phosphorylation Assays. MCF7 cells were plated in 24-well plates as described above for HRG binding experiments. Monoclonal antibodies to ErbB2 were added to each well and incubated for 30 min at room temperature; then rHRGβ1$_{177-244}$ was added to each well for a final concentration of 0.2 nM, and the incubation was continued for 8 min. Media was carefully aspirated from each well, and reactions were stopped by the addition of 100 μl of SDS sample buffer (5% SDS, 25 mM DTT, and 25 mM Tris-HCl, pH 6.8). Each sample (25 μl) was electrophoresed on a 4–12% gradient gel (Novex) and then electrophoretically transferred to polyvinylidene difluoride membrane. Antiphosphotyrosine (4G10, from UBI, used at 1 μg/ml) immunoblots were developed, and the intensity of the predominant reactive band at M_r ~180,000 was quantified by reflectance densitometry, as described previously (10, 29).

Cell Proliferation Determined by [^{3}H]Thymidine Incorporation into DNA. Cells were seeded into 96-well plates at a density of 10^4/well for treatments up to 3 days and 5×10^3/well for the 6-day incubation. Following an overnight incubation to allow for cell adherence, the medium was removed and replaced with medium containing 0.1% FBS to growth arrest the cells. After a 48-h incubation, this medium was replaced with medium containing 0.1, 1.0, or 10% FBS and rHRGβ1 (5 pM-10 nM), 3 nM EGF (Sigma Chemical Co.), or 10 μg/ml (65 nM) of the anti-ErbB2 monoclonal antibody 4D5 (27, 28, 30). The cells were then incubated for 0.5, 1, 3, or 6 days, pulsed with 1 μCi/well [^{3}H]thymidine (Amersham) for 4 h, and harvested onto Unifilter GF/C plates (Packard Instrument Co.) using a Packard Filtermate 196 harvester. After allowing the filter plates to dry, MicroScint 20 liquid scintillation cocktail (Packard) was added to each well; then the plates were heat-sealed and counted in a Packard Topcount.

Cell Cycle Analysis. Cells were plated at a density of 2×10^6 cells/dish in 60 × 15-mm culture dishes and allowed to adhere overnight. The monolayers were then washed with PBS and incubated with medium containing 0.1% FBS for 48 h to arrest cell growth. The medium was removed and replaced with medium supplemented with 0.1, 1.0, or 10% FBS alone or containing 0.3 nM rHRGβ1, 3 nM EGF, or 65 nM 4D5. After a 1-day incubation, the cells were trypsinized, washed with PBS, fixed in ice-cold methanol, and stored at −20°C. The fixed cells were then washed twice with PBS and incubated with 100 μg/ml RNAse (Worthington Biochemical) for 30 min at 37°C. The RNAse was removed by centrifuging the cells, and the pellet was incubated with 50 μg/ml propidium iodide (Molecular Probes, Inc.) in PBS for DNA staining. The samples were then incubated overnight at 4°C and analyzed on an Epics Elite flow cytometer (Coulter Corporation) using Modfit LT software (Verity Software House).

Cell Proliferation Assay with Crystal Violet. Tumor cells were plated at a density of 2×10^4/well in 96-well plates in media containing 0.1% FBS and allowed to adhere for 2 h. Monoclonal antibodies (10 nM) or media alone were added, and the cells were incubated for 2 h at 37°C. rHRGβ1 (0.3 nM) was then added, and the cells were incubated for 3 days. Monolayers were then washed with PBS and fixed/stained with 0.5% crystal violet. Plates were air dried, the dye was eluted with 0.1 M sodium citrate (pH 4.2) in ethanol (50:50), and the absorbance was read at 540 nM to determine cell viability (30).

Determination of Cell Number. Cells were plated as described for the [^{3}H]thymidine assays. After each incubation, the monolayers were washed once with PBS, cells were detached with trypsin, and viable cells were counted by trypan blue dye exclusion.

Quantification of Cell Proliferation by Anchorage-independent Growth in Soft Agar. Cells were seeded in 60 × 15-mm dishes onto a bottom layer of 0.5% Bacto-agar (Difco) at the following densities: 10^4/dish for SK-OV-3; 2×10^4/dish for MCF7, SK-BR-3, MDA-MB-231, BT-474, and HBL-100; and 10^5/dish for MDA-MB-361, MDA-MB-453, and MDA-MB-468. A top layer of 0.25% agar in medium containing 0.3 nM rHRGβ1 or 3 nM EGF or medium alone (as a control) was added to each dish. After 2–4 weeks, colonies were stained with 250 μg/dish 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) and enumerated using an Omnicon 3600 Tumor Colony Image Analysis System (Imaging Products International, Inc.).

RESULTS

Determination of HRG Binding. Using ^{125}I-labeled rHRGβ1$_{177-244}$ to perform direct binding experiments, we determined both the affinity and the number of HRG receptors by Scatchard analysis on 13 different cell lines of breast and ovarian origin. A summary of these data is shown in Table 1. For each of these cell lines, the binding data could be fit to a single class of high affinity binding sites. Only the immortalized human mammary epithelial cell line, HBL-100, lacked detectable HRG binding. Very low (<10,000 receptors/cell) HRG binding could be detected on the ovarian cell line SK-OV-3 and the breast tumor cell lines MDA-MB-175-VII, MDA-MB-468, and BT-20. The following breast cancer cell lines, MCF7, MDA-MB-361, T47D, and BT-483, and the ovarian cancer cell line Caov3 were found to have between 10,000–22,000 receptors/cell, and these cell lines were arbitrarily characterized as containing intermediate levels of HRG binding sites. Cell lines having high numbers of HRG binding sites (*i.e.*, >25,000 receptors/cell) were SK-BR-3, MDA-MB-453, and BT-474. Previously, we have determined that in the absence of ErbB2, ErbB3 binds HRG with a binding affinity of 0.9–2 nM (31). When COS7 cells or NIH3T3 cells coexpress ErbB2 with ErbB3, a higher affinity binding site of ~20 pM is observed (29, 32). Using a similar COS7 cell system, Tzahar *et al.* (33) have reported recently that NDF binds to ErbB4 and ErbB3 with K_ds of 1.5 and 8 nM, respectively. These authors speculated that the difference in the binding constants may be related to cell-specific determinants or are due to the preparation or radiolabeling of the recombinant ligands. Recently, we have determined that ErbB4 or ErbB3 binds HRG with similar affinity, if either is expressed singly in cells of hematopoietic origin in the absence of any other ErbB receptors.[3] The average K_d for the 12 cell lines shown in Table 1 that exhibit HRG binding is 99 ± 14 pM. The binding constants shown in Table 1 are at least an order of magnitude higher in affinity than what has been reported for ErbB3 or ErbB4 alone. These data suggest that other components besides ErbB3 or ErbB4 are contributing to the formation of a high affinity HRG binding site on these cells.

Monoclonal Antibodies to ErbB2 Block HRG Binding to Breast and Ovarian Tumor Cells. Based on our previous observations with COS7 cells expressing ErbB3 and ErbB2, as well as our experience with human Schwann cells, we hypothesized that the high-affinity HRG receptors present on these tumor cells were the result of heterodimer formation of ErbB3 with ErbB2 or possibly ErbB4 with ErbB2 (21, 29). To evaluate whether ErbB2 contributes to the formation of this high-affinity HRG binding site on human tumor cells, we surveyed a panel of well-characterized anti-ErbB2 antibodies (28) for their ability to inhibit HRG-stimulated tyrosine phosphorylation of proteins in the M_r 180,000 range from whole-cell lysates of MCF7 cells. MCF7 cells are reported to express all known ErbB receptors, but at relatively low levels (34). Since ErbB2, ErbB3, and ErbB4 have nearly identical molecular sizes (35, 36), it is not possible to discern which protein is becoming tyrosine phosphorylated when whole-cell lysates are evaluated by Western blot analysis. However, these cells are ideal for HRG tyrosine phosphorylation assays because under the assay conditions used, in the absence of exogenously added HRG, they exhibit low to undetectable levels of tyrosine phosphorylation proteins in the M_r 180,000 range. As shown in Fig. 1*A*, several of these antibodies, including 2C4, 7F3, and 4D5, significantly inhibited

[3] R. W. Akita, N. Chiang, L. Bald, G. Schaefer, B. M. Fendly, and M. X. Sliwkowski. Allosteric modulation of heregulin binding to ErbB3, manuscript in preparation.

Table 1 *HRG receptors on human tumor cell lines*

Direct binding of ^{125}I-labeled rHRGβ1$_{177-244}$ to tumor cells. Breast and ovarian cancer cell lines were plated in 24-well plates at an initial density of 10^5 cells/well and allowed to grow for 48 h. The cultures were washed twice with F12/DMEM and then placed on ice to thermally equilibrate. ^{125}I-labeled rHRGβ1$_{177-244}$ (5 pM to 2 nM) was then added to each well. Binding reactions were performed for 4 h on ice. Scatchard analysis was performed as described previously (10, 29). The values reported are the average and SD for three different binding experiments, each of which consisted of triplicate incubations for each concentration of ^{125}I-labeled rHRGβ1$_{177-244}$ tested. Error values for both K_d and sites per cell were <10% for each binding experiment. The cell number was determined by trypan blue exclusion.

Cell line	K_d (pM)	Sites/cell	ErbB-2 expression[a]
HBL-100	ND[b]	ND	Normal/low
SK-OV-3	84 ± 18	2500 ± 440	+++
MDA-MB-175	232 ± 103	2900 ± 1700	+
MDA-MB-468	68 ± 6	4000 ± 500	ND
BT-20	57 ± 3	7300 ± 400	Normal/low
MCF7	91 ± 16	12,700 ± 1300	Normal/low
MDA-MB-361	87 ± 3	14,500 ± 1000	++
T47D	81 ± 8	15,900 ± 800	+
BT-483	81 ± 6	20,100 ± 1000	Normal/low
Caov3	140 ± 3	21,700 ± 1200	Normal/low
SK-BR-3	54 ± 8	27,300 ± 1500	+++
MDA-MB-453	76 ± 3	29,000 ± 500	++
BT-474	137 ± 25	34,600 ± 2200	+++

[a] Values taken from Lewis *et al.* (30).
[b] ND, not detected.

the generation of a HRG-induced tyrosine phosphorylation signal at M_r 180,000. In the absence of HRG, none of these antibodies were able to stimulate tyrosine phosphorylation of proteins in the M_r 180,000 range (data not shown). Also, these antibodies do not cross-react with EGFR (28), ErbB3, or ErbB4.[4] Antibodies 2C4 and 7F3 significantly inhibited HRG stimulation of p180 tyrosine phosphorylation to <25% of control. However, 4D5 was able to block HRG stimulation of tyrosine phosphorylation by ~50%. Previously, 2C4 and 7F3 were assigned the same ErbB2 epitope as reported in the original characterization of this antibody panel, and this epitope is different than that recognized by 4D5 (28). Fig. 1*B* shows dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of p180 tyrosine phosphorylation as determined by reflectance densitometry. Evaluation of these inhibition curves using a 4-parameter fit yielded an IC_{50} of 2.8 ± 0.7 nM and 29.0 ± 4.1 nM for 2C4 and 7F3, respectively. Varying concentrations of 2C4 or 7F3 were incubated with MCF7 cells in the presence of ^{125}I-labeled rHRGβ1, and the inhibition curves are shown in Fig. 1*C*. Analysis of these data yielded an IC_{50} of 2.4 ± 0.3 nM and 19.0 ± 7.3 nM for 2C4 and 7F3, respectively. A maximum inhibition of ~74% for 2C4 and 7F3 are in agreement with the tyrosine phosphorylation data. We concluded from these experiments that in MCF7 cells, ErbB2 was critical for HRG stimulation of tyrosine phosphorylation and was an important component for high-affinity HRG binding.

It was of interest to determine whether the effect of the anti-ErbB2 antibodies observed on MCF7 was a general phenomenon. To address this question, human tumor cell lines were incubated with 2C4 or 7F3 and the degree of specific ^{125}I-labeled rHRGβ1 binding was determined. The results from this study are shown in Fig. 2. Binding of ^{125}I-labeled rHRGβ1 could be significantly inhibited by either 2C4 or 7F3 in all cell lines, with the exception of the breast cancer cell line MDA-MB-468, which has been reported to express little (37) or no ErbB2 (30, 38). The remaining cell lines are reported to express ErbB2, with the level of ErbB2 expression varying widely among these cell lines (30, 38). In fact, the range of ErbB2 expression in the cell lines tested varies by more than 2 orders of magnitude. For example, BT-20, MCF7, and Caov3 express ~10^4 ErbB2 receptors/cell, whereas BT-474 and SK-BR-3 express ~10^6 ErbB2 receptors/cell (30). Given the wide range of ErbB2 expression in these cells and the data in Fig. 2, it was concluded that the interaction between ErbB2 and ErbB3 or ErbB4, was itself a high-affinity interaction that takes place on the surface of the plasma membrane. At present, it is unclear whether these complexes involving ErbB2-ErbB3 or ErbB2-ErbB4 are preexisting in the absence of HRG or whether the association with

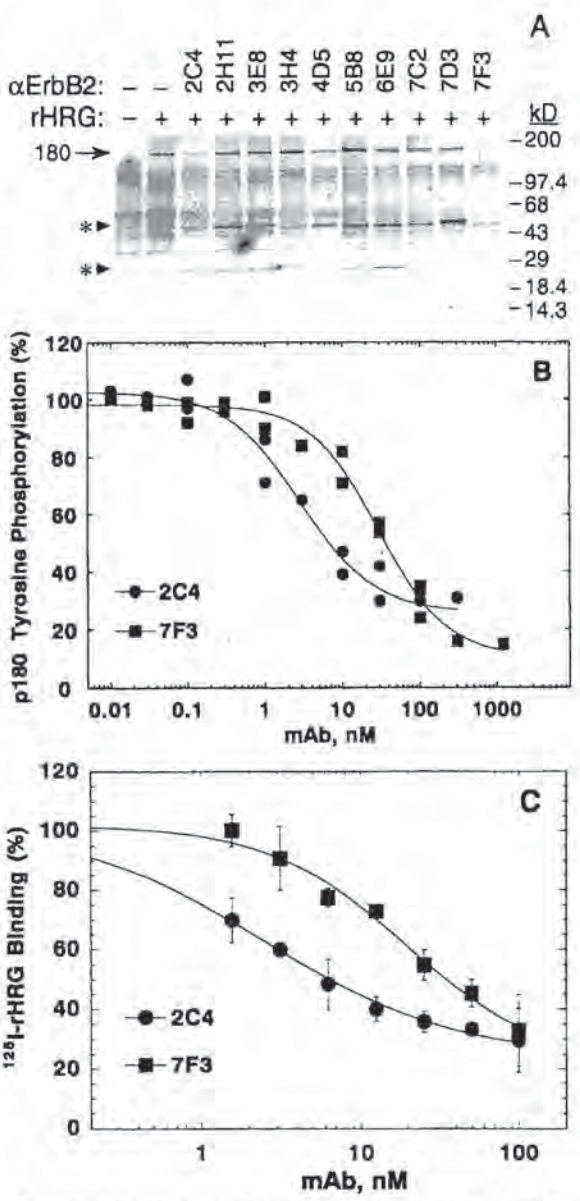

Fig. 1. Effect of anti-ErbB2 antibodies on rHRGβ1 activation of MCF7 cells. *A*, effect of the anti-ErbB2 antibodies on the generation of a phosphotyrosine signal at M_r 180,000. Antiphosphotyrosine immunoblots were developed as described in "Materials and Methods." *, immunoreactive bands that result from the interaction of the murine monoclonal antibody with the secondary antibody detection system. *B*, dose-response curves for 2C4 or 7F3 inhibition of HRG stimulation of tyrosine phosphorylation. *C*, dose-response curves for the inhibition of ^{125}I-labeled rHRGβ1$_{177-244}$ binding to MCF-7 cells by 2C4 or 7F3; *bars*, SE.

[4] L. Bald, M. X. Sliwkowski, R. Akita, N. Chiang, C. Wirth, R. Taylor, N. Dybdal, and B. M. Fendly. Deciphering heregulin-mediated signaling through ErbB4: application of anti-ErbB4 monoclonal antibodies, manuscript in preparation.

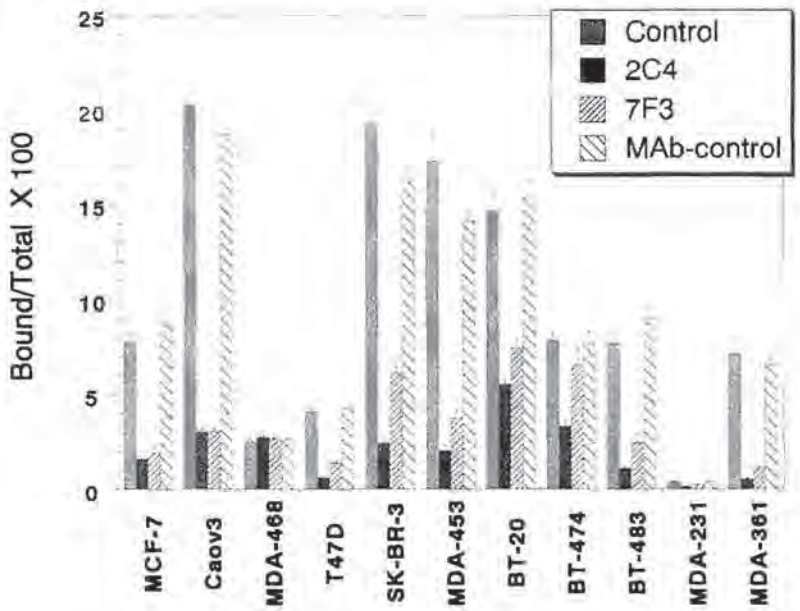

Fig. 2. Inhibition of specific ^{125}I-labeled rHRGβ1$_{177-244}$ binding to a panel of human tumor cell lines by the anti-ErbB2 monoclonal antibodies 2C4 or 7F3. Monoclonal antibody-controls are isotype-matched murine monoclonal antibodies that do not block rHRG binding. Nonspecific ^{125}I-labeled rHRGβ1$_{177-244}$ binding was determined from parallel incubations performed in the presence of 100 nM rHRGβ1. Values for nonspecific ^{125}I-labeled rHRGβ1$_{177-244}$ binding were less than 1% of the total for all of the cell lines tested; *bars*, SE.

ErbB2 is driven by the formation of HRG-ErbB3 or HRG-ErbB4 complexes.

Stimulation of Mitogenesis by HRG in Human Tumor Cells. We initially reported that HRG stimulated the growth of SK-BR-3 breast tumor cells as measured by crystal violet staining (10). Having determined the levels of HRG receptors in a number of different human tumor cell lines, we examined the mitogenic response of these cells to rHRGβ1. Dose-response curves consisting of 5 pM-10 nM rHRGβ1 in different serum concentrations were generated for all of the cell lines listed in Table 1. Prior to treatment with rHRGβ1, cells were growth arrested by incubation in medium containing 0.1% FBS for 48 h. Fig. 3 shows typical curves for three breast tumor cell lines after treatment for 3 days. MCF7 responsiveness to rHRGβ1 was dependent on serum concentration in the assay medium. When assayed in 0.1% FBS, MCF7 cells showed a 4–5-fold stimulation of [^{3}H]thymidine incorporation after treatment for 1 day (data not shown) and 3 days (Fig. 3*a*), with the maximal response occurring between 0.1 and 1 nM rHRGβ1, consistent with the affinity constant shown in Table 1. rHRGβ1 also stimulated DNA synthesis 2–2.5-fold in 1% FBS but had no effect in 10% FBS (Fig. 3*a*). A likely explanation for this effect is that MCF7 cells contain high levels of estrogen receptors (39) and, in the absence of other exogenously added growth factors, requires estrogen for growth (40). Alternatively, the presence of peptide growth factors such as insulin-like growth factor-1 may reduce cellular responses to HRG (41). Sufficient quantities of these factors are present when cells are cultured in 10% serum; however, 0.1% serum is not sufficient. In low serum concentrations, HRG activation of ErbB receptor pathways is then sufficient for stimulating mitogenic activity. These data are consistent with those reported recently by Pietras *et al.* (42), which demonstrate a direct interaction between HRG-mediated activation of ErbB pathways and cross-talk with estrogen receptor pathways. In contrast, the ErbB2 overexpressing breast tumor cell lines, SK-BR-3 and MDA-MB-453, showed enhanced incorporation of [^{3}H]thymidine in a serum-dependent manner after 1 day of treatment with rHRGβ1 (data not shown) and after 3 days of treatment (Fig. 3, *b* and *c*). Although there was no effect in 0.1% FBS, [^{3}H]thymidine incorporation increased in cells exposed to rHRGβ1 in 1 or 10% FBS.

Comparison of the HRG Response to EGF and 4D5. Since responsiveness of a number of tumor cell lines to either EGF or the anti-ErbB2 monoclonal antibody 4D5 has been extensively studied, we compared treatment of these cell lines with either EGF or 4D5 under identical conditions as a comparison to rHRGβ1. *In vitro*, EGF inhibits the growth of tumor cell lines such as MDA-MB-468 (43) and A431 (44, 45), both of which overexpress EGFR. Conversely, cells that express normal/low or moderate levels of EGFR are frequently growth-stimulated by exogenous EGF (39). Inhibition of cell growth by 4D5 is dependent on the expression level of ErbB2 (30), *i.e.*, 4D5 is cytostatic only for cells that overexpress ErbB2. Using a 3-day assay, the data in Fig. 4 show that the changes in [^{3}H]thymidine incorporation in these cell lines were distinct, depending on the treatment conditions. rHRGβ1 stimulated thymidine incorporation in MCF7, SK-BR-3, BT-474, MDA-MB-453, MDA-MB-361, T47D, and BT-20 cells at all serum concentrations tested. However the magnitude of the response varied, depending on the serum concentrations present in the assay. In contrast, EGF (3 nM) stimulated [^{3}H]thymidine incorporation in HBL-100, T47D, BT-20, and SK-OV-3 cells under all conditions tested. Both rHRGβ1 and EGF stimulated MCF7 cells in both 0.1% and 1% FBS, although the magnitude of the response was less with EGF than with rHRGβ1. 4D5 had no effect on this cell line, as reported previously (30). DNA synthesis was inhibited by EGF and, more potently, by 4D5 in SK-BR-3 cells at all serum concentrations tested. Similarly, [^{3}H]thymidine incorporation in BT474, MDA-MB-453, and MDA-MB-361 breast tumor cells was also inhibited by 4D5 since these cell lines overexpress ErbB2. rHRGβ1 and 4D5 did not affect [^{3}H]thymidine incorporation in the MDA-MB-231 and MDA-MB-468 breast tumor lines. Consistent with previous reports (43), EGF was inhibitory for MDA-MB-468 cells. No significant changes in MDA-MB-231 cells were detected with either rHRGβ1, EGF, or 4D5 under the conditions tested.

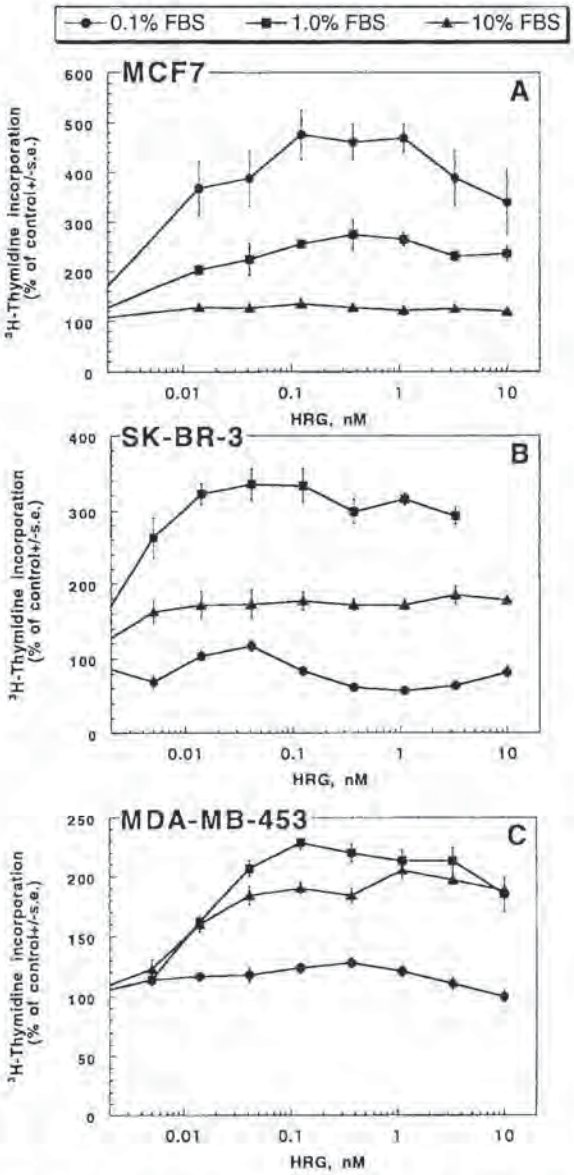

Fig. 3. Stimulation of DNA synthesis by rHRGβ1. MCF7 (*A*), SK-BR-3 (*B*), and MDA-MB-453 (*C*) breast tumor cells were plated at a density of 10^4 cells/well in 96-well microtiter plates and allowed to adhere overnight. The medium was then replaced with low serum medium (0.1% FBS) to growth arrest the cells. After 48 h, this medium was removed, and the cells were treated with different concentrations of rHRGβ1 in medium supplemented with 0.1 (●), 1 (■), or 10% FBS (▲) for 3 days. At the end of each incubation, the cells were radiolabeled with 1 μCi/well [^{3}H]thymidine for 4 h, then harvested onto filter plates for scintillation counting. The data are expressed as the mean cpm of 4–8 replicates; *bars*, SE.

Cell Cycle Changes Induced by rHRGβ1, EGF, and Murine Monoclonal Antibody 4D5. To further characterize the effects of rHRGβ1, EGF, and 4D5 on tumor cell growth, analyses of cell cycle phase fraction distributions were performed. MCF7, SK-BR-3, and MDA-MB-453 breast tumor cells were serum starved and then treated for 1 day with 0.3 nM rHRGβ1, 3 nM EGF, or 66 nM 4D5 in 0.1, 1, or 10% FBS. Fig. 5 shows the results presented as the fraction of cells in S phase as an indicator of proliferative status measured at ~30 h following treatment. For both MCF7 and SK-BR-3 cells, the changes in the percentage of S-phase cells after each treatment correlates well with the effects on [^{3}H]thymidine incorporation (Fig. 3). rHRGβ1 caused a 3-fold increase in the percentage of MCF7 cells in S-phase in 0.1% FBS. EGF treatment induced a smaller proportion of cells to enter S phase in 0.1 and 1% FBS (1.6- and 1.3-fold, respectively),

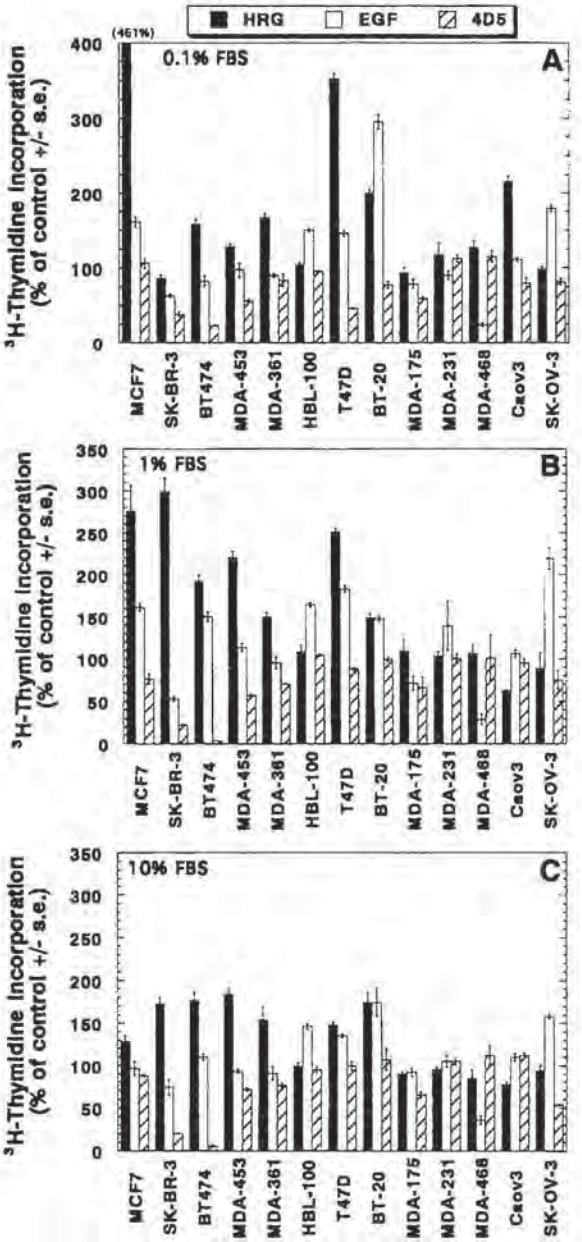

Fig. 4. Comparison of the response of different breast and ovarian tumor cell lines to rHRGβ1, EGF, and 4D5. Mitogenic assays were performed as described in Fig. 3. Cells were treated with 0.37 nM rHRGβ1, 3 nM EGF, or 66 nM 4D5 in medium containing 0.1% (*A*), 1% (*B*), or 10% (*C*) FBS for 3 days. The data are expressed as the percentage of [^{3}H]thymidine incorporation (mean of 4–8 replicates; *bars*, SE) as compared to untreated control cells.

while 4D5 had no effect. With SK-BR-3 cells, rHRGβ1 stimulation of the percentage of cells in S phase was enhanced with increasing serum concentration (1.5–1.8-fold). EGF had an inhibitory effect in 0.1 and 1% FBS, and as expected, 4D5 decreased the percentage of S-phase cells at all concentrations of serum. MCF7 and SK-BR-3 cells were also treated for 3 days with rHRGβ1 with similar results. In contrast, the fraction of cells in S phase in the MDA-MB-453 cell line upon exposure to rHRGβ1 did not differ from controls in the three serum concentrations tested at 30 h. These results are different from the data shown in Fig. 4, where rHRGβ1 increased the incorporation of [^{3}H]thymidine in the MDA-MB-453 cells in 1 and 10% FBS. On the other hand, EGF and 4D5 have similar effects on both [^{3}H]thymidine incorporation and cell cycle progression. As expected, EGF-treated MDA-MB-453 cells were not different from control, since this cell line expresses little or no detectable EGFR (30, 38), whereas treatment with 4D5 reduced the number of S-phase cells. In contrast to previous reports (11, 26, 46), upon HRG treatment, there was no suggestion of a G_2-M growth arrest nor was there evidence for induction of a ploidy abnormality. Explanations for these discrepancies can likely be attributed to differences in the purity of the NDF preparations or the particular cell lines being used in these earlier studies.

Effect of rHRGβ1 on Cell Number. Because of the discrepancy between the MDA-MB-453 [^{3}H]thymidine incorporation and cell cycle experiments, it was necessary to determine actual cell number after rHRGβ1 treatment. Fig. 6 shows that the effects of rHRGβ1 on MCF7 and SK-BR-3 cell number are in agreement with the data in Figs. 3 and 5. The MCF7 breast tumor cells show the greatest increase in cell number to rHRGβ1 after 3 days of treatment in 0.1% FBS, while proliferation of SK-BR-3 cells is increased in higher serum concentrations. There was no change in MDA-MB-453 cell number after exposure to 0.3 nM rHRGβ1 in 0.1, 1, or 10% FBS. Therefore, it appears that the incorporation of [^{3}H]thymidine in this cell line is not an indicator of DNA synthesis and cell proliferation. These results are in agreement with those reported earlier for the response of MDA-MB-453 cells to concentrated conditioned medium containing recombinant NDF (25). Our data for the responsiveness of MDA-MB-453 and SK-BR-3 cells differ from those reported earlier for NDFα (12) or purified natural gp30, which has been shown to be related to HRG/NDF (14, 26).

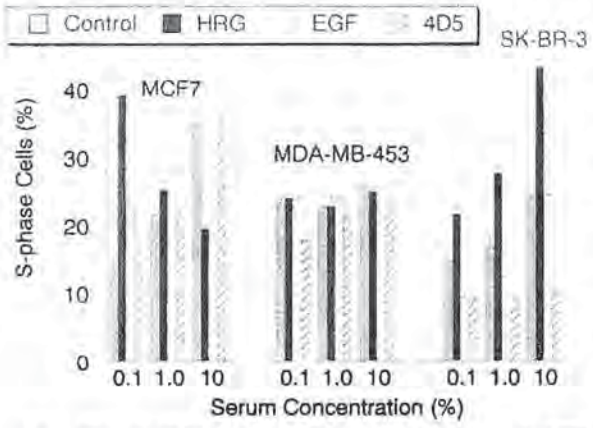

Fig. 5. Effects of rHRGβ1, EGF, and 4D5 on cell cycle progression in MCF7, SK-BR-3, and MDA-MB-453 breast tumor cells. Each treatment was done in triplicate, as described in "Materials and Methods." The data shown are pooled from three to four separate experiments; *bars*, SE.

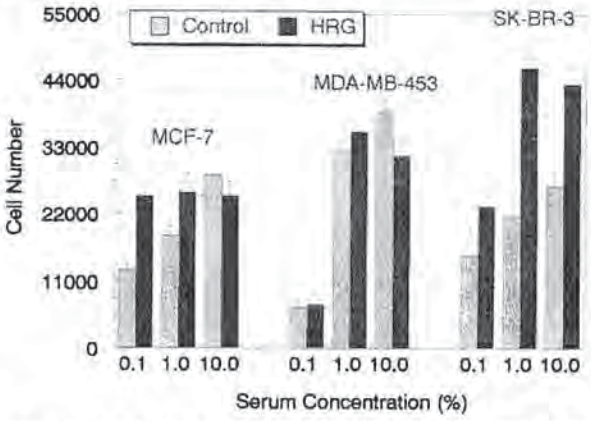

Fig. 6. Effect of rHRGβ1 on MCF7, SK-BR-3, and MDA-MB-453 cell number. Cells were treated as in Fig. 1, except at the end of the incubation, the monolayers were detached with trypsin, and viable cells were counted by trypan blue dye exclusion.

HRG Stimulation of Tumor Cell Growth Is Inhibited by Anti-ErbB2 Monoclonal Antibodies 2C4 and 7F3. Since 2C4 and 7F3 are cytostatic for cells that overexpress ErbB2 (30), we could not test the antagonist effect of 2C4 or 7F3 on HRG-induced growth of cells that overexpress ErbB2. The effect of 2C4 or 7F3 on three cell lines that express low/normal levels of ErbB2 is shown in Fig. 7. MCF7 and T47D are breast cancer cell lines that are known to express low to intermediate levels of all ErbB receptors (34, 36, 39). Although ErbB4 is not expressed in the ovarian cancer cell line Caov3 (35), other ErbB receptors are present (30) (data not shown). The effect of 2C4 or 7F3 on rHRGβ1 stimulation of cell growth using a 3-day crystal violet assay format is shown in Fig. 7. In each case, rHRGβ1-mediated growth was inhibited by 2C4 or 7F3 to levels close to those observed without HRG treatment. The results observed with 2C4 or 7F3 are similar to those reported recently by Graus-Porta *et al.* (22), using a version of T47D cells engineered to sequester ErbB2 in the endoplasmic reticulum with a single-chain anti-ErbB2 antibody. These data are also similar to those obtained when rHRGβ1 was tested in combination with 2C4 on human Schwann cells (21).

Anchorage-independent Growth in Soft Agar. For a final determination of the effects of rHRGβ1 on cell proliferation, we studied the anchorage-independent growth of different breast and ovarian tumor cell lines in soft agar and compared the response with EGF. To allow for enhanced proliferation of colonies in soft agar, cells were seeded at densities resulting in minimal colony formation in untreated cells, and all experiments had to be performed in 10% serum. As shown in Fig. 8, MCF7, T47D, SK-BR-3, BT474, and SK-OV-3 cells showed a greater than 2-fold increase in colony formation in response to treatment with 0.3 nM rHRGβ1. Growth in soft agar of the MDA-MB-468 cell line was also slightly enhanced. Since this cell line does not express ErbB2 (30, 38) and presumably low or undetectable levels of ErbB4 but does express ErbB3, this response might be mediated by ErbB3. Since ErbB3 is an impaired kinase (47, 48), these data suggest that ErbB3 may be heterodimerizing with another ErbB family member other than ErbB2. One plausible candidate for this active signaling complex may be EGFR-ErbB3 since MDA-MB-468 cells overexpress EGFR (49). Colony formation by MDA-MB-453 and MDA-MB-361 cells was inhibited by HRG treatment relative to control plates. In comparison, EGF had little effect on MCF7, MDA-MB-453, MDA-MB-361, or MDA-MB-231 cells. Growth in soft agar was stimulated by EGF in SK-OV-3 cells and was slightly enhanced in SK-BR-3 and BT474 cells. Treatment of MDA-MB-468 cells with EGF completely abolished colony formation.

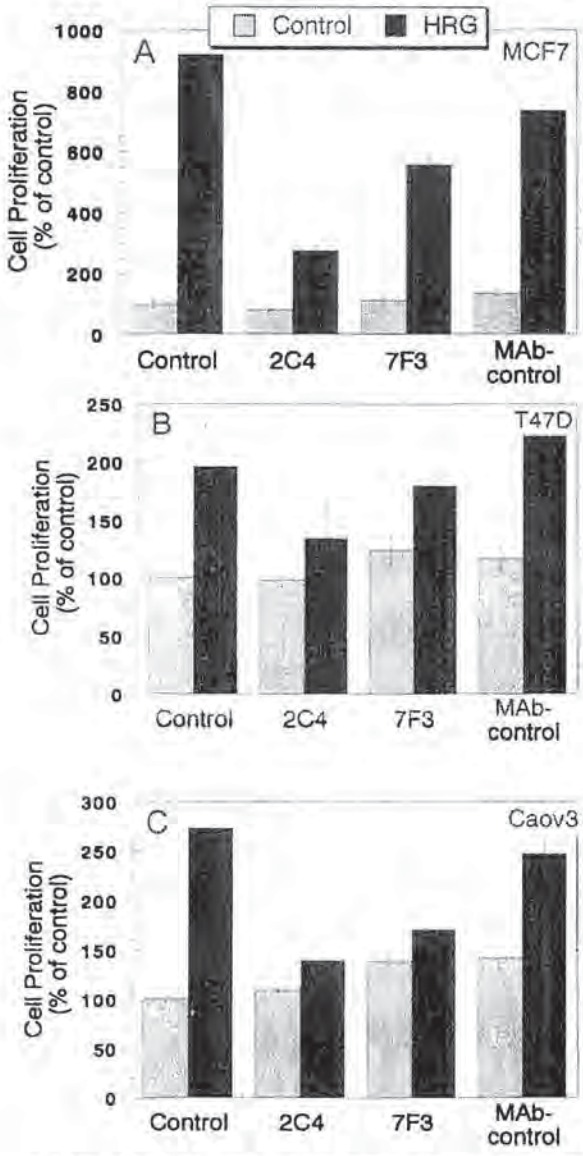

Fig. 7. Effect of 2C4 or 7F3 on rHRGβ1 induced proliferation of MCF7 (*A*), T47D (*B*), and Caov3 (*C*) tumor cell lines. Monoclonal antibodies (10 nM) or medium alone were added, and the cells were incubated for 2 h at 37°C. rHRGβ1 (0.3 nM) was then added, and the cells were incubated for 3 days. Monolayers were then washed with PBS and fixed/stained with 0.5% crystal violet; then the absorbance was read at 540 nM to determine cell proliferation (30).

DISCUSSION

In this study, we describe a systematic analysis of HRG responsiveness in a panel of human breast and ovarian cancer cell lines. High-affinity HRG receptors could be detected on most of these cell lines. However, the range of HRG receptor expression is significantly narrower than that observed for either EGFR or ErbB2. Since HRG binding requires the presence of ErbB3 or ErbB4, these data imply that ErbB3 and ErbB4 expression may be more tightly regulated in breast and ovarian tumors cells than either EGFR or ErbB2. Antibodies directed against ErbB2 inhibited HRG binding in these cell lines, suggesting that the formation of this high-affinity binding site was likely the result of heterodimerization of ErbB2 with ErbB3 or with ErbB4. In addition, the binding affinity of HRG for ErbB4 appears to be considerably lower than the values determined here for this panel of human tumor cell lines (33). Although ErbB4 is expressed in some of these cell lines (34, 35), our analysis of ErbB4 expression in these cells using a panel of ErbB4-specific monoclonal antibodies indicates that the ErbB4 expression level is significantly lower than EGFR, ErbB2, or ErbB3.[4] ErbB4 is a fully functional HRG receptor (35, 50), and it has been demonstrated recently that ErbB4 is required for normal embryonic neural and cardiac development (51). Regardless of the relative expression levels or the activation of particular signal transduction pathways, the abrogation of HRG responsiveness by anti-ErbB2 monoclonal antibodies indicates that ErbB2 is critical in mediating HRG responsiveness. Our conclusion from these studies is that inhibition of HRG response by these blocking anti-ErbB2 antibodies is the result of inhibiting the recruitment of ErbB2 to a high-affinity complex with ErbB3 or ErbB4. Using different experimental approaches similar conclusions have also been drawn regarding the role of ErbB2 in mediating HRG responses in the breast cancer cell lines T47D (22) and MCF7 (52).

At present, more is known about the interaction of ErbB2 with ErbB3 than ErbB2 with ErbB4. Although ErbB3 can function as a HRG-binding protein, it is a unique receptor in that its intrinsic tyrosine kinase activity appears to be impaired (47, 48). When coexpressed with ErbB2, ErbB3 becomes phosphorylated on tyrosine residues in its cytoplasmic domain that generate a signal primarily through the PI3-kinase pathway (32, 53). Thus, a functional HRG receptor may be viewed as a heterodimer of ErbB3 with ErbB2, where ErbB2 modulates the affinity of ErbB3 for HRG binding and contributes an active tyrosine kinase domain. Conversely, ErbB3 is required for HRG binding and upon phosphorylation of its unique tyrosine residues in the cytoplasmic domain, ErbB3 is capable of coupling to PI3-kinase. This latter adapter function is analogous to the interaction of IRS-1 with insulin receptor. IRS-1 is the principal substrate of the activated insulin receptor tyrosine kinase. After becoming tyrosine phosphorylated, IRS-1 couples with PI3-kinase and other SH2-proteins for the propagation of downstream signaling (54). Coopera-

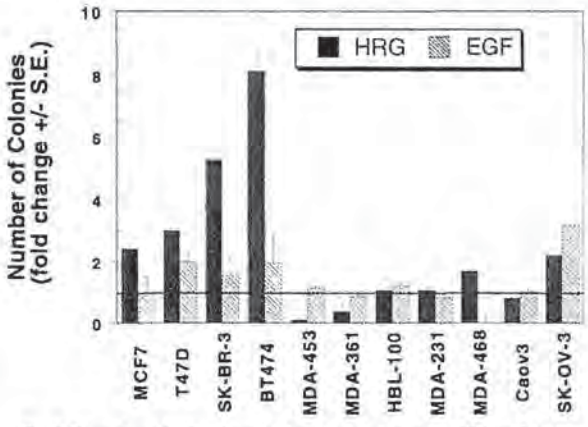

Fig. 8. Colony formation in soft agar by breast and ovarian tumor cell lines. Cells were seeded in 60 × 15-mm dishes onto a bottom layer of 0.5% agar and overlaid with 0.25% agar in Ham's F12:DMEM (50:50) medium supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine. Treatment groups for each cell line were 0.3 nM rHRG, 3 nM EGF, or an untreated control (designated by *horizontal bar*). After 2–4 weeks, the colonies were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide and counted using an Omnicon 3600 Image Analysis System. The data are represented as the fold change in the number of colonies per dish (*bars*, SE) that were greater than 80 μm relative to untreated control dishes. Each treatment group consisted of three replicate dishes.

tion between ErbB3 and ErbB2 in neoplastic transformation of NIH3T3 cells has also been demonstrated recently (53, 55).

We also studied the effects of rHRG on the growth of these cell lines using several different assay formats. In a number of these formats, it was found that the presence of serum profoundly influenced the magnitude of the HRG response on a number of these cell lines. In general, the results from these experiments agreed among the formats used. MCF7, Caov3, T47D, BT-20, BT-474, and SK-BR-3 cells were growth stimulated in a number of assays under defined serum concentrations. Our results with the stimulation of growth of these breast tumor cell lines are similar to those recently obtained with the mouse mammary epithelial cell line HC11 (56, 57) and the immortalized human epithelial cell line MCF-10A (41). Two notable exceptions in the present study were MDA-MB-453 and MDA-MB-361 cells. Although these cell lines were stimulated by rHRG treatment in the mitogenic assay, this incorporation of [^{3}H]thymidine did not appear to lead to cell division. Indeed, anchorage-independent growth assays suggested that HRG treatment was growth inhibitory at high serum concentrations. There are several possible explanations for the apparent discrepancy between the stimulation of [^{3}H]thymidine incorporation in the MDA-MB-453 and MDA-MB-361 cells and the negative results obtained with cell cycle, cell count, and soft agar experiments. Incorporation of [^{3}H]thymidine into DNA occurs exclusively via the salvage pathway for DNA synthesis. Differences in rates of transport of labeled nucleotide into the cell, in activity of thymidine kinase or other synthetic or degradative enzymes, or in the size of intracellular nucleotide pools can lead to inconsistent results (58). Extranuclear labeling would also lead to enhanced incorporation independent of DNA synthesis. Studies showing increased [^{3}H]thymidine incorporation without subsequent cell division have been reported (59, 60).

We compared the HRG response in these cell lines to 4D5, a cytostatic monoclonal antibody directed against ErbB2. Growth inhibitory response of cell lines to 4D5 is strictly dependent on ErbB2 expression levels (30) and appears to be independent of serum concentrations. The growth of cell lines such as BT-474 and SK-BR-3 is inhibited by 4D5, whereas under the same conditions, they are growth stimulated by HRG. Additionally, HRG-mediated growth responses in cell lines known to express low/normal levels of ErbB2 such as MCF7, T47D, and Caov3 were inhibited by two other anti-ErbB2 antibodies, 2C4 and 7F3. To explain these results with HRG and 4D5, we speculate that ErbB2 heterodimerization with other ErbB family members is itself a high-affinity interaction and is preferred to ErbB2 homodimerization. This hypothesis is supported by the observation that HRG activation of ErbB2 occurs at a wide range of ErbB2 expression levels. In the present study, ErbB2 activation occurs by at least two different mechanisms, heterodimerization or homodimerization. In addition to ErbB2, the heterodimerization pathway requires HRG and a receptor for HRG, *i.e.*, ErbB3 or ErbB4. Monoclonal antibodies directed against ErbB2, such as 2C4 or 7F3 and to a lesser extent 4D5, are capable of disrupting these ErbB2-ErbB3/4 interactions so that HRG activation of ErbB2 is ablated. Constitutive activation of ErbB2, which is likely the result of ErbB2 homodimerization or oligomerization, occurs at high ErbB2 receptor densities and is independent of HRG, ErbB3, and ErbB4 expression. In agreement with this hypothesis is the observation that a threshold of ErbB2 expression must be surpassed to transform rodent fibroblast (61). Moreover, reversion of this transformed phenotype can occur by treatment with anti-ErbB2 monoclonal antibodies (27). Additionally, the growth inhibitory properties of anti-ErbB2 monoclonal antibodies are observed only on human tumor cell lines that overexpress ErbB2 (30). Thus, activation of ErbB2 by HRG or ErbB2 overexpression can result in the proliferation of tumor cell growth. As observed, antibodies that disrupt either of these activation pathways will have growth-inhibitory effects.

In comparison to HRG, treatment of these tumor cell lines with EGF under identical conditions produced only modest effects on growth proliferation. An exception was MDA-MB-468 cells, which were significantly growth inhibited by EGF (44, 45). Interestingly, the *in vitro* growth-inhibitory properties of EGF do not translate into a therapeutic benefit when the same cell lines are examined as xenografts *in vivo* (62, 63). *In vivo* experiments with MDA-MB-453 and MDA-MB-361 cells may be warranted given the growth-inhibitory effects observed with HRG treatment in the anchorage-independent growth assays. Recently, it has been demonstrated that HRG administration to athymic mice bearing MCF7 tumor xenografts is growth stimulatory to the tumors (42).

We conclude that expression of ErbB3 or ErbB4 with ErbB2 allow breast and ovarian tumor cells to be HRG responsive. The cell lines demonstrated to be HRG responsive do not express any known HRG isoform transcripts. HRG transcripts can be detected in fibroblasts as well as nontransformed breast cells and stromal elements in human breast tumors (53, 55, 64). These observations set the stage for potential paracrine stimulation of ErbB3/ErbB2- and ErbB4/ErbB2-expressing tumor cells and suggests that the interaction (53, 64) of HRG and its receptors may be important in transformation, tumor cell growth, or the maintenance of a transformed phenotype. Determination of the role that endogenous HRG plays in these processes may allow for therapies that target these receptors or this family of ligands.

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EXHIBIT E

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ErbB2 Potentiates Breast Tumor Proliferation through Modulation of $p27^{Kip1}$-Cdk2 Complex Formation: Receptor Overexpression Does Not Determine Growth Dependency

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Overexpression of the ErbB2 receptor, a major component of the ErbB receptor signaling network, contributes to the development of a number of human cancers. ErbB2 presents itself, therefore, as a target for antibody-mediated therapies. In this respect, anti-ErbB2 monoclonal antibody 4D5 specifically inhibits the growth of tumor cells overexpressing ErbB2. We have analyzed the effect of 4D5-mediated ErbB2 inhibition on the cell cycle of the breast tumor cell line BT474. 4D5 treatment of BT474 cells resulted in a G_1 arrest, preceded by rapid dephosphorylation of ErbB2, inhibition of cytoplasmic signal transduction pathways, accumulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, and inactivation of cyclin-Cdk2 complexes. Time courses demonstrated that 4D5 treatment redirects $p27^{Kip1}$ onto Cdk2 complexes, an event preceding increased $p27^{Kip1}$ expression; this correlates with the downregulation of c-Myc and D-type cyclins (proteins involved in $p27^{Kip1}$ sequestration) and the loss of $p27^{Kip1}$ from Cdk4 complexes. Similar events were observed in ErbB2-overexpressing SKBR3 cells, which exhibited reduced proliferation in response to 4D5 treatment. Here, $p27^{Kip1}$ redistribution resulted in partial Cdk2 inactivation, consistent with a G1 accumulation. Moreover, $p27^{Kip1}$ protein levels remained constant. Antisense-mediated inhibition of $p27^{Kip1}$ expression in 4D5-treated BT474 cells further demonstrated that in the absence of $p27^{Kip1}$ accumulation, $p27^{Kip1}$ redirection onto Cdk2 complexes is sufficient to inactivate Cdk2 and establish the G_1 block. These data suggest that ErbB2 overexpression leads to potentiation of cyclin E-Cdk2 activity through regulation of $p27^{Kip1}$ sequestration proteins, thus deregulating the G_1/S transition. Moreover, through comparison with an ErbB2-overexpressing cell line insensitive to 4D5 treatment, we demonstrate the specificity of these cell cycle events and show that ErbB2 overexpression alone is insufficient to determine the cellular response to receptor inhibition.

The ErbB family of type I receptor tyrosine kinases has four members, ErbB1/epidermal growth factor receptor, ErbB2/Neu, ErbB3, and ErbB4. Although these receptors share common structural elements, including an extracellular ligand-binding domain and an intracellular tyrosine kinase domain, ligands have been identified only for ErbB1, ErbB3, and ErbB4 (for a review, see reference 16). ErbB2 remains an orphan receptor, with no diffusible ErbB2-specific ligand identified. However, ErbB2 can be transactivated through heterodimerization with other ErbB family members (11, 62) and appears to be their preferred heterodimerization partner (23, 30). ErbB2-containing heterodimers couple potently to major mitogenic signaling cascades, such as the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3-kinase) pathways (16). Moreover, ErbB2 plays a role in the potentiation and prolongation of ErbB receptor signaling (4, 22, 30, 49).

The role of growth factors and their cognate receptors in cell growth and differentiation is now well established. Additionally, deregulation of growth factor receptors and/or elements of their signaling pathways occurs during the stepwise progression of a normal cell to a malignant phenotype. In this respect, two ErbB family members, ErbB1 and ErbB2, are involved in the development of many human cancers, including ovary and breast cancers. Indeed, amplification of the gene encoding ErbB2, leading to overexpression of the receptor, was one of the first consistent genetic alterations found in primary human breast tumors (6, 70, 71). Furthermore, overexpression of ErbB2 correlates with a poor patient prognosis not only in breast cancer (24, 59, 70, 71) but also in other malignancies, such as ovarian (71) and gastric (84) cancers. These observations suggest that ErbB2 overexpression provides tumor cells with a growth advantage leading to a more aggressive phenotype. It seems likely, therefore, that an ErbB2-dependent sustained mitogenic stimulus may contribute to the uncontrolled cell growth associated with tumor progression. This phenomenon is presumably due to the formation of active receptor dimers which signal even in the absence of ligand. In agreement with this hypothesis, treatment with ErbB2-specific antibodies has been shown to selectively inhibit the growth of tumor cells which overexpress ErbB2 (26, 27, 29, 37, 38). However, despite the obvious involvement of ErbB2 in tumor progression, the underlying mechanisms by which overexpression of this receptor potentiates tumor cell growth remain poorly understood.

In addition to perturbations in signal transduction networks, aberrant expression of key cell cycle regulators also contributes to deregulated cell proliferation during tumor development (reviewed in references 18 and 28). In nonimmortalized, somatic cells genetic integrity during cell division is maintained through the proper execution of an intrinsic cell cycle machinery. The replication, repair, and segregation of DNA must be accurately performed in order to prevent the genetic changes associated with malignant transformation. The major regulators of cell cycle progression are the cyclin-dependent kinases (Cdks), the periodic activation and inactivation of which reg-

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ulate not only progression through each cell cycle stage but also transitions from one cell cycle stage into another (for a review, see reference 47). In G_1, for example, passage of cells from growth factor dependency to growth factor independence (through the restriction point) is mediated by the sequential activation of cyclin D-dependent kinases Cdk4 and -6 and cyclin E-dependent kinase Cdk2 (for a review, see reference 68). These kinases phosphorylate and inactivate growth suppressor proteins of the retinoblastoma protein (pRb) family, allowing the expression of genes whose activities are required for S-phase entry (75). The importance of this pathway in growth control is highlighted by the fact that many of its components are commonly mutated, deleted, or aberrantly expressed in human cancer (for reviews, see references 18 and 28).

The activity of G_1 Cdks is stringently regulated, not only by association with specific regulatory cyclin subunits and phosphorylation/dephosphorylation events but also through association with specific Cdk inhibitors (CKIs) (47, 67, 68). There are two classes of CKIs, the INK4 proteins (INK4a to -d), which act specifically on cyclin D-dependent kinases, and the CIP/KIP family (p21$^{Cip1/Waf1}$, p27^{Kip1}, and p57^{Kip2}), which bind all G_1 cyclin-Cdk complexes (67, 68). In fibroblasts, regulation of p27^{Kip1} function is an essential step in the pathway linking mitogenic signals to passage through the restriction point (14). This is thought to be due to p27^{Kip1}-mediated regulation of cyclin E-Cdk2 activity. Indeed, in vitro, p27^{Kip1} is a more effective inhibitor of cyclin E-Cdk2 than of cyclin D-Cdk4 (56, 76). Additionally, in vivo, p27^{Kip1} mediates inhibition of cyclin E-Cdk2 in cells that are exposed to growth-inhibitory agents (57, 72).

Although it was first assumed that CKIs act solely as inhibitors of Cdk complexes, members of the CIP/KIP family also promote the assembly of Cdk4-cyclin D complexes (34). Indeed, both p21$^{Cip1/Waf1}$ and p27^{Kip1} are essential for Cdk4-cyclin D activity (13), being found in active kinase complexes in proliferating cells (8, 34, 73, 85). Furthermore, the sequestration of p27^{Kip1} (and p21$^{Cip1/Waf1}$) into higher-order complexes with cyclin D-dependent kinases appears to play a role in the activation of cyclin E-Cdk2 as cells progress through late G_1. In this respect, the proto-oncogene *c-myc*, which is clearly involved in the regulation of cyclin E-Cdk2 activity (7, 36), has been shown to play a major role in p27^{Kip1} sequestration through modulation of cyclin D protein levels (10, 54), as well as possibly other unknown p27^{Kip1} sequestration proteins (2, 77). This suggests that a number of p27^{Kip1}-sequestering proteins may exist. The relative contribution of each to cell cycle control may depend on cellular context.

In this study, we have addressed the question of why ErbB2 overexpression in tumors is associated with more aggressive growth characteristics. In this regard, an anti-ErbB2 monoclonal antibody (MAb 4D5), directed to the extracellular domain of the receptor, has been previously shown to specifically inhibit the growth of tumor cells overexpressing the ErbB2 receptor (27, 37, 38). These observations suggest that the growth of tumors overexpressing ErbB2 may be potentiated by increased ErbB2 receptor signaling. To gain insight into the consequence of ErbB2 overexpression for tumor development, we have examined how receptor overexpression impinges on cytoplasmic signaling pathways and elements of cell cycle control by analyzing the molecular mechanism of action of this growth-inhibitory antibody. We show that 4D5 treatment of BT474 cells, a human breast carcinoma cell line overexpressing ErbB2, results in a stable G_1 accumulation. This correlates with rapid downregulation of ErbB2 receptor signaling, increased p27^{Kip1} levels, and inactivation of the cyclin E-Cdk2 complex. We further demonstrate that ErbB2 receptor inhibition leads to a redistribution of p27^{Kip1} protein onto Cdk2 complexes. This event precedes increases in p27^{Kip1} expression, paralleling the loss of proteins involved in p27^{Kip1} sequestration, and is sufficient to totally inhibit Cdk2 activity and establish the G_1 block. These data suggest that in breast tumor cells ErbB2 overexpression provides an essential signaling element, leading to the potentiation of cyclin E-Cdk2 activity through sequestration of the CKI p27^{Kip1}. Analysis of a second overexpressing cell line (SKBR3), which exhibits reduced proliferation in response to 4D5 treatment, supports this hypothesis. Furthermore, through comparison with an ErbB2-overexpressing, gastric carcinoma cell line (MKN7) insensitive to 4D5 treatment, we demonstrate that the growth response to 4D5-mediated inhibition of ErbB2 receptor function is tumor specific and may correlate with ErbB receptor expression profiles and/or the absence of compensatory mitogenic signaling pathways.

MATERIALS AND METHODS

Cell culture, growth assays, lysate preparation, and flow cytometry. Breast carcinoma (BT474, T47D, and SKBR3) cells were obtained from the American Type Culture Collection (Manassas, Va.) and grown in Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, Md.), supplemented with 10% fetal calf serum, at 37°C and 5% CO_2. Gastric carcinoma (MKN7) cells were kindly provided by C. Benz (University of California, San Francisco) and cultured as described above except that the Dulbecco's modified Eagle's medium was mixed 1:1 with Ham's F-12 (GIBCO BRL). BT474, SKBR3, and MKN7 cells were considered ErbB2 overexpressors by the criterion that they express approximately 0.5×10^6 to 1.0×10^6 receptors/cell (I. Harwerth and N. E. Hynes, unpublished results; see also reference 37). For growth assays, cells were plated at a density of 2,000 cells/cm^2 or as stated in the text. After 24 h of incubation, the medium was changed and either the purified mouse MAb 4D5 (kindly supplied by Genentech, Inc., South San Francisco, Calif.) or FRP5 (25) was added to a final concentration of 10 μg/ml. Both of these antibodies are of the isotype immunoglobulin G1. Cells were trypsinized at the times stated and counted in a hemocytometer. Cells grown for more than 4 days were refed with fresh medium, with or without antibody, on day 4.

For direct measurements of DNA synthesis, cells were seeded onto acid-washed glass coverslips and cultured in the presence of antibody as described above. After the times stated, bromodeoxyuridine (BrdU) was added for 4 h, the cells were fixed, and BrdU incorporation into nuclei was revealed by immunofluorescence as previously described (35). Cells were counted, and the percentage with BrdU-labeled nuclei was calculated.

For preparation of protein lysates, cells were plated at a density of 3×10^4 cells/cm^2. After 24 h of incubation, the medium was changed and 4D5 or FRP5 was added to a final concentration of 10 μg/ml. At the times indicated, cells were first washed with ice-cold phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and then with buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 25 mM β-glycerophosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 15 mM pyrophosphate, 2 mM sodium orthovanadate, 10 mM sodium molybdate, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and 1 mM phenylmethylsulfonyl fluoride (protease inhibitors from Sigma Chemical, St. Louis, Mo.). Cells were extracted in the same buffer containing 1% NP-40. After homogenization, lysates were clarified by centrifugation and frozen at −80°C. Protein concentrations were determined with the Bio-Rad (Munich, Germany) protein assay reagent.

To analyze the cell cycle profile of cells, cultures were seeded and treated with antibody as for the preparation of protein lysates. At the times indicated, cells were trypsinized, washed twice with ice-cold PBS, and resuspended in propidium iodide buffer (1 mM sodium citrate [pH 4.0], 1.5 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40, 4 μg of propidium iodide/ml, and 80 μg of RNase A/ml in PBS). After 30 min of incubation on ice, cell cycle distribution was monitored with a Becton Dickinson FACScan flow cytometer.

Immunological techniques. For immunoblot analysis of cell cycle regulators and signal transducers, clarified protein lysates (30 to 50 μg) were electrophoretically resolved on denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gels (7.5 to 14%), transferred to polyvinylidene difluoride (Boehringer Mannheim GmbH, Mannheim, Germany), and probed with the following primary antibodies: anti-cyclin A and -p45^{SKP2} (kindly supplied by W. Krek, Friedrich Miescher Institute, Basel, Switzerland); anti-c-Myc (9E10), -cyclin E (C-19), -cyclin D2 (C-17), -cyclin D3 (C-16), -Cdk2 (M2), and -Cdk4 (C-22; from Santa Cruz Biotechnology, Santa Cruz, Calif.); anti-cyclin D1 (DCS-6; Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom); anti-pRb (G3-245; Pharmingen, San Diego, Calif.); anti-p27^{Kip1} (Transduction Laboratories, Lexington, Ky.); and anti-protein kinase B (PKB), -phospho-PKB (serine 473), -Erk1/2, and -phospho-Erk1/2 (threonine 202/tyrosine 204; New England Biolabs, Inc., Bev-

erley, Mass.). Decorated proteins were revealed using horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins followed by enhanced chemiluminescence (ECL kit; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

For Cdk2-$p27^{Kip1}$ and Cdk4-$p27^{Kip1}$ coimmunoprecipitation experiments, clarified protein lysates (70 to 150 μg for Cdk2 and 350 μg for Cdk4) were precleared with 10 μl of protein A Sepharose (Sigma) and then precipitated with 2 μg of anti-Cdk2 (M2) or anti-Cdk4 (H-22; Santa Cruz Biotechnology) antibody previously coupled to protein A-Sepharose. Beads were washed thoroughly in extraction buffer, and Cdk-$p27^{Kip1}$ levels were analyzed by immunoblotting as described above.

For analysis of ErbB receptor protein and phosphotyrosine levels, protein extracts were either precipitated with an antibody specific for ErbB1 (500 μg with antibodies 528 and R-1 mixed 1:1), ErbB3 (400 μg with antibody C-17), ErbB4 (500 μg with antibody C-18; from Santa Cruz Biotechnology), or ErbB2 (60 to 200 μg with antibody 21N; specific for the intracellular domain of the receptor [49]). ErbB protein and tyrosine phosphorylation levels were analyzed by immunoblotting as described above, using anti-ErbB1 (1005; Santa Cruz Biotechnology), ErbB3 (C-17), ErbB4 (C-18), or ErbB2 (21N) antibody and a phosphotyrosine-specific MAb as previously described (49). Stripping of membranes for reprobing was performed as previously described (49).

Histone H1 kinase assays. Histone H1 kinase assays were performed using Cdk2 (M-2) immunoprecipitates (from 50 μg of lysate protein) or cyclin E (C-19) immunoprecipitates (from 75 μg of lysate protein) as previously described (81) except that the amount of histone H1 (Boehringer Mannheim) per assay was increased to 5 μg and the final reaction volume was reduced to 20 μl. Phosphorylated proteins were resolved by SDS-polyacrylamide gel (10%) electrophoresis (PAGE) and analyzed by autoradiography and scintillation counting.

$p27^{Kip1}$ antisense assays. Antisense and mismatch $p27^{Kip1}$ phosphorothioate oligonucleotides, modified by the addition of a propynyl group to the pyrimidine bases, were prepared and purified by reversed-phase chromatography by Microsynth (Balgach, Switzerland). Due to sequence conservation between the murine and human $p27^{Kip1}$ genes, the antisense and mismatch sequences utilized were the same as those previously used for the inhibition of $p27^{Kip1}$ expression in murine fibroblasts (14). BT474 cells were plated as stated above for the preparation of cell lysates. After 24 h of incubation, cells were treated with 50 nM oligonucleotides mixed with LipofectAMINE (or LipofectAMINE alone as a control) for 5.5 h as instructed by the manufacturer (GIBCO BRL). After this time, cells were washed and refed with normal culture medium. The cells were then allowed to recover for 3 to 5 h before the addition of antibody 4D5 (10 μg/ml). At the times stated (i.e., subsequent to antibody addition), cells were either extracted for lysate production or trypsinized for flow cytometry as described above.

In vivo [^{32}P]orthophosphate-labeled tryptic phosphopeptide mapping of the ErbB2 receptor. Cells were cultured as stated above for the preparation of cell lysates. After 24 h of incubation, cells were deprived of phosphate for 12 h (using phosphate-free medium supplemented with 10% normal phosphate-containing medium) and labeled with [^{32}P]orthophosphate (Amersham) for 4 h. In the continued presence of [^{32}P]orthophosphate, cells were subsequently treated with antibody 4D5 or FRP5 (10 μg/ml) for 1 h; equal cell numbers were extracted for each treatment (twice as many MKN7 as BT474 cells were extracted due to the lower stoichiometry of ErbB2 phosphorylation in this cell line), and the ErbB2 receptor was immunoprecipitated as described above. Phosphorylated ErbB2 was excised from the gel, and tryptic phosphopeptide mapping was performed as previously described (49).

RESULTS

Treatment of BT474 cells, but not MKN7 cells, with MAb 4D5 induces a stable growth arrest. Primary tumors overexpressing ErbB2 show a more aggressive phenotype which is associated with poor patient prognosis. The precise role of ErbB2 overexpression in tumor development, however, is not determined. To address this question, we have screened a number of ErbB2-overexpressing tumor cell lines for MAb 4D5 sensitivity. An isotype-matched MAb (FRP5), which recognizes the extracellular domain of ErbB2 but is not growth inhibitory (25, 26), was used as a negative control. From this analysis, and in agreement with previous work (37, 38), the growth of the breast carcinoma cell line BT474 was found to be drastically inhibited by 4D5 treatment over a 7-day period (Fig. 1A, bottom). This correlated with a 10-fold decrease in S-phase fraction, as determined by pulse-labeling of antibody-treated cells with BrdU followed by immunofluorescence (Fig. 1B). In contrast, the growth of MKN7 cells, an ErbB2-overexpressing, gastric carcinoma cell line, was unaffected by 4D5 (Fig. 1A, top; see also reference 37). In both cell lines, FRP5

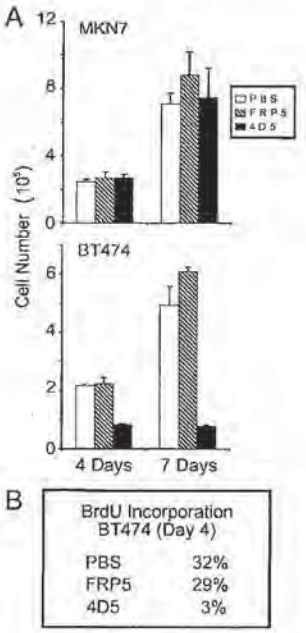


FIG. 1. Proliferation assays of BT474 and MKN7 cells treated with anti-ErbB2 antibodies. MKN7 and BT474 cells were seeded at a density of 2,000 cells/cm^2; after 24 h of incubation, the medium was changed and either MAb FRP5 or MAb 4D5 was added to a final concentration of 10 μg/ml, or an equal volume of PBS was added. After 4 and 7 days of incubation, cells were trypsinized and total cell number was calculated (A), or after 4 days, cells were pulse-labeled with BrdU for 4 h and BrdU incorporation into nuclei was revealed by immunofluorescence (B). Shown are the percentages of nuclei labeled with BrdU during the pulse period.

had little effect; after 7 days of incubation, FRP5-treated cells displayed slightly increased cell numbers compared to untreated controls (Fig. 1A), possibly due to the partially agonistic effects of this antibody on the ErbB2 receptor (25, 41). These data indicate, therefore, that although both of these cell lines overexpress ErbB2 (Fig. 2A; see Materials and Methods), they exhibit quite different responses to 4D5 treatment. This variability between cellular responses to 4D5 treatment has been previously reported (37, 38) and may reflect different dependencies on ErbB2 overexpression among tumor cell lines.

MAb 4D5-mediated growth inhibition is independent of effects on receptor phosphorylation. Both MAb FRP5 and 4D5 efficiently bind the extracellular domain of ErbB2 (25, 37). The growth defect seen, therefore, is presumably caused by a sustained antibody-specific effect on the ErbB2 receptor, which in BT474 cells is manifested by growth inhibition. Immunoblot analysis of ErbB receptor immunoprecipitates revealed that BT474 and MKN7 cells express quite different ErbB receptor complements (Fig. 2A). While both clearly overexpressed ErbB2, in contrast to a low-expressing breast tumor cell line (T47D), ErbB2 derived from BT474 cell extracts was highly phosphorylated in comparison with that derived from MKN7 (Fig. 2A, compare top and bottom panels). This elevated ErbB2 tyrosine phosphorylation correlated with coexpression of ErbB3 (Fig. 2A, top), the preferred and most potent heterodimerization partner of ErbB2 (55, 79), and suggests that ErbB2 is more active as a tyrosine kinase in BT474 cells than in MKN7 cells. Interestingly, although ErbB3 protein could

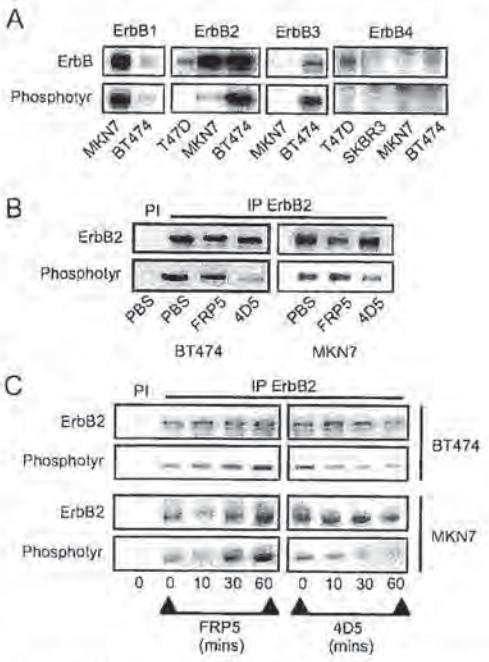


FIG. 2. Screen of ErbB receptor protein and tyrosine phosphorylation levels and effects of anti-ErbB2 antibody treatment on tyrosine phosphorylation of the ErbB2 receptor in BT474 and MKN7 cells. Cells were seeded at a density of 3×10^4 cells/cm^2. After 24 h of incubation, extracts were made and ErbB receptor protein (ErbB) and phosphotyrosine (Phosphotyr) levels were analyzed following immunoprecipitation of the appropriate receptor (A). Cells were seeded as described above, and either MAb FRP5 or MAb 4D5 was added to a final concentration of 10 μg/ml or an equal volume of PBS was added. Extracts were prepared, and ErbB2 protein and phosphotyrosine levels were assessed after 48 h of incubation (B) or at the times indicated (C) following immunoprecipitation (IP) with the ErbB2-specific polyclonal antibody 21N. Preimmune control precipitations are indicated (PI). In panels B and C, longer exposures were required for MKN7 cells due to the lower ErbB2 tyrosine phosphorylation levels in these cells than in BT474 cells. The exposures shown, therefore, do not represent a quantitative comparison of the two cell lines but were chosen to most clearly represent phosphorylation changes induced as a result of 4D5 treatment.

not be detected by immunoblotting in MKN7 cells, ErbB1 was highly expressed and highly phosphorylated in this cell line (Fig. 2A, top and bottom panels). In BT474 cells, however, ErbB1 expression was equivalent to that of a moderately expressing cell line (data not shown). ErbB4 protein, in contrast, was just detectable in BT474 cells compared to a known nonexpressing (SKBR3) and moderately expressing (T47D) cell line. No ErbB4 was detected in MKN7 cells, and in all cell lines, tyrosine phosphorylation was undetectable (Fig. 2A, compare top and bottom panels), indicating that ErbB4 is not a major signaling element in these cells in the culture conditions used. These observations suggest that the relative activity of overexpressed ErbB2 may depend on the coexpression of other ErbB receptors, such as ErbB3. Furthermore, in MKN7 cells, overexpression of ErbB1 alone is insufficient to fully activate ErbB2.

To address the question of whether differences in cellular response to 4D5 treatment are reflected by differential effects on ErbB2 receptor signaling capacity, immunoblot analyses of ErbB2 receptor protein and phosphotyrosine levels after antibody treatment were performed. After 48 h of 4D5 treatment, a dramatic decrease in ErbB2 tyrosine phosphorylation was observed in BT474 cells (Fig. 2B, left). This was accompanied by a resultant increase in the electrophoretic mobility of the receptor. However, no significant decrease in ErbB2 receptor levels was observed, even after these long treatment times. Furthermore, a similar analysis of 4D5-treated MKN7 cells also revealed receptor dephosphorylation with no effect on protein levels (Fig. 2B, right). To analyze the kinetics of receptor dephosphorylation, time courses of 4D5 treatment of BT474 and MKN7 cells, with FRP5 as a control, were performed. Strikingly, decreased ErbB2 tyrosine phosphorylation was observed within 10 min of 4D5 treatment in both cell lines, and this lower level was maintained for 1 h (Fig. 2C). Decreases in total receptor phosphorylation were also observed if cells were cultured in medium containing $^{32}P_i$ and subsequently treated for 1 h with 4D5, followed by immunoprecipitation of the ErbB2 receptor (Fig. 3A). Additionally, in agreement with previous reports (25, 41), FRP5 treatment of both cell lines rapidly induced ErbB2 phosphorylation (Fig. 2C and 3A). Taken together, these data indicate that treatment of BT474 or MKN7 cells with MAb 4D5 or FRP5 results in comparable effects on ErbB2 phosphorylation.

To determine whether the 4D5-induced changes in ErbB2 receptor phosphorylation was due to dephosphorylation of specific sites or represented a general effect on receptor phosphorylation, tryptic phosphopeptide mapping of $^{32}P_i$-labeled ErbB2 immunoprecipitates from MAb-treated cell lines, as in Fig. 3A, was performed. In vivo-labeled ErbB2 from untreated, asynchronously growing BT474 cells exhibited phosphopeptide maps similar to those observed in MKN7 cells (Fig. 3B, compare top panels). As expected from the above results, FRP5 treatment of BT474 cells induced increases in the phosphorylation of a number of phosphopeptides (i.e., a through d) while not affecting the relative phosphorylation state of others (i.e., e and f; Fig. 3B, bottom left). In contrast, 4D5 treatment of BT474 cells led to the disappearance of b, c, and d and to a decrease in the intensity of a, e, and f, suggesting that it caused a general dephosphorylation of the ErbB2 receptor (Fig. 3B, bottom right). Quantitatively similar results were observed in MKN7 cells (data not shown). In conclusion, therefore, treatment with 4D5 induces general receptor dephosphorylation in both BT474 and MKN7 cells.

MAb 4D5 treatment inhibits cytoplasmic signaling in BT474 cells but not in MKN7 cells. ErbB2 plays a pivotal role in ErbB receptor-mediated activation of the major cytoplasmic, mitogenic signaling pathways, such as the MAP kinase and PI3-kinase pathways (4, 16, 22, 30, 49). We therefore investigated the effects of 4D5 treatment on these pathways in both BT474 and MKN7 cells (Fig. 4). As a readout for the MAP kinase and PI3-kinase pathways, the activation states of the Erk1/2 protein kinases and PKB, respectively, were measured by immunoblotting with antibodies specific for activating phosphorylation sites (see Materials and Methods). Consistent with effects on ErbB2 receptor phosphorylation (Fig. 2C), a dramatic decrease in PKB phosphorylation was observed within 10 min of 4D5 treatment in BT474 cells (Fig. 4, middle). This reduction was maintained for at least 4 h (Fig. 4 and data not shown). Equivalent effects on Erk1/2 phosphorylation were not observed, although a reduction in phosphorylation was seen at later times (Fig. 4, bottom, and data not shown). In this respect, however, in contrast to PKB phosphorylation, Erk1/2 phosphorylation appeared to be comparatively low in BT474 cells and was dramatically induced by FRP5 treatment (Fig. 4, middle and bottom; compare BT474 and MKN7). This indicates that the MAP kinase pathway may not be optimally activated under normal growth conditions in these cells. Surprisingly, a similar analysis of MKN7 cells gave no indication of 4D5-mediated downregulation of either of these pathways (Fig. 4, middle and bottom). A slight increase in Erk1/2 and

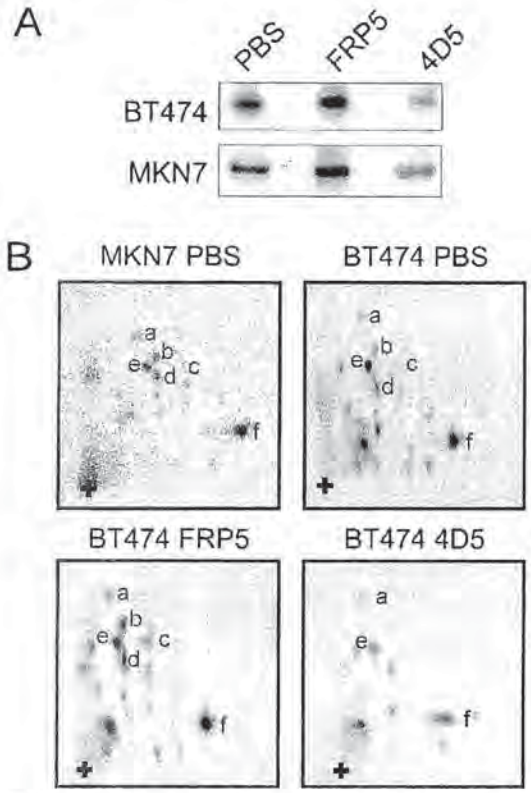


FIG. 3. In vivo [^{32}P]orthophosphate labeling of the ErbB2 receptor in BT474 and MKN7 cells after anti-ErbB2 antibody treatment; tryptic phosphopeptide mapping. Cells were seeded as in Fig. 2. Twice as many MKN7 cells as BT474 cells were seeded (see Materials and Methods). After 24 h of incubation, cells were deprived of phosphate for 12 h and then prelabeled with [^{32}P]orthophosphate followed by addition of MAb FRP5, MAb 4D5, or an equal volume of PBS for 1 h. Equal amounts of BT474 cell extracts (doubled in the case of MKN7 extracts) were immunoprecipitated with the anti-ErbB2 antibody 21N, and labeled proteins were identified by separation using SDS-PAGE (7.5% gel) (A). The labeled ErbB2 receptor protein was excised and analyzed by tryptic phosphopeptide mapping (B). Specific tryptic phosphopeptides are indicated by letters, and the origin is shown by a plus sign. Gels and tryptic phosphopeptide maps were analyzed with a phosphorimager. Although MKN7 samples were exposed for longer than BT474 samples, due to lower stoichiometry of phosphorylation, the same exposure is shown for each treatment protocol. The amount of label incorporated into the immunoprecipitated ErbB2 protein in panel A was quantified by scintillation counting. BT474 cells treated with MAb FRP5 and MAb 4D5 had 158 and 60% ^{32}P incorporation, respectively, compared to the control (PBS)-treated cells. MKN7 cells treated with MAb FRP5 and MAb 4D5 had 139 and 75% ^{32}P incorporation, respectively, compared to the control (PBS)-treated cells.

PKB phosphorylation was observed after 10 min of 4D5 treatment. However, this was not apparent at later times and was shown to be due to the medium change at the beginning of the experiment (Fig. 4 and data not shown). These data demonstrate that despite similar effects of 4D5 treatment on receptor phosphorylation levels in both BT474 and MKN7 cells, only BT474 cells exhibit downstream effects on cytoplasmic signaling molecules. This suggests that receptor dephosphorylation alone is insufficient to determine the cellular response of ErbB2-overexpressing tumor cells to 4D5-induced receptor inhibition.

MAb 4D5 treatment induces a G_1 arrest in BT474 cells which correlates with accumulation of $p27^{Kip1}$ and Cdk2 inactivation. To more accurately define the 4D5-specific growth

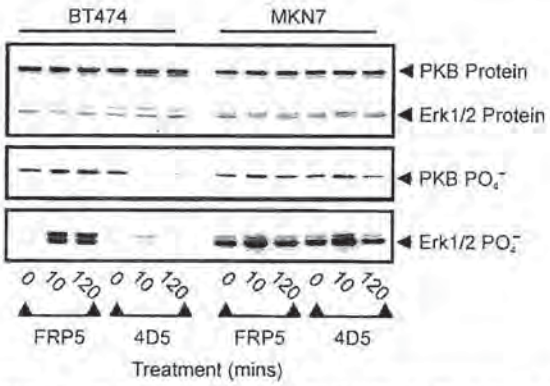
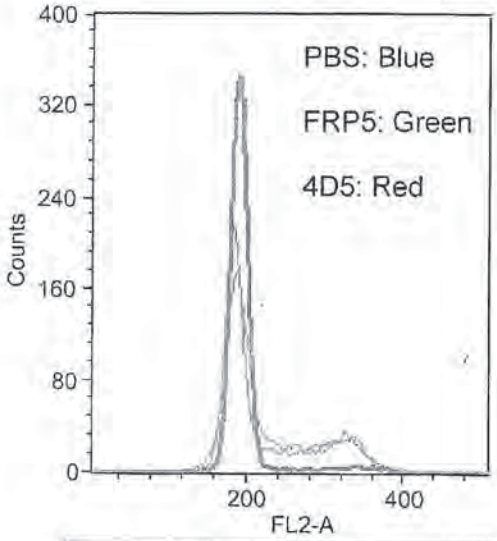


FIG. 4. Analysis of PKB and Erk1/2 phosphorylation after treatment of BT474 and MKN7 cells with anti-ErbB2 antibodies. Cells were seeded and treated with MAb FRP5 or MAb 4D5 as in Fig. 2. At the times indicated, cells were extracted and the protein levels (top) and phosphorylation (PO_4^-) states of PKB (middle) and Erk1/2 (bottom) were evaluated by immunoblotting. Untreated cells ($t = 0$) were included as controls.

suppression of BT474 cells, the cell cycle profiles of antibody-treated cells were analyzed after 48 h treatment using flow cytometry. As expected from previous proliferation assays (Fig. 1), FRP5 had no effect on cell cycle distribution compared to untreated controls (Fig. 5). However, 96% of 4D5-treated cells accumulated in G_1 (Fig. 5). This G_1 arrest was stable for up to a week (data not shown), indicating that these cells were blocked in the ability to progress into S phase of the cell cycle. No evidence of apoptosis was observed. Indeed, if 4D5 was removed from the culture medium, the cells were able to reenter the cell cycle (data not shown), confirming that this is a cytostatic rather than cytotoxic agent.

To identify the molecular basis of this G_1 arrest, an initial screen of effects on the levels and activity of G_1 regulators was performed. For this, BT474 cell extracts were prepared after 48 h treatment with MAb 4D5 or FRP5 and analyzed by immunoblotting (Fig. 6A and B). As expected, markers of S-phase progression (cyclin A and $p45^{SKP2}$) as well as G_2/M (cyclin B1) were almost completely absent in 4D5-treated cells. Additionally, pRb was found exclusively in its hypophosphorylated, active, growth suppressor state. FRP5 treatment, in contrast, had no effect on the expression of these proteins or on pRb phosphorylation (Fig. 6A). Analysis of G_1 Cdk and cyclin levels demonstrated no changes in Cdk4, Cdk6, cyclin E, or cyclin D1 expression, whereas cyclin D2 and D3 levels were reduced in 4D5-treated cells (Fig. 6A and B). Most strikingly, however, the CKI $p27^{Kip1}$ was dramatically increased in 4D5-treated cells, a phenomenon correlating with the disappearance of the faster-migrating, active form of Cdk2 (Fig. 6B). Immunoprecipitation of Cdk2, followed by either immunoblotting for associated $p27^{Kip1}$ or by histone H1 kinase assay, showed an increase in Cdk2-$p27^{Kip1}$ association and almost complete Cdk2 inactivation (Fig. 6C). No effects on the protein levels of other CKIs (including $p21^{Cip1/Waf1}$, $p57^{Kip2}$, $p15^{INK4b}$, and $p16^{INK4a}$) were observed (data not shown). Surprisingly, multiple independent kinase assays revealed that Cdk4 activity (the major cyclin D-dependent kinase expressed in BT474 cells) remained little affected in these experiments despite decreased cyclin D levels (data not shown). As cyclin D proteins are not completely lost, this maintenance of activity could be due to there being sufficient cyclin D to maintain Cdk4 activity. It should also be noted that FRP5-treated cells showed reproducible increases in cyclin D1 protein expression as well



% Cell Cycle Stage

	G1	S	G2/M
PBS	67	12	21
FRP5	65	16	20
4D5	96	1	3

FIG. 5. Analysis of cell cycle distribution after treatment of BT474 cells with anti-ErbB2 antibodies. BT474 cells were seeded as in Fig. 2. After 24 h of incubation, the medium was changed, and MAb FRP5, MAb 4D5, or PBS was added as in Fig. 2. After 48 h, cells were harvested by trypsinization and nuclei were stained with propidium iodide. Shown is flow cytometry analysis of antibody-treated cells compared to PBS-treated controls (top). Percentages of cells in each cell cycle stage are indicated (bottom).

as increased Cdk2 activity (Fig. 6A and C). The partial agonistic effect of this antibody could explain this phenomenon (25, 41). Taken together, these data indicate that 4D5 treatment of BT474 cells induces increased p27^{Kip1} protein levels, leading to the inhibition of Cdk2, events which do not occur with a noninhibitory control antibody.

To further determine a possible relationship between increased p27^{Kip1} expression and the G_1 block, BT474 cells were treated for 4 to 48 h with 4D5 and, at the times shown (Fig. 7), either extracted and analyzed for markers of G_1 arrest by Western blotting (Fig. 7B) or trypsinized, and the cell cycle profile was determined by flow cytometry (Fig. 7A). Untreated cells (time [t] = 0 h) and cells treated for 48 h with FRP5 were used as controls. The results showed that 4D5-induced effects on pRb phosphorylation, as well as on cyclin A, cyclin B1, and p45^{SKP2} levels, became apparent only between 24 and 36 h (Fig. 7B), coincident with a large proportion of cells having accumulated in G_1 (Fig. 7A; 87 and 96% in G_1 at 24 and 36 h, respectively). In contrast, p27^{Kip1} levels increased after 8 h, well before the G_1 block was evident (77.5 and 74% in G_1 at 0 and 8 h, respectively). The timing of p27^{Kip1} accumulation would, therefore, suggest that p27^{Kip1}-mediated inhibition of Cdk2-cyclin E activity is the direct cause of the G_1 block.

MAb 4D5-induced Cdk2 inactivation parallels increased p27^{Kip1}-Cdk2 association, downregulation of D-type cyclins and the c-Myc transcription factor, and loss of p27^{Kip1} from Cdk4 complexes. The above data support the hypothesis that in BT474 cells inhibition of ErbB2 receptor function through 4D5 treatment induces p27^{Kip1} protein accumulation by an unknown mechanism. However, a closer analysis of the kinetics of Cdk2 inactivation demonstrated that after only 2 h of 4D5 treatment, Cdk2 activity had already decreased (Fig. 8A) despite no increase in p27^{Kip1} levels at this time (Fig. 8B). Indeed, after 8 h, when p27^{Kip1} levels were just starting to increase, total Cdk2 activity had decreased to approximately 50% of normal levels, reaching minimum levels after 24 h when p27^{Kip1} protein levels were not yet maximal (Fig. 8). Furthermore, similar kinetics of inactivation were seen if Cdk2 activity was measured after immunoprecipitation of cyclin E (Fig. 8A). Subsequent analysis of Cdk2-p27^{Kip1} association, in immunoprecipitates from the same samples, revealed that p27^{Kip1} started to accumulate on Cdk2 within 2 h of 4D5 treatment, reaching a peak after 16 to 24 h (Fig. 8B). At later times (36 to 48 h), less p27^{Kip1} appeared to be associated with Cdk2. However, this was coincident with decreased Cdk2 levels which, along with reduced pRb expression (Fig. 6A), was always observed after longer treatments with 4D5 and may represent a delayed program of adaptation to the G_1 block.

Increased association of p27^{Kip1} with Cdk2 correlates with Cdk2 inactivation in 4D5-treated BT474 cells, and both events begin prior to the accumulation of p27^{Kip1} protein. One must consider, therefore, that 4D5 treatment results in the rapid release of an intracellular pool of sequestered p27^{Kip1} protein. Cyclin D-dependent kinase complexes, as well as the c-Myc transcription factor, play major roles in the regulation of p27^{Kip1} sequestration (see the introduction). With this in mind, therefore, we performed a more detailed time course of 4D5 treatment and analyzed the extracts for correlations between changes in p27^{Kip1}-Cdk2 association and c-Myc or cyclin D protein expression (Fig. 9A). Strikingly, c-Myc protein levels decreased within 1 h of 4D5 addition, reaching a minimum level within 2 h. This decrease correlated exactly with the initial accumulation of p27^{Kip1} on Cdk2 in the same samples (Fig.

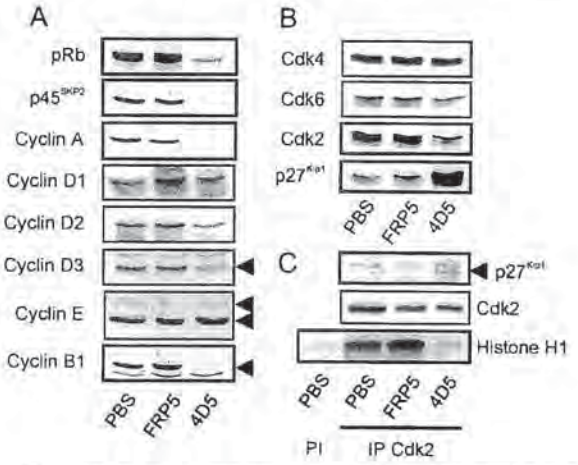


FIG. 6. Analysis of G_1 regulators in anti-ErbB2 antibody-treated BT474 cells. BT474 cells were seeded and treated with MAb FRP5, MAb 4D5, or PBS as in Fig. 2. After 48 h, cells were extracted and the protein levels of G_1 regulators were evaluated by immunoblotting (A and B). Additionally, p27^{Kip1} association with Cdk2 complexes, and Cdk2 activity, was assessed through immunoprecipitation (IP) of Cdk2 followed by immunoblotting for associated p27^{Kip1} protein or in vitro histone H1 kinase assay (C). Preimmune control precipitations are indicated (PI).

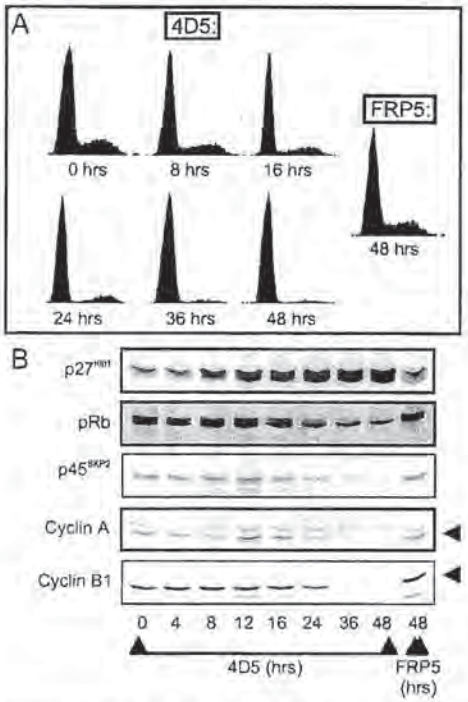


FIG. 7. Time course of the effect of MAb 4D5 treatment on the cell cycle distribution and the levels of cell cycle markers in BT474 cells. BT474 cells were seeded and treated with MAb FRP5 or MAb 4D5 as in Fig. 2. At the times shown, cells were trypsinized, and half of the sample was either stained with propidium iodide and analyzed for cell cycle distribution using flow cytometry (A) or extracted for immunoblot analysis of the proteins indicated (B). Untreated cells (t = 0) and cells treated for 48 h with MAb FRP5 were included as controls.

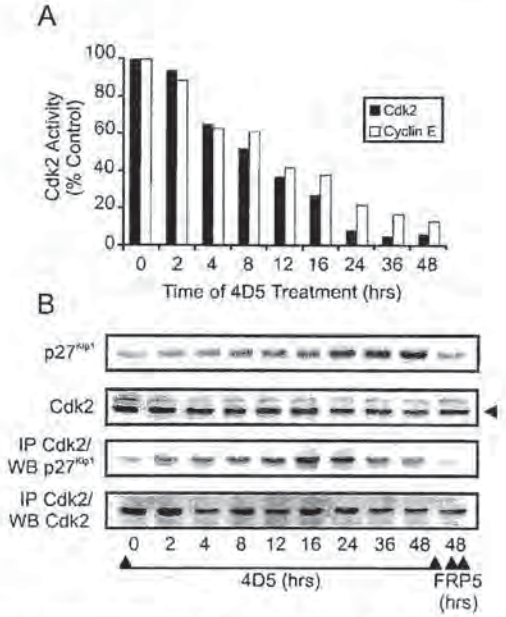


FIG. 8. Time course of Cdk2 inactivation and Cdk2-p27Kip1 complex formation in MAb 4D5-treated BT474 cells. BT474 cells were seeded and treated with MAb FRP5 or MAb 4D5 as in Fig. 2. At the times indicated, cell extracts were prepared and immunoprecipitated with Cdk2-specific or cyclin E-specific antibodies followed by in vitro histone H1 kinase assay (A). Additionally, the levels of $p27^{Kip1}$ and Cdk2 protein in the same extracts were either analyzed directly by immunoblotting (WB) or after immunoprecipitation (IP) with Cdk2-specific antibodies (B). Untreated cells (t = 0) and cells treated with MAb FRP5 for 48 h were included as controls. Cdk2 kinase activity is expressed as percentage of control (t = 0) cells.

9A) but was transient, as c-Myc protein levels began to recover after 16 h, reaching normal levels after 36 to 48 h (Fig. 9A and data not shown). Cyclin D levels also decreased with similar kinetics (Fig. 9A). The previous immunoblot analysis (Fig. 6A) demonstrated that after 48 h of 4D5 treatment, cyclin D1 was present at normal levels. Subsequent analysis of longer time courses indicated that, as with c-Myc, cyclin D1 levels did indeed recover at later times, reaching normal levels by 48 h (not shown). Taken together, the rapid downregulation of c-Myc and cyclin D proteins provides an explanation for the redistribution of $p27^{Kip1}$ onto Cdk2 complexes after 4D5 treatment. Indeed, in this context, loss of $p27^{Kip1}$ from cyclin D-Cdk4 complexes was observed after 4D5 treatment and correlated with increased $p27^{Kip1}$-Cdk2 complex formation (Fig. 9B).

Redirection of $p27^{Kip1}$ protein onto Cdk2 complexes occurs in the absence of increased $p27^{Kip1}$ expression in SKBR3 cells: correlation with partial Cdk2 inactivation and reduced proliferation levels. To extend the observations made in BT474 cells, a similar cell cycle analysis was performed on a second ErbB2-overexpressing cell line (SKBR3) sensitive to 4D5 treatment. Intriguingly, growth assays demonstrated that the proliferation rate of these cells was reproducibly reduced by approximately 50% as a result of 4D5 treatment (Fig. 10A), indicating that these cells were less sensitive to antibody-mediated ErbB2 receptor inhibition than BT474 cells. This decrease in proliferation rate was not dependent on cell density (Fig. 10A, compare Expt. 1 with Expt. 2) and correlated with a 10 to 15% increase in the proportion of cells in G_1 as judged by flow cytometry (Fig. 10B and data not shown), suggesting a delay in G_1-to-S progression. This possibility was supported by the observation that the phosphorylation state of pRb was reduced after 24 h of 4D5 treatment, with a higher proportion being found in the hypophosphorylated, growth suppressor state (Fig. 10C). Additionally, cyclin A protein expression was reduced, but not completely absent, consistent with the fact that these cells were still proliferating at a lower rate in the presence of 4D5 (Fig. 10C). As expected, treatment with the control antibody FRP5 had no growth-inhibitory effects and again appeared to be partially agonistic (Fig. 10).

Further analysis of 4D5-treated cells demonstrated that $p27^{Kip1}$ protein was indeed redirected onto Cdk2 complexes (Fig. 10C). This correlated with a reduction in c-Myc and cyclin D protein levels, a reduction in the intensity of the faster migrating, active form of Cdk2 and a reduction in Cdk2 activity to 43% of that seen in untreated cells (Fig. 10C). Interestingly, no increase in $p27^{Kip1}$ levels was observed after either 24 h (Fig. 10C) or 48 h (not shown) of 4D5 treatment. SKBR3 cells, therefore, exhibited 4D5-induced effects on $p27^{Kip1}$ sequestration protein levels and $p27^{Kip1}$-Cdk2 complex formation similar to those observed in BT474 cells. In contrast, redirection of $p27^{Kip1}$ onto Cdk2 complexes in SKBR3 cells was insufficient to totally inactivate Cdk2, a situation reflected in the growth characteristics of these cells. These data suggest that the extent of growth inhibition elicited by 4D5 treatment is cell type specific and correlates with the degree of Cdk2 inactivation. Moreover, an increase in $p27^{Kip1}$ expression is not a universal response to ErbB2 inhibition in 4D5-sensitive cells.

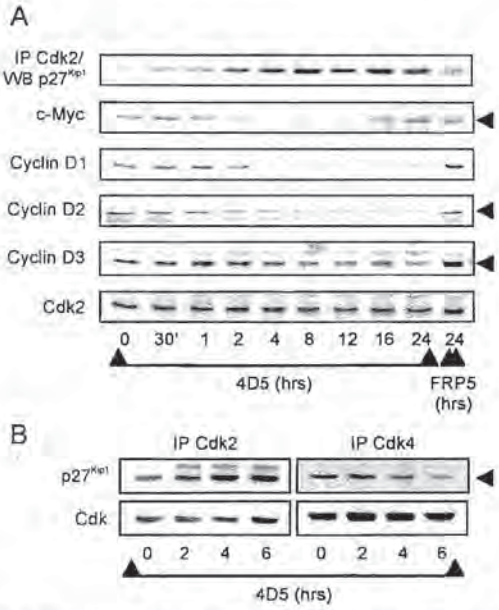


FIG. 9. Time course of MAb 4D5-induced effects on Cdk/p27Kip1 association and levels of proteins involved in p27Kip1 sequestration in BT474 cells. BT474 cells were seeded and treated with MAb FRP5 or MAb 4D5 as in Fig. 2. At the times indicated, cell extracts were prepared and analyzed by immunoblotting (WB) either directly or after immunoprecipitation (IP) with Cdk2-specific antibodies (A). Additionally, p27Kip1 association with Cdk2 and Cdk4 was analyzed by immunoblotting after immunoprecipitation (IP) with Cdk2- or Cdk4-specific antibodies (B). Untreated cells (t = 0) and cells treated with MAb FRP5 for 24 h were included as controls.

An equivalent response to MAb 4D5 treatment is not observed in MKN7 cells. If the molecular events observed in BT474 and SKBR3 cells were indeed related to growth inhibition, it would be expected that they would not occur in MKN7 cells, as these cells were not growth inhibited by treatment with 4D5 (Fig. 1A). To address this question, a time course of 4D5 treatment was performed with MKN7 cells. Here, no increase in $p27^{Kip1}$ protein levels or $p27^{Kip1}$-Cdk2 association was observed, even after 24 h (Fig. 11) or 48 h (not shown) of 4D5 treatment. Furthermore, c-Myc and cyclin D protein levels were little affected (Fig. 11). These data suggest that the cell cycle effects observed in BT474 and SKBR3 cells are related to growth inhibition, rather than being nonspecific events.

Increased $p27^{Kip1}$ levels are not required for MAb 4D5-induced $p27^{Kip1}$-Cdk2 association, Cdk2 inactivation, and G_1 arrest in BT474 cells. In BT474 cells, increased $p27^{Kip1}$-Cdk2 complex formation correlated with Cdk2 inactivation and preceded increased $p27^{Kip1}$ expression. This observation prompted the question of whether increased $p27^{Kip1}$ expression is an essential component in mediating the G_1 block induced by ErbB2 receptor inhibition in BT474 cells, or whether it is simply a consequence of 4D5-induced Cdk2 inactivation. This question was particularly relevant considering the absence of $p27^{Kip1}$ induction in SKBR3 cells, which displayed only partial Cdk2 inhibition in response to 4D5 treatment (Fig. 10C). To address this issue, therefore, we used an antisense approach to assess the cell cycle effects of preventing 4D5-specific increases in $p27^{Kip1}$ protein levels in BT474 cells. For this, a specific 15-base $p27^{Kip1}$ antisense oligonucleotide and a mismatch control oligonucleotide were constructed as previously described (14) (see Materials and Methods) and introduced into BT474 cells by lipofection. Immunoblots of lipofection-treated BT474 cells, subsequently treated for 24 or 36 h with 4D5, revealed that although $p27^{Kip1}$ levels increased as a result of 4D5 treatment in both untreated and mismatch controls, $p27^{Kip1}$ protein levels were unaffected by 4D5 in cells treated with antisense oligonucleotide (Fig. 12A). These data demonstrate the efficacy of antisense-mediated inhibition of $p27^{Kip1}$ protein expression in this system. Strikingly, when the cell cycle profile of the same cells was analyzed by flow cytometry, antisense-treated cells were still found to be blocked in G_1, as a result of 4D5 treatment, to an extent similar to that for untreated or

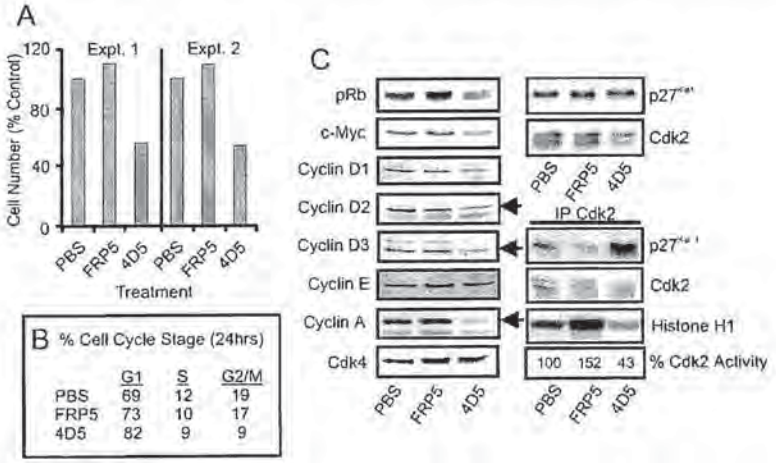


FIG. 10. Effects of anti-ErbB2 MAb treatment on SKBR3 cell proliferation and the expression and activity of G_1 regulators. SKBR3 cells were seeded as in Fig. 2. (A, Expt. 1; B and C) or at half the density (A, Expt. 2). After 24 h of incubation, PBS, MAb FRP5, or MAb 4D5 was added as in Fig. 2, and cells were treated as follows: (A) incubated for 4 days and trypsinized, after which total cell number was calculated; (B) incubated for 24 h, trypsinized, and treated with propidium iodide, after which cell cycle distribution was analyzed by flow cytometry; (C) incubated for 24 h, after which cell extracts were prepared and the protein levels of G_1 regulators were evaluated by immunoblotting, or $p27^{Kip1}$ association with Cdk2 complexes, and Cdk2 activity, was assessed through immunoprecipitation (IP Cdk2) of Cdk2 followed by immunoblotting for associated $p27^{Kip1}$ protein or in vitro histone H1 kinase assay. Cdk2 activity is indicated as a percentage of that in control (PBS)-treated cells.

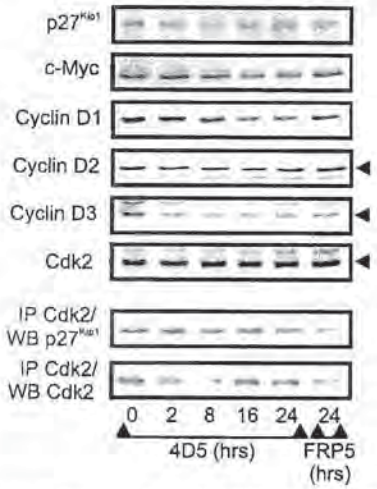


FIG. 11. Time course of the effects of MAb 4D5 treatment on G_1 regulators in MKN7 cells. MKN7 cells were seeded at a density of 3×10^4 cells/cm^2. After 24 h of incubation, the medium was changed and MAb FRP5 or MAb 4D5 was added to a concentration of 10 μg/ml for the times indicated. Cell extracts were prepared and analyzed by immunoblotting (WB) either directly or after immunoprecipitation (IP) with Cdk2-specific antibodies. Untreated cells (t = 0) and cells treated with MAb FRP5 for 24 h were included as controls.

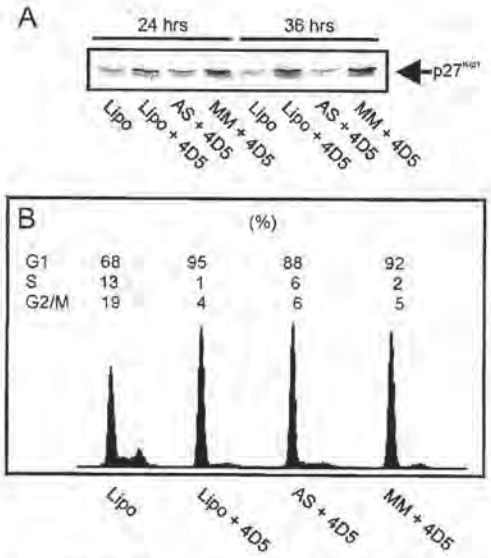


FIG. 12. Effect of antisense-mediated inhibition of MAb 4D5-induced $p27^{Kip1}$ accumulation on the cell cycle of BT474 cells. BT474 cells were seeded at a density of 3×10^4 cells/cm^2. After 24 h of incubation, cells were treated with LipofectAMINE alone (Lipo), $p27^{Kip1}$ antisense oligonucleotide (AS), or a mismatch control oligonucleotide (MM) as outlined in Materials and Methods. Cells were subsequently refed with normal growth medium; after 3 to 5 h, MAb 4D5 (+ 4D5) was added (10 μg/ml) for 24 or 36 h. After these times, cell extracts were prepared and $p27^{Kip1}$ protein levels were examined by immunoblotting (A). After 36 h, cells were trypsinized and treated with propidium iodide, and cell cycle distribution was analyzed by flow cytometry (B). Cells treated with LipofectAMINE alone followed by no addition of MAb 4D5 were included as controls. The LipofectAMINE procedure itself had no effect on cell cycle distribution compared to untreated controls, as assessed after 36 h of incubation (not shown).

mismatch control-treated cells (Fig. 12B). Importantly, cells treated with oligonucleotide displayed a normal cell cycle profile when cultured for the same time in the absence of 4D5 (data not shown).

Consistent with the presence of a G_1 block, Cdk2 activity was also decreased as a result of 4D5 treatment in all cases (Fig. 13A). Indeed, although Cdk2 inactivation appeared to be slightly delayed in antisense-treated cells (38%, compared to 18 and 12% in untreated and mismatch-treated cells, respectively, after 24 h incubation with 4D5), by 36 h almost total Cdk2 inactivation had occurred (4%, compared to 5 and 2.5% in untreated and mismatch-treated cells, respectively). Further analysis of Cdk2 immunoprecipitations for $p27^{Kip1}$ association indicated that after 24 h of treatment with 4D5 (a time when Cdk2 levels were unaffected), similar levels of $p27^{Kip1}$ protein became associated with Cdk2 in antisense-treated cells compared to controls (Fig. 13B). These data confirm that 4D5 treatment of BT474 cells induces the relocation of $p27^{Kip1}$ protein onto Cdk2 complexes. Furthermore, this movement is independent of increased $p27^{Kip1}$ protein levels and is sufficient to potentiate Cdk2 inactivation and, hence, establish a G_1 block. Additionally, no induction of the expression of the CKI $p21^{Cip1/Waf1}$ was observed after lipofection of either the antisense or mismatch control oligonucleotide (data not shown). This attests to the specificity of this effect, ruling out nonspecific effects of single-stranded DNA on Cdk2 activity.

DISCUSSION

ErbB2 overexpression potentiates cyclin E-Cdk2 activity in breast tumor cells. Examination of primary tumors overexpressing the ErbB2 receptor tyrosine kinase has revealed more aggressive tumor phenotypes, associated with poor patient prognosis (24, 59, 70, 71, 84). The extracellular accessibility and involvement in tumor malignancy suggest ErbB2, therefore, as an appropriate target for tumor-directed therapies. For this reason, elucidating the molecular mechanisms by which ErbB2 overexpression potentiates tumor cell growth is a priority. In this work, we have shown that MAb 4D5 treatment of the ErbB2-overexpressing breast tumor cell line BT474 results in a rapid reduction in ErbB2 receptor phosphorylation. Consistent with the relationship between tyrosine phosphorylation and receptor activity, a concomitant decrease in the activity of downstream cytoplasmic signaling pathways was also demonstrated. These observations imply antibody-mediated interference of receptor function in 4D5-treated cells. Consequently, BT474 cells respond to antibody treatment by growth arrest, suggesting that they are dependent on elevated ErbB2 receptor activity for proliferation. More specifically, 4D5 treatment results in a block of the G_1/S transition, characterized by a rapid increase in $p27^{Kip1}$ levels and inactivation of the cyclin E-Cdk2 complex.

Increased $p27^{Kip1}$ protein levels, with an associated G_1 accumulation, have been previously observed in ErbB1-overexpressing human carcinoma cell lines after treatment with the ErbB1 growth-inhibitory MAb 225 (53, 81, 83), as well as in an ErbB2-overexpressing ovarian carcinoma cell line treated with 4D5 (83). This relationship could point to a general role for ErbB receptor overexpression in maintaining cyclin E-Cdk2 activity by directly controlling $p27^{Kip1}$ protein levels, thus deregulating control mechanisms regulating the G_1/S transition. The consequences of such a role for tumor development are obvious and would explain the more aggressive growth characteristics of tumors overexpressing ErbB2 receptors. However, through the work presented here, we provide evidence

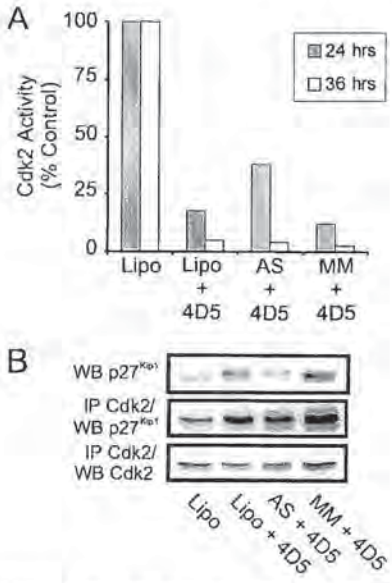


FIG. 13. Effect of antisense-mediated inhibition of MAb 4D5-induced p27^{Kip1} accumulation on Cdk2 activity and p27^{Kip1}-Cdk2 complex formation in BT474 cells. BT474 cells were seeded, treated with either LipofectAMINE alone (Lipo), antisense p27^{Kip1} oligonucleotide (AS), or mismatch (MM) control oligonucleotide, and treated with MAb 4D5 (+ 4D5) as outlined in Fig. 12. After 24 and 36 h of incubation, cells were extracted and immunoprecipitated with Cdk2-specific antibodies followed by in vitro histone H1 kinase assay (A). After 24 h of incubation, the same extracts were analyzed for p27^{Kip1} protein levels by immunoblotting (WB) directly or after immunoprecipitation (IP) of Cdk2 complexes. Cells treated with LipofectAMINE alone followed by no addition of MAb 4D5 were used as controls, and Cdk2 activity is expressed as a percentage of this control.

disputing this simple interpretation. First, a second ErbB2-overexpressing cell line (SKBR3), which is also growth inhibited as a result of 4D5 treatment, did not exhibit increased p27^{Kip1} expression. Additionally, a more in-depth analysis of the effects of 4D5 treatment on p27^{Kip1} function in BT474 cells demonstrated that the most immediate effect (within 2 h) of 4D5 treatment was to increase the availability of p27^{Kip1}, allowing it to interact with cyclin-Cdk2 complexes. This occurred prior to increases in p27^{Kip1} protein levels and paralleled Cdk2 inactivation kinetics. A similar shift of p27^{Kip1} protein onto Cdk2 complexes was also observed in SKBR3 cells, correlating with reduced Cdk2 activity. We postulate, therefore, that elevated ErbB2 receptor signaling in overexpressing tumor cells potentiates G_1/S progression by impeding p27^{Kip1} association with cyclin E-Cdk2 complexes. This hypothesis is supported by further experiments, using an antisense p27^{Kip1} oligonucleotide to prevent 4D5-induced increases in p27^{Kip1} protein levels in BT474 cells. Here, increased p27^{Kip1}-Cdk2 complex formation was still observed after 4D5 treatment, correlating with Cdk2 inactivation. Additionally, although increased p27^{Kip1} levels may have contributed to the stability of the G_1 arrest at later times, this event was found not to be required to establish the G_1 block induced by 4D5 treatment in these cells.

It is known that p27^{Kip1} levels are regulated principally by degradation (51). Furthermore, (i) phosphorylation of p27^{Kip1} on threonine 187 by Cdk2 kinase and (ii) stable trimeric complex formation with cyclin-Cdk2 complexes act as signals for ubiquitination and hence target p27^{Kip1} to the proteasome degradation machinery (43, 44, 66). From the results presented here, p27^{Kip1} accumulation in BT474 cells appears to be a secondary effect of 4D5-induced p27^{Kip1}-Cdk2 complex formation, as the latter was sufficient to almost totally inhibit Cdk2 activity. In the absence of Cdk2 activity, p27^{Kip1} protein would be inefficiently phosphorylated and stabilized. This supposition is supported by two observations. First, no p27^{Kip1} induction was observed in 4D5-treated SKBR3 cells, which exhibited only partial Cdk2 inactivation as a result of p27^{Kip1} redirection. Second, using [^{35}S]methionine pulse-chase techniques after 16 h of 4D5 treatment, we have shown a doubling of p27^{Kip1} protein half-life, with no specific effect on p27^{Kip1} translation (unpublished data).

With these data in mind, therefore, we propose that the initial effect of inhibiting ErbB2 receptor function in 4D5-sensitive breast carcinoma cells is to redirect p27^{Kip1} onto Cdk2 complexes resulting in inhibition of G_1/S progression. The extent of Cdk2 inactivation following receptor inhibition appears to be cell type specific and in some cases is sufficient to instigate a complete G_1 block and induce increased p27^{Kip1} protein expression. More specifically, we speculate that in some tumors ErbB2 overexpression promotes constitutive intracellular signaling leading to sequestration of p27^{Kip1} away from Cdk2 complexes.

ErbB2 overexpression regulates the expression of proteins involved in p27^{Kip1} sequestration. The D-type cyclins and the transcription factor c-Myc are involved in regulating p27^{Kip1} sequestration in proliferating cells (8, 34, 73, 77, 85). In this respect, a reduction in the level of these proteins was observed in 4D5-treated BT474 and SKBR3 cells, correlating with p27^{Kip1}-Cdk2 complex accumulation. Loss of p27^{Kip1} sequestration proteins would provide an explanation for 4D5-induced p27^{Kip1} relocation onto Cdk2 complexes. However, we acknowledge that alternative mechanisms of regulating p27^{Kip1} availability in tumor cells may also be affected (50). Previous reports have shown that activation of the MAP (Erk1/2) kinase pathway leads to stabilization of the c-Myc protein (65) and increased cyclin D transcription (42). Furthermore, activation of the PI3-kinase/PKB pathway has been implicated in the translational induction of c-Myc (80) and stabilization of the D-type cyclins (13, 17). Here, we have shown that 4D5-treated BT474 cells exhibit a rapid and dramatic reduction in PKB phosphorylation, with less dramatic effects on Erk1/2, suggesting an effect on the activation state of these kinases. Additionally, effects on both PKB and Erk1/2 phosphorylation have also been demonstrated in SKBR3 cells (data not shown). Based on the literature, therefore, these observations could account for the decreased expression of c-Myc and D-type cyclins in cells sensitive to 4D5 treatment. Recently, the role of c-Myc in the regulation of cyclin D1 and D2 expression has been demonstrated (10, 54). Whether in our experiments downregulation of the D-type cyclins after 4D5 treatment was purely a consequence of reduced c-Myc protein levels is a matter for debate, particularly as in BT474 cells c-Myc protein levels recovered at later times (36 to 48 h) of 4D5 treatment, whereas cyclin D2 (and D3) levels did not. Intriguingly, changes in cyclin D1 protein levels did mirror fluctuations in c-Myc protein, suggesting that cyclin D1 expression is downstream of the c-Myc transcription factor in BT474 cells.

It should also be noted that no effect of 4D5-induced c-Myc downregulation was seen at the level of expression of cyclin E or the G_1 Cdk-regulatory phosphatase Cdc25A (Fig. 6A and data not shown). Both of these proteins have been postulated to be downstream transcriptional targets of c-Myc (2, 48). For Cdc25A, this now seems unlikely (48). Moreover, the exact relationship between c-Myc and cyclin E expression is not established. Indeed, the cyclin E promoter has no consensus

c-Myc binding sites, and in some systems c-Myc has been shown to increase cyclin E-Cdk2 activity in the absence of changes in cyclin E expression (61, 74). An additional point to consider is that cyclin E is constitutively overexpressed in breast tumor cells (31, 32). It is possible, therefore, that cyclin E levels were maintained after 4D5 treatment as a result of tumor-specific deregulation of cyclin E expression.

Differential responses to MAb 4D5 treatment indicate differences in growth dependency in ErbB2-overexpressing tumor cells. In vitro screening of ErbB2-overexpressing cell lines for 4D5-mediated growth inhibition has revealed variability in the response of tumor cells to antibody treatment (37, 38). Here, we have also shown that two overexpressing breast tumor cell lines, BT474 and SKBR3, respond to antibody-mediated inhibition of ErbB2 signaling to differing extents. Moreover, effects on cell proliferation appear to correlate with the extent of Cdk2 inhibition induced by antibody treatment. In this context, cyclin E-Cdk2 kinase activity is known to be heavily deregulated in breast tumor cells (31, 32). Indeed, increased cyclin E expression has been associated with a high proliferative capacity, highly aggressive tumors, and poor patient prognosis (45, 46, 58). Furthermore, cyclin E-Cdk2 activation levels in primary breast tumors correlate with the phosphorylation status of pRb and with proliferation rates (40), a finding which corroborates the observations presented here. The reason for the differences between the overall responses of BT474 and SKBR3 cells to 4D5 treatment is not known. However, we note that SKBR3 cells express approximately fourfold less $p27^{Kip1}$ protein than BT474 cells, as well as significantly higher levels of cyclin D2 (sevenfold), cyclin D3 (twofold), Cdk6 (fourfold), and c-Myc (twofold) proteins (unpublished data). It is possible, therefore, that cell-type-specific differences in the expression of $p27^{Kip1}$ and $p27^{Kip1}$ sequestration proteins may determine the potency of the growth response to ErbB2 receptor inhibition. This important question will be addressed in the future.

An additional consideration is that ErbB2-overexpressing tumor cells may exhibit graded responses to ErbB2 inhibition due to different dependencies on elevated ErbB2 receptor expression for the maintenance of mitogenic signaling pathways (discussed below). This possibility is exemplified in MKN7 cells, which also overexpress ErbB2 but are not growth inhibited by 4D5 treatment. Indeed, 4D5 treatment of MKN7 cells had no effect on $p27^{Kip1}$ sequestration or $p27^{Kip1}$ protein levels. Accordingly, major cytoplasmic signaling pathways, as well as c-Myc and cyclin D protein levels, remained essentially unchanged. These data indicate that the effects on the cell cycle machinery observed in 4D5-treated BT474 and SKBR3 cells are indeed related to growth inhibition. Furthermore, despite the fact that MKN7 cells dramatically overexpress ErbB2 to levels similar to those observed in BT474 cells (Fig. 2A; see also references 21 and 37) and also exhibit a general reduction in receptor phosphorylation as a result of 4D5 treatment, impaired ErbB2 receptor signaling does not seem to affect the maintenance of $p27^{Kip1}$ sequestration proteins or stimulate $p27^{Kip1}$-Cdk2 complex formation in this case. The role of ErbB2 overexpression in the potentiation of Cdk2 activity in tumors, therefore, is not universal.

ErbB2 receptor overexpression alone does not determine growth dependency. Consistent with downstream effects on cytoplasmic signaling pathways, we have demonstrated that 4D5 treatment of BT474 cells results in a rapid and general reduction in ErbB2 phosphorylation. In contrast to a previous report examining the effect of 4D5 treatment on ovarian cancer cells (83), we observed no gross downregulation of ErbB2 protein levels. It is possible that slight decreases in ErbB2 expression were not detected by the immunoblotting approach that we used. With this in mind, therefore, we cannot rule out the possibility that partial receptor downregulation may have occurred after prolonged 4D5 treatment, as previously shown (27, 33). Until now, receptor analyses showing 4D5-induced reductions in ErbB2 phosphorylation were performed after long treatment periods, and tryptic phosphopeptide mapping was not carried out (27, 33). Moreover, it has also been suggested that 4D5 induces ErbB2 phosphorylation in BT474 cells (63). From detailed time courses of 4D5 treatment of BT474 cells, however, we have detected decreased receptor phosphorylation levels within 10 min of 4D5 addition. Furthermore, through phosphopeptide mapping, receptor dephosphorylation was shown to be general, including sites stimulated by treatment with MAb FRP5, a known ErbB2 partial agonist (25, 41). The lack of correlation between our observations and those of Scott and coworkers (63) could be due to differences in cell culture conditions before 4D5 addition. In our experiments, cells were treated with antibody at low densities (see Materials and Methods) when a normal cell cycle profile was evident (Fig. 7A). In contrast, 80 to 100% confluent cells were used by the above authors, which could have resulted in differences in response to antibody treatment.

Taken together, our data show that 4D5-induced inhibition of ErbB2 receptor signaling in BT474 cells affects downstream signaling events required for the maintenance of $p27^{Kip1}$ sequestration proteins and, hence, Cdk2 activity. It is tempting to propose, therefore, that the general dephosphorylation of ErbB2 induced by 4D5 treatment is sufficient to inhibit growth in ErbB2-overexpressing cell lines. However, as 4D5 treatment of the insensitive tumor cell line MKN7 also induced receptor dephosphorylation in a similar fashion, this event cannot be considered a marker for cellular response to 4D5 treatment. Consequently, one has to consider that other cell-type-specific factors may determine whether tumor cells become dependent on elevated ErbB2 signaling for proliferation.

It has been suggested that long-term resistance to 4D5 treatment may be due to intracellular expression of the extracellular domain of ErbB2, which interferes with internalized ErbB2-4D5 complexes (64). However, from the rapid kinetics of receptor dephosphorylation observed in both MKN7 and BT474 cells after addition of 4D5, this hypothesis would not explain the resultant differential effects on nuclear proteins. From the data presented in this paper, therefore, we speculate on a number of more plausible explanations for why there are differences between cellular responses to 4D5-induced ErbB2 receptor inhibition. First, although ErbB2 is overexpressed in MKN7 cells to levels similar to those in BT474 cells, the receptor is minimally phosphorylated in the former case (Fig. 2A). This implies that ErbB2 is not as active a signaling moiety in MKN7 cells as in BT474 cells. Of all ErbB receptor interactions, the ErbB2-ErbB3 heterodimer is considered the preferred and most potent signaling module (55, 79), coupling efficiently to the PI3-kinase pathway (20, 60). It is significant, therefore, that ErbB3 is overexpressed and active in BT474 cells but is undetectable in MKN7 cells. Moreover, the PI3-kinase pathway, as measured by PKB phosphorylation, is dramatically downregulated in BT474 as a result of 4D5 treatment but remains unaffected in MKN7 cells. Previously, ErbB3 expression has been shown to enhance ErbB2-mediated transformation and tumorigenic growth of NIH 3T3 cells (1, 78, 88). Furthermore, ErbB2-ErbB3 coexpression has been observed in human breast tumors (9, 69), where it has been postulated to play a critical role in tumor progression (69). ErbB3 may, therefore, collaborate with ErbB2, contributing to tumor development. The above data, together with the finding that

4D5-sensitive SKBR3 cells express similar levels of ErbB3 protein (as well as ErbB1 and ErbB2) as BT474 cells (data not shown) and reports that ErbB2-overexpressing cell lines with no or low ErbB3 expression are minimally growth inhibited by 4D5 treatment (37, 38), make it tempting to postulate that the strong proliferative signal resulting from an ErbB2-ErbB3 collaboration could lead to growth dependency during tumor development.

A second possible explanation for resistance to 4D5 treatment is the presence of alternative signaling pathways with the capacity to override ErbB2 receptor inhibition. In MKN7 cells a likely candidate is the ErbB1 receptor, which, in contrast to the situation in BT474 and SKBR3 cells, is overexpressed (see Fig. 2A and reference 37) and highly activated in these cells (Fig. 2A). The observations that ErbB-mediated signaling pathways overlap (5, 16), that epidermal growth factor rescues growth inhibition caused by 4D5 treatment (reference 83 and our unpublished data), and that the anti-ErbB1 MAb 225 augments 4D5-induced growth inhibition (83) provide compelling evidence for alternative routes by which tumor cells ensure maintenance of their proliferative capacity. With these two possibilities in mind, therefore, the involvement of all ErbB receptor family members in determining cellular responses to 4D5 treatment should be considered.

Implications for tumor development. Alterations targeting and, therefore, deregulating the G_1 Cdk/pRb phosphorylation pathway are commonly found in human cancers. In this respect, numerous correlations between abnormal p27^{Kip1} expression and advanced tumor grade have been made (for examples, see references 12, 19, 39, 58, and 82). Enhanced proteasome-dependent p27^{Kip1} degradation has been postulated to be a major influencing factor in these tumors (19, 39). Here, we show that ErbB2 overexpression can provide an additional level of p27^{Kip1} deregulation during tumor development; maintaining p27^{Kip1} sequestration proteins and, thus, potentiating cyclin E-Cdk2 activity. However, we further demonstrate that receptor overexpression levels alone cannot predict to what extent elevated ErbB2 receptor signaling will contribute to deregulation of the G_1/S transition. Bearing this in mind, we note that even though all patients treated with a humanized version of 4D5 (Herceptin) presented with metastatic breast carcinomas overexpressing ErbB2, not all responded to treatment (3, 15, 52). It is tempting to speculate, therefore, that the ability of a given tumor cell to elicit p27^{Kip1} relocation and, hence, Cdk2 inactivation in response to ErbB2 receptor inhibition may determine the potency of the clinical response to Herceptin. This ability may depend on the relative contribution of other growth factor receptors to ErbB2 activation and to the maintenance of specific intracellular, mitogenic signaling pathways. In some tumor cells, ErbB1 or ErbB3 expression is coamplified with ErbB2, leading to the suggestion that they may collaborate in the induction of human malignancies. The determination of whether such relationships do, indeed, exist has an impact not only on elucidating the mechanisms by which ErbB2 overexpression contributes to malignant transformation but also on therapeutic and screening strategies used in the clinic.

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EXHIBIT F

Antagonism of Chemotherapy-Induced Cytotoxicity for Human Breast Cancer Cells by Antiestrogens

By C. Kent Osborne, Libbey Kitten, and Carlos L. Arteaga

In a prior National Surgical Adjuvant Breast and Bowel Project (NSABP) adjuvant study, the addition of the antiestrogen tamoxifen to chemotherapy with melphalan and fluorouracil adversely affected survival in several patient subsets, suggesting an antagonistic drug interaction. To investigate this possibility, we studied the interaction of tamoxifen and other antiestrogens with several cytotoxic drugs in cultured human breast cancer cell lines. Clinically relevant concentrations of tamoxifen and melphalan reduced colony survival of estrogen receptor (ER)-positive breast cancer cells when used alone in a colony-forming assay. However, pretreatment of cells with tamoxifen followed by exposure to melphalan resulted in antagonism, with more colonies surviving treatment with the combination than with melphalan alone. Identical effects were seen using several other triphenylethelene antiestrogens. An antagonistic interaction was observed even with a brief preincubation with tamoxifen that had no effect on cell proliferation, indicating that antagonism was not due to tamoxifen's known cell kinetic effects. Tamoxifen even antagonized melphalan cytotoxicity in ER-negative breast cancer cells and in cultured liver cells. An additive drug interaction occurred when melphalan was combined with pharmacologic concentrations of estradiol or medroxyprogesterone acetate, but antagonism was also observed with dexamethasone. Tamoxifen also antagonized the cytotoxicity of fluorouracil in these cells. However, an additive interaction occurred when the antiestrogen was combined with doxorubicin or 4-hydroxycyclophosphamide, an alkylating agent that is transported into the cell by a different carrier-mediated mechanism than melphalan. To avoid potential antagonism in the clinic, combinations of tamoxifen with melphalan and/or fluorouracil should be avoided.

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CLINICAL TRIALS of chemo-endocrine treatment for breast cancer have been disappointing. The addition of the antiestrogen tamoxifen to standard chemotherapy regimens has resulted in little or no benefit compared with the sequential use of the two modalities in patients with advanced breast cancer or in the adjuvant setting.[1] In fact, in one large adjuvant study comparing melphalan and fluorouracil with or without tamoxifen, an adverse effect of tamoxifen was observed in several patient subsets.[2] These clinical studies suggest that tamoxifen could interact antagonistically with certain cytotoxic drugs.

Recent laboratory studies of antiestrogen action suggest several possible mechanisms by which tamoxifen could interact with a cytotoxic drug to either enhance or antagonize its effects. In breast cancer cells containing estrogen receptor (ER), tamoxifen binds to the receptor, competitively blocks the binding of estrogen, and inhibits cell proliferation. Cell kinetic studies have shown that a major effect of antiestrogens is to reversibly slow cell cycle transit by causing a G_1 transition delay.[3-5] After several days of incubation with clinically relevant concentrations of tamoxifen, cells accumulate in G_1-phase at the expense of S- and G_2M-phase, conceivably rendering them less vulnerable to S-phase specific cytotoxic agents. This "cytostatic" effect is reversible with estrogen replenishment, which results in a synchronous wave of cells leaving G_1-phase and entering S-phase.[4] The ability to hormonally synchronize breast cancer cells in this fashion has led to the development of clinical trials of hormonal synchronization combined with cytotoxic chemotherapy in an attempt to enhance the drugs' lethal effects.[6,7] Antiestrogens have no significant effects on proliferation of cultured ER-negative breast cancer cells except with very high concentrations that result in non–receptor-mediated cytotoxicity.[5]

Antiestrogens have other biochemical effects on cells that could influence drug cytotoxicity.

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Tamoxifen inhibits CA^{++}-dependent cellular processes. It antagonizes calmodulin- and phospholipid-activated protein kinase and is an effective Ca^{++} channel antagonist, effects that could alter drug uptake.[8-10] Tamoxifen inhibits protein kinase C in non-breast cancer tissue, apparently via non–ER-mediated mechanisms. It has also been hypothesized that lipophilic tricyclic triparanol analogues including tamoxifen could interact with the cell membrane lipid domain, thereby altering packing density and, secondarily, diffusion rates of certain drugs.[11]

The effects of tamoxifen on the cytotoxicity of several drugs have been investigated in cell culture model systems. A brief incubation with very high concentrations of tamoxifen (3 μmol/L) increased the sensitivity of Adriamycin (doxorubicin; Adria Laboratories, Columbus, OH)-resistant P388 leukemia cells to Adriamycin.[11] In contrast, brief preincubation of cultured breast cancer cells with clinically relevant tamoxifen concentrations (< 1 μmol/L) attenuated the cytotoxicity of both Adriamycin and fluorouracil.[12] This result contradicts another report that tamoxifen and fluorouracil were synergistically cytotoxic for breast cancer cells by a mechanism involving modulation of cellular RNA.[13,14] Finally, exceedingly high concentrations of tamoxifen antagonized the cytotoxicity of melphalan for cultured breast cancer cells, probably by inhibiting drug uptake.[15]

Full interpretation of these sometimes contradictory studies is further confounded by the fact that the target cells were often exposed to very high (supraphamacologic) concentrations of tamoxifen for only a short (one- to two-hour) duration. This brief preincubation is insufficient to achieve an antiestrogen-induced effect on tumor cell kinetics before exposure to the cytotoxic agent. Furthermore, this strategy may not accurately depict in vivo chemo-endocrine therapy in which tumor cells are exposed to a prolonged clinically relevant tamoxifen concentration with the cytotoxic chemotherapy given in intermittent "bursts" over the course of treatment.

In the present study, we examined the effects of triphenylethelene antiestrogens on the cytotoxicity of several antineoplastic agents used in breast cancer treatment. Using a variety of experimental conditions we found that antiestrogens antagonize melphalan and fluorouracil but not cyclophosphamide or doxorubicin cytotoxicity. Melphalan antagonism by tamoxifen is independent of cell cycle kinetic changes and independent of tumor ER status.

MATERIALS AND METHODS

Human Breast Cancer Cell Lines

The ER-positive cell lines, MCF-7 and ZR75-1, and the ER-negative MDA-231 cells were the gift of Dr Marc Lippmann (National Cancer Institute, Bethesda, MD). The ER-negative BT-20 cells and a long-term culture of buffalo rat liver cells (BRL-3A) were obtained from the American Type Culture Collection (Rockville, MD). All cell lines were grown in monolayer in improved minimum essential medium (IMEM, Irvine Scientific, Alamosa, CA) supplemented with 5% to 10% fetal bovine serum (FBS, GIBCO Laboratories, Grand Island, NY), 10 nmol/L insulin (Eli Lilly Research Laboratories, Indianapolis), and antibiotics, and subcultured as previously described.[16]

Drugs and Hormones

4-OH-cyclophosphamide, an active metabolite of cyclophosphamide, was the gift of Dr David Alberts (University of Arizona, Tucson). Melphalan was obtained from Burroughs Wellcome, Research Triangle Park, NJ, and fluorouracil and doxorubicin were purchased from commercial sources. The synthetic antiestrogens tamoxifen citrate (Stuart Pharmaceuticals, Wilmington, DE), toremifene (Adria Laboratories), LY156758 (Eli Lilly), and nafoxidine (Sigma-Tau, Holmdel, NJ) were dissolved in ethanol prior to use. 17β Estradiol (Calbiochem, San Diego), dexamethasone (Sigma), and medroxyprogesterone acetate (Farmitalia Carlo Erba, Milan, Italy) were also diluted in ethanol.

Experimental Design

To investigate the effects of hormonal treatments on drug cytotoxicity, cell lines were suspended in trypsin (.02%) to EDTA (0.1%) in phosphate buffered saline (PBS) and replicately plated in 100-mm culture dishes in complete culture medium. After 24 hours the medium was exchanged for IMEM supplemented with 5% dextran-coated charcoal-stripped calf serum to reduce the endogenous estrogen concentration. Antiestrogens or other hormones were then added to the culture medium at the indicated concentrations and the cultures maintained at 37°C in a humidified CO_2 incubator for 72 hours, sufficient time to cause a marked reduction in S-phase and accumulation in G_1-phase of cells that express ER.[3,4] In some experiments, cells were preincubated with antiestrogens for only one hour. Control dishes were incubated with vehicle alone (0.1% ethanol) for an identical duration.

Following preincubation with antiestrogens or hormones, cytotoxic drugs were added directly to the cultures at the indicated concentrations, and the cultures were incubated for one hour at 37°C. The monolayers were then washed once with serum-free IMEM, once with trypsin-EDTA, and then suspended in EDTA/PBS. A single-cell suspension was prepared by gentle pipetting and then the suspension was

diluted in IMEM supplemented with 5% stripped serum to give the appropriate cell concentration.

Cloning Assays

Cytotoxicity was assessed by determining the surviving fraction of drug treated cultures compared with control (vehicle only) cultures in a clonogenic assay. The MCF-7, ZR75-1, and BRL-3A cell lines were cloned in a monolayer cloning assay in which single cells (650/mL) were plated in 35-mm Petri dishes. In cultures that had been preincubated for 72 hours with an antiestrogen, the antiestrogen was also added to the cloning assay culture medium to provide the cells with a continuous exposure analogous to the situation in patients treated with tamoxifen. Colonies (> 50 cell aggregates) were counted manually using an inverted stage microscope after ten days of growth at 37°C in a 5% CO_2 humidified incubator.

Since the ER-negative human breast cancer cell lines did not form tight round colonies when grown in monolayer, an anchorage-independent colony growth assay was used. In 35-mm culture dishes a 1 mL top layer containing a single-cell suspension (3×10^3 cells) in 0.8% agarose (Sea-Plaque; FMC Corporation, Rockland, ME), IMEM/10% FBS, without or with an antiestrogen was added to an already hardened bottom layer of 1 mL of 0.8% agarose, IMEM/10% FBS, and 10 mmol/L Hepes (GIBCO). Colonies were manually counted with an inverted microscope after ten to 14 days.

Statistical Analysis of Drug Interactions

In vitro drug interactions were quantified by the method of Valeriote and Lin.[17] The colony surviving fractions (SFs) for tamoxifen or the cytotoxic drug used alone (SF_A or SF_B) and the SF for the drugs used in combination (SF_{A+B}) were determined in the colony-forming assay. The SF was calculated as the ratio between the mean number of colonies surviving on six drug-treated plates and the mean number of colonies formed on six separate control plates. The approximate SE of the SF was computed as previously described.[18] A theoretical predicted SF for the combination of the two drugs assuming a simple additive effect was calculated by multiplying the actual SFs observed with each drug alone ($SF_A \times SF_B$). The net drug interaction of the combination was then assessed by comparing the SF actually observed experimentally with the drugs in combination (SF_{A+B}) with that predicted for an additive effect ($SF_A \times SF_B$). If SF_{A+B} actually observed experimentally is less than the predicted value, the drug interaction is defined as synergistic. If SF_{A+B} is similar to the predicted, the interaction is additive. When SF_{A+B} is greater than the predicted, an antagonistic interaction occurred.

RESULTS

Dose-Survival Curves of ER-Positive MCF-7 Cells Treated With Melphalan and/or Tamoxifen

In initial experiments, the effect of tamoxifen preincubation (72 hours) on the cytotoxic effects of melphalan was examined in the ER-positive MCF-7 cells. We have previously shown that under these conditions tamoxifen induces a G_1 block resulting in a marked reduction in the percentage of S-phase cells.[3,4] In Fig 1, dose-survival curves for cells treated with melphalan alone or with melphalan and a clinically relevant tamoxifen concentration of 1 μmol/L are compared with a theoretical predicted curve assuming an additive interaction. A dose-response effect on colony survival is evident with melphalan concentrations between 0.1 and 1.0 μg/mL. The SF of cells treated with tamoxifen alone was 0.50. Colony survival with melphalan plus tamoxifen was consistently greater than that predicted for an additive effect. At the highest melphalan dose more colonies survived treatment with the combination (SF = 0.33) than with melphalan alone (SF = 0.15). Thus, the addition of tamoxifen to melphalan was antagonistic.

In a reciprocal experiment the effect of tamoxifen dose without or with melphalan (0.5 μg/mL) was examined (Fig 2). A 72-hour incubation with tamoxifen alone reduced the SF in a dose-response fashion with a SF of .21 observed at a concentration of 1 μmol/L in this experiment. The SF observed with melphalan alone was 0.3. When the cells were treated with a combination of the two drugs, the dose-survival curve was consistently above that predicted for an additive effect and more colonies actually survived the drug combination than melphalan alone, again demonstrating an antagonistic interaction even with a very low tamoxifen concentration (0.1 nmol/L).

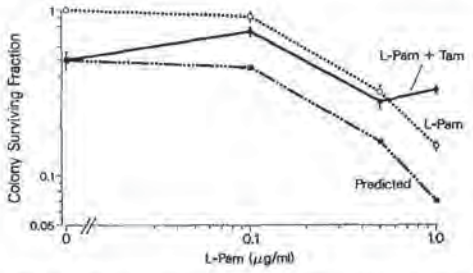

Fig 1. Dose-survival curves of melphalan without or with tamoxifen in MCF-7 cells. Cells were treated with melphalan (L-PAM) alone for one hour, tamoxifen (Tam, 1 μmol/L) for 72 hours, or the combination. The cells were cloned, and the surviving fraction was determined for each drug used alone and for the combination, and the predicted curve was calculated as described in Methods. Values represent the means ± SD of six separate determinations.

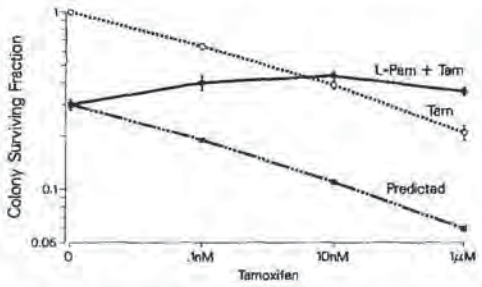

Fig 2. Dose-survival curves of tamoxifen without or with melphalan in MCF-7 cells. Cells were treated with various doses of tamoxifen (Tam) for 72 hours, melphalan (L-PAM; 0.5 μg/mL) for one hour or the combination, and the surviving fractions determined as described in Fig 1. Values represent the means ± SD of six determinations.

Effect of Other Antiestrogens

The antagonistic interaction with melphalan was observed with a variety of antiestrogens; preincubation of these cells with three other triphenylethelene derivatives had identical effects (Table 1). Each of the antiestrogens used alone significantly inhibited colony survival although to a variable degree. However, when the antiestrogens were combined with melphalan, the observed SF in each case was greater than that predicted for an additive effect. Furthermore, the actual number of colonies surviving with the antiestrogen-melphalan combinations was greater than that seen with melphalan alone, a clearly antagonistic interaction.

Table 1. Effect of Triphenylethelene Antiestrogens on Melphalan Cytotoxicity in MCF-7 Cells

Treatment*	No. of Colonies ± SE	Surviving Fraction†	
		Observed ± SD	Predicted
Controls	150 ± 4	—	—
L-PAM	66 ± 3	.44 ± .05	—
Tamoxifen	79 ± 2	.52 ± .04	—
+ L-PAM	106 ± 5	.71 ± .07	.23
LY156758	86 ± 4	.57 ± .06	—
+ L-PAM	101 ± 3	.67 ± .06	.25
Toremifene	116 ± 3	.77 ± .06	—
+ L-PAM	125 ± 5	.83 ± .08	.34
Nafoxidine	90 ± 7	.60 ± .09	—
+ L-PAM	76 ± 1	.50 ± .03	.26

Abbreviation: L-PAM, melphalan.

*Cells were incubated with vehicle alone (controls), antiestrogens alone (0.5 μmol/L for 72 hours), melphalan alone (0.5 μg/mL for one hour), or the combination of the two agents. The observed and predicted surviving fractions were determined as described in Methods.

†Values represent the means of six separate determinations.

Duration of Tamoxifen Preincubation

All of the previous experiments used a 72-hour tamoxifen preincubation to allow cells to accumulate in G_1-phase prior to melphalan treatment. To assess whether this G_1 arrest is necessary for the antagonistic effect, MCF-7 cells were incubated with either tamoxifen for 72 hours or for only one hour prior to the addition of melphalan (Table 2). A one-hour preincubation is insufficient to cause a significant perturbation of cell cycle phase distributions,[4] and it did not reduce colony number. However, tamoxifen for only one hour did antagonize the effects of melphalan, as indicated by comparison of the observed and predicted SFs. Thus, the antagonistic interaction cannot be explained on a cell kinetic basis, and it does not require prolonged exposure to the antiestrogen.

Effects of Tamoxifen on Melphalan Cytotoxicity in Other Cell Lines

To further examine the potential mechanism of melphalan antagonism by tamoxifen, we studied several other cell lines (Table 3). The ER-positive ZR75-1 cell line, like MCF-7, is inhibited by tamoxifen and by melphalan alone. When tamoxifen was combined with melphalan, the observed SF (0.63) was nearly twice that predicted for an additive interaction for these agents (0.33), again indicating antagonism. As expected, tamoxifen had no effect on the colony SF of the two ER-negative human breast cancer cell lines (BT-20 and MDA-231; data not shown). Nevertheless, it did antagonize melphalan cytotoxicity in each of these lines since the observed

Table 2. Effect of Duration of Tamoxifen Pretreatment on Melphalan Cytotoxicity in MCF-7 Cells

Treatment*	No. of Colonies ± SE	Surviving Fraction	
		Observed ± SD	Predicted
Control	124 ± 6	—	—
L-PAM	22 ± 2	.18 ± .02	—
Tam 72 hours	39 ± 14	.31 ± .11	—
+ L-PAM	39 ± 7	.32 ± .95	.06
Tam 1 hour	156 ± 8	1.25 ± .09	—
+ L-PAM	72 ± 3	.58 ± .04	.22

Abbreviation: TAM, tamoxifen.

*Cells were incubated with tamoxifen (1 μmol/L) for either 72 hours or only one hour (Tam), melphalan (L-PAM, 0.5 μg/mL for one hour) or the combination and were then processed as described in Methods.

Values are the means of six determinations.

Table 3. Interaction of Tamoxifen and Melphalan in Other ER-Positive and ER-Negative Cell Lines

Cell Line	ER	Surviving Fraction for Tam + L-PAM*	
		Observed ± SD	Predicted
ZR-75-1	+	.63 ± .05	.33
BT20	—	.81 ± .03	.69
MDA-231	—	.79 ± .15	.38
BRL-3A	—	.45 ± .03	.31

*Each cell line was treated with tamoxifen (1 μmol/L) for 72 hours, melphalan for one hour (0.5 μg/mL for ZR75-1, BT20, and MDA-231; 2 μg/mL for BRL-3A), or the combination. Cytotoxicity was assessed in a cloning assay and the observed and predicted surviving fractions calculated as described in Methods. Values are the means of triplicate determinations.

SF with the combination was greater than that predicted for an additive effect. Antagonism was even observed with tamoxifen and melphalan in a non-breast cancer cell line (BRL-3A), an ER-negative line derived originally from rat liver.

Interaction With Other Endocrine Agents

To determine if melphalan antagonism is specific for the triphenylethelene antiestrogens, we examined the effects of pharmacologic concentrations of several other endocrine agents in MCF-7 cells (Table 4). Dexamethasone, 17β-estradiol, and medroxyprogesterone acetate all inhibited colony formation slightly when used alone. When the latter two agents were combined with melphalan, the SF was actually less than with melphalan alone, and the observed values were similar to those predicted, suggesting an additive effect. On the other hand, an antagonistic interaction was observed with dexamethasone, an agent commonly used at high doses in breast cancer patients as an antiemetic.

Table 4. Effects of Other Hormonal Agents on Melphalan Cytotoxicity in MCF-7 Cells

Treatment*	No. of Colonies ± SE	Surviving Fraction	
		Observed ± SD	Predicted
Control	369 ± 13	—	—
L-PAM	70 ± 4	.19 ± .01	—
Dexamethasone	256 ± 7	.69 ± .03	—
+ L-PAM	92 ± 4	.25 ± .01	.13
17β-estradiol	261 ± 14	.71 ± .04	—
+ L-PAM	35 ± 8	.09 ± .02	.13
Medroxyprogesterone	325 ± 10	.88 ± .04	—
+ L-PAM	50 ± 4	.14 ± .01	.17

*Cells were treated with 1 μmol/L of each hormone for 72 hours, melphalan (L-PAM; 0.5 μg/mL) for one hour, or the combination. Cytotoxicity was assessed in a cloning assay and the surviving fractions determined. Values shown are the mean of six determinations.

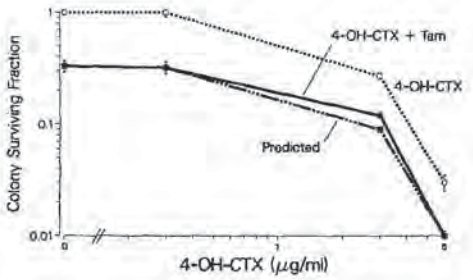

Fig 3. Dose-survival curves of 4-hydroxy-cyclophosphamide without or with tamoxifen in MCF-7 cells. Cells were treated with various doses of 4-hydroxy-cyclophosphamide (4-OH-CTX) for one hour, tamoxifen (1 μmol/L) for 72 hours, or the combination, and the surviving fractions determined as described in Fig 1. Values represent the means ± SD of six determinations.

Tamoxifen and Other Cytotoxic Drugs

Dose-survival curves for three other commonly used agents in breast cancer without or with tamoxifen are shown in Figs 3 to 5. 4-Hydroxycyclophosphamide, the active metabolite of cyclophosphamide, is an alkylating agent that is transported into the cell by a different carrier-mediated mechanism than melphalan.[19] In contrast to melphalan, this drug shows an additive effect when combined with tamoxifen in MCF-7 cells (Fig 3). The SF observed with the combination of the two drugs was identical to that predicted by an additive interaction at each concentration of the cytotoxic agent tested. Similarly, antagonism was not observed when MCF-7 cells were treated with tamoxifen and doxorubicin (Fig 4). In fact, the observed SFs with the

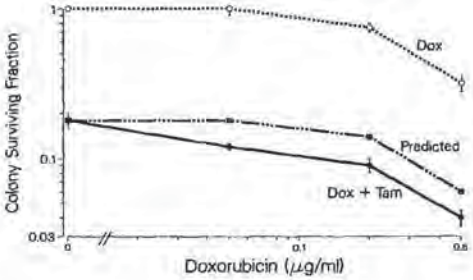

Fig 4. Dose-survival curves of doxorubicin without or with tamoxifen in MCF-7 cells. Cells were treated and processed as described in Figs 1-3.

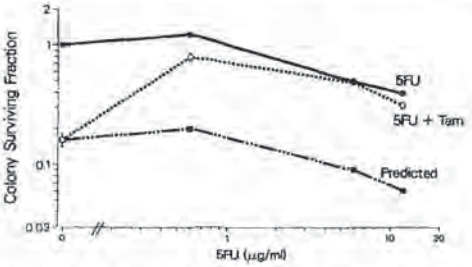

Fig 5. Dose-survival curves of fluorouracil (5FU) without or with tamoxifen in MCF-7 cells. Cells were treated and processed as described in previous figures.

combination were slightly less than those predicted at each dose, suggesting a modest synergistic interaction in this experiment. In another similar experiment, the interaction was simply additive. However, similar to melphalan, the combination of tamoxifen and fluorouracil was antagonistic (Fig 5). At each concentration tested, the SF observed with the combination of tamoxifen plus FU was much greater than that predicted for an additive effect, indicating that these drugs are also antagonistic.

DISCUSSION

These data demonstrate that treatment of cultured breast cancer cells with combinations of a cytotoxic agent and an antiestrogen can result in an antagonistic interaction. The antagonism is drug-specific and even alkylating-agent specific, since it was observed with melphalan and fluorouracil but not with 4-hydroxy-cyclophosphamide or doxorubicin. The antagonistic interaction was not unique to tamoxifen, but was observed with all four triphenylethelene antiestrogens tested. Interestingly, antiestrogen antagonism of melphalan cytotoxicity is not a consequence of the ability of these agents to induce a G_1 arrest, since antagonism was still observed under conditions in which tamoxifen has no antiproliferative or cell kinetic effects. Furthermore, tamoxifen and melphalan were antagonistic in ER-negative breast cancer cells and even in non-breast cancer tissue (rat liver cells) in which the antiestrogen has no effect on cell proliferation.

Our results confirm and extend the previous report by Goldenberg and Froese who showed that tamoxifen and melphalan were antagonistic.[15] This study also reported that the antiestrogen reduced the cellular uptake of melphalan, suggesting a possible mechanism of action. However, this study used extraordinarily high concentrations of tamoxifen ranging from 5 μmol/L to 25 μmol/L, the latter a concentration that is more than 25-fold that usually observed in patients treated for breast cancer, and a concentration that could have nonspecific and clinically irrelevant membrane effects. Nevertheless, using much lower tamoxifen concentrations, we have also found antagonism of melphalan cytotoxicity. Whether these lower tamoxifen doses antagonize melphalan by inhibiting drug uptake will require additional study.

In our study, tamoxifen did not antagonize the effects of 4-hydroxy-cyclophosphamide, the active metabolite of cyclophosphamide, despite its effects on another alkylating agent melphalan. It is interesting to speculate that tamoxifen inhibits specific carrier-mediated drug uptake processes, and that its failure to antagonize 4-hydroxy-cyclophosphamide is due to the fact that melphalan and cyclophosphamide are transported into the cell by independent transport mechanisms.[19]

Other investigators have examined the interaction of tamoxifen with fluorouracil and doxorubicin. Benz et al[13,14] reported a synergistic interaction between fluorouracil and high doses of tamoxifen in 47-DN human breast cancer cells that may have been due to tamoxifen-enhanced RNA-mediated toxicity of the antimetabolite. On the other hand, Hug et al[12] reported that tamoxifen antagonized the cytotoxic effects of fluorouracil and doxorubicin in MCF-7 and MDA-468 breast cancer cells. Our results are in partial agreement with the latter study, since under our experimental conditions we find that tamoxifen and fluorouracil are antagonistic in MCF-7 cells. However, we cannot confirm an antagonistic interaction between tamoxifen and doxorubicin; in our studies these drugs were additive or slightly synergistic. These results are consistent with other reports demonstrating that tamoxifen can increase doxorubicin sensitivity in leukemia cells, perhaps by altering membrane lipid packing density[11] or by its calcium channel antagonist properties.[8] The discrepancies reported in different studies on the interaction of tamoxifen with fluorouracil and doxorubicin cannot readily be explained, although they could be related to differences in cultured cell lines, differ-

ences in experimental conditions, or to differences in the drug doses and schedules employed.

We also examined the effect on melphalan cytotoxicity of pharmacologic concentrations of other endocrine agents used in breast cancer treatment. Medroxyprogesterone acetate and 17β estradiol, both at 1 μmol/L, had additive effects when combined with melphalan. Dexamethasone, on the other hand, had an antagonistic interaction. These data suggest caution when empirically combining cytotoxic agents with other drugs such as dexamethasone, which is commonly used as an antiemetic. Additional study is required to determine if this agent or other glucocorticoid derivatives can antagonize other commonly used cytotoxic drugs.

In any event, these in vitro data have potentially important clinical implications, and they suggest that the empirical combining of drugs such as an antiestrogen and a cytotoxic agent may not always be desirable. The data reported here indicate that combinations of tamoxifen and melphalan and/or fluorouracil should be avoided due to potential antagonism if the drugs are administered simultaneously. Furthermore, these data provide a potential explanation for the unique results reported in the National Surgical Adjuvant Breast and Bowel Project (NSABP) study comparing melphalan plus fluorouracil alone, or combined with tamoxifen.[2] Postmenopausal patients, especially those with receptor-positive tumors "benefited" from the addition of tamoxifen to chemotherapy. In this subset, tamoxifen could well have antagonized any small disease-free or overall survival advantage provided by the chemotherapy, but the net effect was beneficial because tamoxifen alone is beneficial in this subset. In contrast, in patients with receptor-negative tumors, especially if premenopausal, tamoxifen could have antagonized the beneficial effects of chemotherapy resulting in a net deleterious effect since it has not shown disease-free or overall survival benefit by itself in this subset.

The potential deleterious effects of combinations of tamoxifen with chemotherapy could be avoided by using drugs such as cyclophosphamide or doxorubicin that do not clearly demonstrate in vitro antagonism. However, an even better solution might be to sequence the two modalities by completing adjuvant chemotherapy before starting prolonged tamoxifen treatment. This hypothesis requires confirmation by future clinical trials.

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EXHIBIT G

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Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line

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Abstract—Tamoxifen, an antiestrogen, and doxorubicin, an anthracycline antibiotic, are each utilized alone and in combination in the treatment of breast carcinoma. In view of conflicting reports relating to the interaction between these drugs, studies were undertaken to characterize the influence of tamoxifen on growth inhibition by doxorubicin in the MCF-7 breast tumor cell line *in vitro*. Studies combining 5 μM tamoxifen, a clinically relevant concentration, with various concentrations of doxorubicin, indicated that this drug combination produces antiproliferative effects that appear to be less than additive. Concentration-dependent growth inhibition was analyzed further using various concentrations of tamoxifen and doxorubicin by the combination index–isobologram method; this quantitative approach provided clear evidence of antagonism between these agents, a finding with potential relevance to the treatment of breast cancer.

Key words: doxorubicin; tamoxifen; MCF-7; breast cancer; antagonism

Tamoxifen is the drug of choice in the treatment of estrogen-receptor positive breast cancer [1, 2], while doxorubicin may be utilized after relapse in patients treated with tamoxifen or in estrogen-receptor negative tumors [3]. Although these drugs have been utilized in combination, the advantage of this combination, in terms of therapeutic efficacy, remains controversial [4].

In vitro studies using doxorubicin-sensitive, estrogen-receptor positive breast tumor cell lines have been reported to show: (i) a slightly additive interaction between tamoxifen and doxorubicin [5]; (ii) a lack of interaction [6]; or (iii) protection by tamoxifen from doxorubicin toxicity [7]. Consequently, it appeared to be of importance to further evaluate this interaction, which could be of clinical relevance in the treatment of breast carcinoma.

Materials and Methods

Doxorubicin was purchased from the Sigma Chemical Co., St. Louis, MO, maintained as a frozen stock solution in distilled water, and protected from light [8]. Tamoxifen citrate (Sigma) was dissolved as a stock solution in 95% ethanol. The final concentration of ethanol for experimental conditions using 5 μM tamoxifen was 0.05%. MTT*, used in the cellular proliferation assay, was obtained from the Sigma Chemical Co.

MCF-7 human breast cancer cells were provided by Dr. Kenneth H. Cowan of the National Cancer Institute, National Institutes of Health, Bethesda, MD. Cells were maintained in high glucose Dulbecco's minimal essential medium (Hazelton Biologics, Lenexa, KS) supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY) and 5% defined bovine serum (Hyclone Laboratories, Logan, UT). The medium also contained glutamine (29.2 mg/100 mL) and penicillin/streptomycin (0.5 mg/100 mL).

While some laboratories utilize charcoal-stripped medium to eliminate the estrogenic effects of the pH indicator, phenol red [9, 10], previous studies have demonstrated that the responsiveness of MCF-7 breast tumor cells to tamoxifen is reduced in charcoal-stripped medium [11]. Consequently, the studies in this manuscript utilize "regular" medium in order to simulate a physiological milieu, where breast tumor cells are exposed to estradiol.

Cellular proliferation studies. The effects of tamoxifen and doxorubicin on cellular proliferation were monitored using a modification of the MTT tetrazolium dye assay [12–14], as described previously [15]. All growth studies were performed utilizing a continuous (72 hr) exposure to drug. The IC_{50} values generated using this type of analysis are comparable with those using cytotoxicity (clonogenic) assays [13, 14].

Intracellular accumulation of doxorubicin. Cellular doxorubicin levels were assessed using high performance liquid chromatography, as previously described [16, 17]. This methodological approach also serves to monitor drug metabolism [16, 18].

Ribonuclease protection assay. Expression of mRNA for the estrogen receptor was determined by the RNase protection assay [19] where 0.001 mg of pGEM-4Z-HEO plasmid DNA linearized with *Eco*RI was utilized in standard *in vitro* transcription reactions with [α-^{32}P]GTP, RNasin, and T7 RNA polymerase, respectively. RNase digestion of the non-hybridized sequences followed, and protected RNA was recovered by phenol/choroform extraction and ethanol precipitation.

Analysis of data. Analysis of statistical data was performed using the unpaired Student's *t*-test. Effects resulting in P values of 0.05 or below were considered to be statistically significant. For growth analysis and intracellular doxorubicin accumulation, values are expressed as means ± SEM, or means ± range for experiments with two replicates. The IC_{50} values were calculated by graphic extrapolation.

Computer software [20, 21] based on the median-effect principle and the combination index-isobologram equations [22, 23] was used for analysis of the interaction(s) between tamoxifen and doxorubicin at a 50:1 ratio.

Results and Discussion

Competitive radioligand binding studies [24] have established the presence of the estrogen receptor in MCF-7 breast tumor cells. Prior to evaluating the interaction of tamoxifen with doxorubicin in MCF-7 cells, expression of message for the estrogen receptor was confirmed by RNase protection (not shown).

MCF-7 breast tumor cells were exposed to 5 μM tamoxifen for 24 hr prior to doxorubicin as well as during the subsequent 48-hr incubation with doxorubicin. Exposure

* Abbreviations: IC_{50} drug concentration inhibiting 50% of cellular growth; and MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

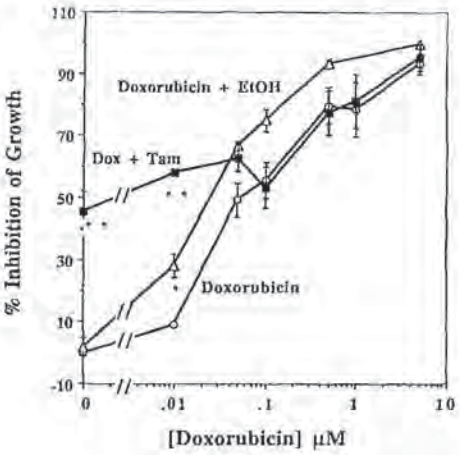

Fig. 1. Interaction between the antiproliferative activities of tamoxifen and doxorubicin in MCF-7 breast tumor cells. Cells in logarithmic growth were pretreated with 5 μM tamoxifen or 0.05% ethanol for 24 hr. At this time, cells were treated with various concentrations of doxorubicin (0.01 to 5.0 μM) with or without a simultaneous incubation with 5 μM tamoxifen or 0.05% ethanol, respectively. The number of viable cells was determined after 72 hr utilizing the MTT tetrazolium dye assay as described in Materials and Methods. The data presented are mean values for percent inhibition of growth ± SEM (as compared with untreated control) or as mean ± range (when only two replicate experiments were performed) for 2–15 individual experiments. Key: (*) significantly different from doxorubicin alone at $P \leq 0.01$, as determined by Student's *t*-test; (**) different from doxorubicin alone at $P \leq 0.001$, as determined by Student's *t*-test; (○) doxorubicin alone; (△) doxorubicin + ethanol; and (■) doxorubicin + tamoxifen.

of MCF-7 cells to 5 μM tamoxifen alone for 72 hr resulted in a 45.6 ± 6.3% inhibition of growth (Fig. 1 at 0 concentration of doxorubicin). Figure 1 further suggests that the combination of tamoxifen and doxorubicin produced less than additive effects on MCF-7 cell growth. At doxorubicin concentrations of 0.05 and 0.1 μM, growth inhibition never significantly exceeded that produced by tamoxifen alone, despite the fact that doxorubicin at these concentrations inhibited growth by 49.1 ± 5.5 and 55.6 ± 5.8%, respectively. At concentrations of 0.05 μM doxorubicin and above, growth inhibition by the combination of tamoxifen and doxorubicin was essentially identical to that produced by doxorubicin alone. (Ethanol, the vehicle in which the tamoxifen was dissolved, at a final concentration of 0.05%, demonstrated minimal effects on the growth of MCF-7 cells, although a small but significant increase in growth inhibition was observed at 0.01 μM doxorubicin.)

Drug accumulation studies failed to show significant differences in intracellular doxorubicin levels in cells exposed to doxorubicin alone or in the presence of tamoxifen (Table 1). High pressure liquid chromatographic analysis of drug extracted from MCF-7 cells after 2 hr of incubation indicated the absence of any doxorubicin metabolites (data not shown).

To perform a more quantitative evaluation of the interaction between tamoxifen and doxorubicin, MCF-7 cells were exposed to various concentrations of tamoxifen alone, doxorubicin alone, or to tamoxifen + doxorubicin in a 50:1 ratio, and growth inhibition was analyzed using the dose–effect analysis program developed by Chou and Talalay [22, 23]. Figure 2 presents a computer extrapolation of the combination index–dose–effect relationship (based on data

Table 1. Lack of effect of 5 μM tamoxifen on the intracellular accumulation of doxorubicin in MCF-7 breast tumor cells

	Intracellular doxorubicin concentration (pmol/10^6 cells)	
Doxorubicin [μM]	Doxorubicin alone	Doxorubicin and tamoxifen
5	1222 ± 167 (4)	1194 ± 154 (4)
10	2098 ± 751 (4)	2807 ± 721 (3)

Cells in logarithmic growth were grown in medium alone or in the presence of 5 μM tamoxifen for 24 hr. At this time, cells were incubated for 2 hr with doxorubicin at concentrations of 5 and 10 μM with or without a simultaneous incubation with 5 μM tamoxifen. Following drug treatments, cells were harvested and intracellular doxorubicin concentrations were determined as described under Materials and Methods. Results were normalized for cellular protein content. The data presented are means ± SEM for the number of replicate experiments indicated in parentheses. No significant differences in doxorubicin accumulation were noted between cells exposed to doxorubicin alone or those exposed to the combination of doxorubicin and tamoxifen.

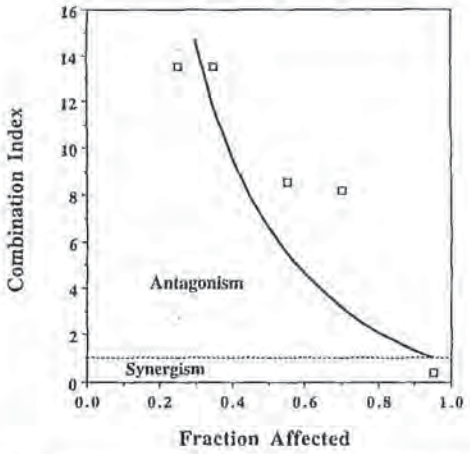

Fig. 2. Fractional inhibition of growth–combination index plot for the combination of tamoxifen + doxorubicin at a 50:1 ratio. Symbols indicate the actual combination data points obtained when MCF-7 human breast tumor cells were exposed to tamoxifen (for 24 hr) followed by doxorubicin for an additional 48 hr. The total incubation period was 72 hr. The interrupted line is derived from a computer simulation of this drug combination assuming that the actions of the two drugs are mutually non-exclusive. A more extensive discussion of this approach to analysis of drug interaction is found in Ref. 25.

derived from a representative experiment), which indicates that the combination of tamoxifen + doxorubicin was antagonistic over virtually the entire range of growth inhibition (up to approximately 95% growth inhibition). This conclusion is based on the fact that the combination index is greater than one over the entire range [20, 21]. Also shown are calculated combination index values for the five experimental conditions where tamoxifen + doxorubicin were combined at a ratio of 50:1 (from which the extrapolated curve was derived).

The basis for antagonism at the lower range of concentrations of doxorubicin may be related to the capacity of tamoxifen to decrease growth of estrogen-receptor positive MCF-7 cells, and to stabilize these cells in the G_0-G_1 phase of the cell cycle [26, 27], resulting in decreased sensitivity to cytotoxic drugs [28]. In this context, DNA synthesis inhibition has been shown to protect against cytotoxicity mediated by topoisomerase II inhibitors such as doxorubicin [29]. Antagonism at elevated concentrations of doxorubicin may be related to the findings of Clarke *et al.* [30], indicating a reduction in estrogen binding capacity in MCF-7 cells following a 24-hr exposure to 0.018 to 1.8 μM doxorubicin, presumably due to a decrease in the rate of estrogen-receptor cycling or its synthesis. In this case, the presence of doxorubicin would serve to abrogate any antiproliferative effects of the antiestrogen tamoxifen. Consequently, both cell cycle arrest and alterations in estrogen-receptor content may contribute to antagonism between doxorubicin and tamoxifen over a relatively wide range of doxorubicin concentrations.

Although tamoxifen has been shown to increase the effectiveness of doxorubicin against multidrug-resistant breast tumor cells *in vitro* [6], the fact that the combination of tamoxifen and doxorubicin in MCF-7 cells is antagonistic may contribute to the observation that the utilization of conventional doses of these agents in combination has not demonstrated markedly greater clinical utility than either drug administered alone [4]. However, it should be noted that a number of different drugs are used in these combination regimens, and that other factors such as the extent of hormone dependency, innate drug resistance, and the clinical pharmacokinetics of the administered drugs may also influence the ultimate response to antitumor drugs in combination regimens.

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EXHIBIT H

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Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials

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Summary An analysis of the activity of compounds tested in pre-clinical in vivo and in vitro assays by the National Cancer Institute's Developmental Therapeutics Program was performed. For 39 agents with both xenograft data and Phase II clinical trials results available, in vivo activity in a particular histology in a tumour model did not closely correlate with activity in the same human cancer histology, casting doubt on the correspondence of the pre-clinical models to clinical results. However, for compounds with in vivo activity in at least one-third of tested xenograft models, there was correlation with ultimate activity in at least some Phase II trials. Thus, an efficient means of predicting activity in vivo models remains desirable for compounds with anti-proliferative activity in vitro. For 564 compounds tested in the hollow fibre assay which were also tested against in vivo tumour models, the likelihood of finding xenograft activity in at least one-third of the in vivo models tested rose with increasing intraperitoneal hollow fibre activity, from 8% for all compounds tested to 20% in agents with evidence of response in more than 6 intraperitoneal fibres ($P < 0.0001$). Intraperitoneal hollow fibre activity was also found to be a better predictor of xenograft activity than either subcutaneous hollow fibre activity or intraperitoneal plus subcutaneous activity combined. Since hollow fibre activity was a useful indicator of potential in vivo response, correlates with hollow fibre activity were examined for 2304 compounds tested in both the NCI 60 cell line in vitro cancer drug screen and hollow fibre assay. A positive correlation was found for histologic selectivity between in vitro and hollow fibre responses. The most striking correlation was between potency in the 60 cell line screen and hollow fibre activity; 56% of compounds with mean 50% growth inhibition below $10^{-7.5}$ M were active in more than 6 intraperitoneal fibres whereas only 4% of compounds with a potency of 10^{-4} M achieved the same level of hollow fibre activity ($P < 0.0001$). Structural parameters of the drugs analysed included compound molecular weight and hydrogen-bonding factors, both of which were found to be predictive of hollow fibre activity. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: anticancer drug discovery; in vitro-to-in vivo correlations; clinical trials

Since its inception in 1955, the National Cancer Institute's (NCI) Developmental Therapeutics Program (DTP) has utilized various experimental screening models to select agents for evaluation as clinical candidates. The philosophical position from which this endeavour proceeded was that elucidation of empirically defined anti-tumor activity in a model would translate into some likelihood of activity in human cancer. The choice of specific screening models was based primarily on response of the models to agents already identified as clinically active (Gellhorn and Hirschberg, 1955; Zubrod et al, 1966). Initially, 3 transplanted rodent models were used: Sarcoma 180, Carcinoma 755 and Leukaemia L1210. The spectrum of models was then broadened, retaining L1210, which was discerned to be the most predictive of clinical activity, and adding a series of transplanted rodent models. This scheme was replaced in 1975 by the murine P388 leukaemia model, which was utilized as a pre-screen and followed by a panel of tumours. This panel first included only rodent tumours but was later enhanced to include human tumour xenografts (Venditti, 1981; Venditti et al, 1984). The human tumour xenografts were employed with the intent of their serving as potentially better predictors of clinical activity against solid human tumours.

In early 1990, the P388 pre-screen was replaced by an in vitro human tumour cell line assay comprised of 60 different cell types (Alley et al, 1988; Monks et al, 1991; Paull et al, 1995). Agents selected on the basis of potency, selective activity against a particular disease category, and/or differential activity against a few specific cell lines were then evaluated against a small number of sensitive human tumours in the nude mouse xenograft model (Dykes et al, 1992; Plowman et al, 1997) as a basis for selecting compounds for further preclinical development. Owing to the large numbers of molecules emerging from the in vitro screen as candidates for xenograft testing, in 1995 this development path was further modified to include a hollow fibre (HF) assay (Hollingshead et al, 1999), activity in which was a prerequisite for study in classical xenograft models. The HF model, where cells are introduced from tissue culture into semi-permeable fibres in mouse intraperitoneal (i.p.) or subcutaneous (s.c.) space and exposed to test agents, is a rapid and efficient means of selecting compounds with the potential for in vivo activity in conventional xenografts in pilot and 'training' sets of compounds.

This drug screening and development scheme remains an empirical one, as compounds are prioritized for development based on the definition of anti-proliferative in vitro and in vivo responses. Owing to an emerging understanding of the molecular

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basis for human cancer, great interest exists in transitioning from an empirical to a potentially more rational, molecular-targeted approach to the discovery and development of novel cancer therapeutics (Sausville and Feigal, 1999). Of particular interest will be the design of models to detect the action of compounds on particular predefined targets. The 'performance features' of compounds that have been evaluated in the 'empirical' development scheme may be of value in serving as a baseline against which newer compounds and models may be compared.

To this end, we present here an experience attempting to correlate activity in the clinic with antecedent activity in preclinical models. In addition, we present NCI's cumulative experience with the performance of the HF assay in relation to in vitro activity and to certain chemical characteristics of the compounds.

MATERIALS AND METHODS

Agents used

Data for 39 agents which had completed phase II trials (Table 1) and which had also been evaluated against in vivo tumour models were compiled. 13 of these compounds were 'standard' agents and the remaining 26 were compounds for which NCI INDs were filed between 1980 and 1996.

Table 1 NSC numbers and common names for 39 phase II clinical agents

NSC Number	Common name
740	Methotrexate
3053	Actinomycin-D
3088	Chlorambucil
8806	Melphalan
19893	5-FU
26271	Cyclophosphamide
26980	Mitomycin C
45388	Dacarbazine
49842	Vinblastine
105014	2-CDA
119875	Cisplatin
123127	Adriamycin HCL
125066	Bleomycin
125973	Paclitaxel
141633	Homoharringtonine
172112	Spiromustine
253272	Caracemide
264880	Dihydro-5-azacytidine
267469	Deoxydoxorubicin
269148	Menogaril
281272	Fazarabine
286193	Tiazofurin
308847	Amonafide
312887	Fludarabine phosphate
325319	Didemnin B
332598	Rhizoxin
336628	Merbarone
337766	Bisantrene
339004	Chloroquinoxaline sulfonamide
347512	Flavone acetic acid
349174	Piroxantrone hydrochloride
352122	Trimetrexate
356894	Deoxyspergualin
361456	Pyrazine diazohydroxide
366140	Pyrazoloacridine
409962	BCNU
609699	Hycamptamine
616348	CPT-11 (irinotecan)
628503	Taxotere (docetaxel)

For an analysis of factors affecting xenograft outcome, 1228 compounds for which both current xenograft data and activity in NCI's 60 cell line in vitro anti-cancer drug screen were available. The compounds were tested in a median of 5 xenograft experiments (range 1–131) and a median of 4 different histologies (range 1–14). In addition, 564 of these had been evaluated in the HF assay. Compounds were selected for xenograft testing at least partially on the basis of performance in the 60 cell line assay and/or the HF assay. Of these, 756 are open, publicly available compounds and 472 are discreet, confidential structures.

A set of 2304 compounds which was evaluated in the 60 cell line assay (1252 open, 1052 discreet) and referred for evaluation and testing in the HF assay was analysed. The compounds represented a wide variety of structural types, and ranged in in vitro potency from GI_{50}s (concentration of drug which achieved 50% growth inhibition averaged over all 60 cell lines) of 100 μM to less than 10 nM. Reasons for referring the compounds for HF evaluation included selective activity against cell lines of a particular histologic type, 'non-standard' mechanism of action as determined by the COMPARE pattern recognition algorithm (Paull et al, 1989), as well as compounds which were structurally novel and possibly representing novel chemotypes directed against defined mechanisms (e.g. topoisomerase inhibition). Lists of the non-proprietary compounds in these data sets are available at http://dtp.nci.nih.gov/docs/bjcwebsup.html.

Clinical response

Clinical response rate of particular histologic tumour types to the 26 NCI IND agents was reviewed in patients from all completed phase II trials with at least 9 patients; the trials were conducted under NCI sponsorship or otherwise included in Cancer Therapy Evaluation Program records, and were carried out with appropriate ethical committee approval. A 'positive' phase II trial involved an objective 50% reduction in tumour size in at least 20% of patients. For the 13 standard agents, response rates were taken from DeVita (De Vita et al, 1982, 1985, 1989, 1993, 1997). All abstractions were done by professional abstractors from EMMES Corporation (Potomac, MD).

Preclinical models

Compounds were tested in a variety of xenograft models according to methods described by Dykes and Plowman (Dykes et al, 1992; Plowman et al, 1997). Two levels of response were considered. For 'survival' models, the 2 thresholds were increased life span of 25% or 50%. In other subcutaneous xenograft models, estimation of tumour weight allowed calculation of treated/control weight ratios (T/C), with thresholds of 40% and 10% indicative of a level or degree of activity in this analysis. Agents were in all cases studied at or below the maximum tolerated dose, defined as the greatest quantity of test agent given as an acute dose which a mouse is able to survive for 11 days.

Agents were tested in the HF assay as described by Hollingshead (Hollingshead et al, 1999). A standard panel of 12 tumour cell lines are used for routine HF screening, including non-small cell lung carcinoma lines NCI-H23 and NCI-H522, breast carcinoma lines MDA-MB-231 and MDA-MB-435, colon sarcoma lines SW-620 and COLO 205, melanoma lines LOX and

UACC-62, ovarian carcinoma lines OVCAR-3 and OVCAR-5, and glioma lines U251 and SF-295. The cells, at densities of 2–10 $\times 10^6$ cells ml^{-1} are flushed into polyvinylidine fluoride fibres having an internal diameter of 1 mm. The fibres are heat-sealed at 2 cm intervals, and the samples are placed into tissue culture medium and incubated for 24 to 48 hours prior to implantation. On the day of implantation, samples of each tumour cell line preparation are quantitated for viable cell mass by a stable endpoint MTT assay so that the time zero cell mass is known. Each mouse receives 3 i.p. implants representing 3 of the tumour cell lines, and 3 s.c. implants of the same 3 tumour cell lines. Mice are treated with experimental agents, which have been solubilized using 10% DMSO in saline and tween 80 (0.05%), on day 3 or 4 following fibre implantation and continuing daily for 4 days. Each agent is administered by i.p. injection at 2 dose levels. The dose levels are determined from the single dose i.p. maximum tolerated dose (MTD) for each test agent; the high dose being the MTD × 0.375 and the low dose being the MTD × 0.25. The fibres are collected from the mice on the day following the fourth treatment and viable cells estimated by the MTT assay. A 50% or greater reduction in percent net growth in the treated samples compared to the vehicle control samples is considered a positive result. A total of 48 fibres is treated in a standard experiment (12 cell lines × 2 implant sites × 2 dose levels). Each treated fibre exhibiting at least a 50% net reduction in cell growth is assigned an arbitrary score of 2 points, and the number of fibres reaching this level in the i.p. and s.c. compartments are recorded separately. Any cell lines in which cell kill is observed are also recorded.

Criteria were initially established for compound activity using a training set of 80 randomly selected compounds which were evaluated in both the HF and xenograft assays. The goal of the training set was to define a scoring system which allowed bias in favour of detecting all compounds with xenograft activity, defined as ≤40% T/C in at least one tumour xenograft. Criteria for activity in the HF assay which accomplished this goal were thus: 20 or more total points (any combination of points in the i.p. and s.c. fibres), 8 or more points in s.c. fibres, or an observation of cell kill in any fibre in either the i.p. or s.c. compartment.

The NCI in vitro anti-cancer drug screen has been described in detail previously (Alley et al, 1988; Monks et al, 1991). It utilizes 60 different human tumour cell lines representing leukaemia, melanoma, lung, colon, brain, ovary, breast, prostate and kidney cancers. For a typical screening experiment, cells are inoculated into 96-well microtitre plates and allowed to incubate for 24 hours. Experimental drugs are solubilized in dimethyl sulfoxide and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted with complete medium. 4,10-fold or 1/2 log additional serial dilutions are made to provide a total of 5 drug concentrations plus control. Following drug addition, the plates are incubated for an additional 48 hours. Sulforhodamine B solution is added to each well, and bound stain is solubilized and the absorbance read. The percentage growth is calculated at each of the drug concentration levels. 3 dose response parameters are calculated for each experimental agent: growth inhibition of 50% (GI_{50}), total growth inhibition (TGI), and the concentration of drug resulting in a 50% reduction in measured protein (LC_{50}).

Structural characteristics

Chem-X, a product of Chemical Design, Ltd (Oxfordshire, UK), was used to quantitate aspects of structural characteristics such as the number of hydrogen bonds, hydrogen bond donors and acceptors. Molecular weights were taken as calculated from molecular formulas from the DTP Drug Information System.

Statistical tests

χ^2 analyses were conducted to examine the effect of various factors on in vivo outcomes; where insufficient data were available for a χ^2 analysis, the Fisher's exact test was employed. The Spearman rank correlation coefficient was used to correlate in vivo activity with clinical responses; this statistic is defined as the more familiar Pearson correlation of the data, after the data values for each measure of activity have been replaced by their respective ranks. The Spearman rank correlation is more appropriate than the Pearson correlation in situations where the data are not normally distributed. A Student's *t*-test was used in comparing mean MW and means for hydrogen-bonding characteristics. Statistical significance was set at the 99% confidence level ($P < 0.01$).

RESULTS

Indicators of clinical activity

For 39 clinical agents, relationships between xenograft response levels, averaged by histology, and the phase II response rates were investigated. The Spearman rank correlation coefficients (r) for all histologies are plotted in Figure 1. Only non-small cell lung (NSCL) xenografts were predictive of clinical activity in the same histology ($r = 0.814$, $P = 0.004$). Breast xenograft models were the most useful for predicting clinical response against any disease, correlating with clinical activity against NSCL ($r = 0.565$, $P = 0.008$), melanoma ($r = 0.540$, $P = 0.007$) and ovarian ($r = 0.611$, $P = 0.003$) cancer, but interestingly, not with clinical breast cancer. Activity in colon xenografts predicted for clinical melanoma response ($r = 0.532$, $P = 0.005$). There were no other correlations that met the criteria for statistical significance. The number of xenograft models with a T/C ≤40% or ILS ≥25% was compared with the presence of clinical activity. Clinical activity was found in only 2/6 agents (33%) with activity in fewer than one-third or more of tested xenograft models, but was found in 21/33 agents (64%) with activity in one-third or more of tested xenograft models (Figure 2A); undoubtedly due to the small number of agents, this difference is not statistically significant ($P = 0.14$). The comparison was also repeated using a more stringent definition of clinical activity, demanding that agents tested in multiple disease demonstrate response in at least 2 diseases. Applying this more strigent definition, clinical activity was found in 0/6 agents (0%) which had activity in fewer than one-third of tested pre-clinical xenograft models, whereas 15/33 agents (45%) with activity in one-third or more of tested pre-clinical xenograft models had clinical activity ($P = 0.04$) (Figure 2B). The analyses were repeated, increasing the threshold for xenograft activity to a T/C ≤10% or ILS ≥50%, a comparison which yielded no useful correlations between overall xenograft activity and either definition of clinical activity.

Indicators of xenograft activity

To assess how HF activity might be related to predicting activity in some xenograft model, we considered the likelihood of responses in xenograft models as a function of HF activity. Table 2A shows

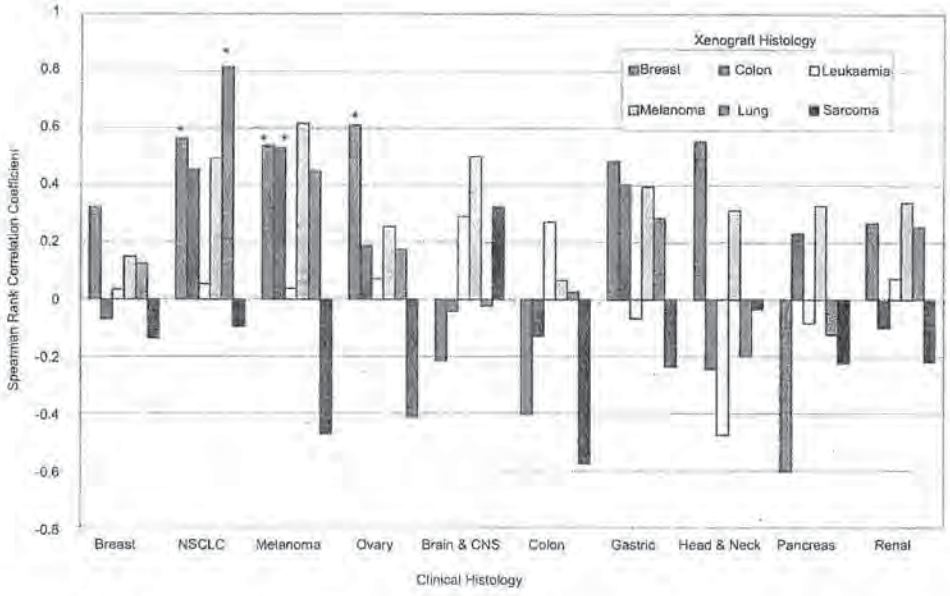

Figure 1 In vivo activity and clinical activity by disease type The median number of agents per correlation was 12 (range 2–27). For the statistically significant correlations, the median was 21 (range 10–26). The number of xenograft systems per histology ranged between 7 and 9 for all histologies except for sarcoma which included only one xenograft model. * – statistically significant correlation

Table 2A IP HF activity vs xenograft activity (at least 4 tumours tested)

	% of xenograft models active			
Responsive IP fibres	<33%	≥33%	Total	% active in 33% of xenografts
0–6 fibres	179	6	185	3%
≥7 fibers	57	14	71	20%
Total	236	20	256	8%
			$\chi^2 = 19.3$, $P < 0.0001$	

Table 2B IP HF activity vs activity in IP xenograft models

Responsive IP fibres	Agents active in IP xenograft	Agents inactive in IP xenograft	Total	% active
0–3 fibres	32	231	263	12%
4–6 fibres	23	85	108	21%
7–9 fibres	16	30	46	35%
≥10 fibres	19	23	42	45%
Total	90	369	459	20%
			$\chi^2 = 33.7$, $P < 0.0001$	

Compounds were studied in a median of one ip xenograft model, with some compounds being tested in as many as 8 models.

that using compounds with studies in at least 4 different tumour types, there exists a strong correlation ($P < 0.0001$) between activity in HF placed in the peritoneal compartment and ultimate xenograft activity, so that compounds with activity in >6 peritoneal fibres had a 20% likelihood of subsequent xenograft activity while compounds with activity in ≤6 fibres had a 3% rate of xenograft activity. One criticism of this correlation is that it would be

Table 2C IP HF activity vs activity in any xenograft model

Responsive IP fibres	Agents active in any xenograft	Agents inactive in all xenograft	Total	% active
0–3 fibres	84	212	296	28%
4–6 fibres	43	84	127	34%
7–9 fibres	26	31	57	46%
≥10 fibres	36	21	57	63%
Total	189	348	537	35%
			$\chi^2 = 28.4$, $P < 0.0001$	

relevant only to intraperitoneal xenografts. Table 2B does in fact show that activity in ≥10 peritoneal fibres does, perhaps not surprisingly, show activity in 45% of peritoneal xenografts. However, Table 2C reinforces the correlation of activity in peritoneal fibres to activity in any xenograft model, including subcutaneous models. Note that for this comparison, if the threshold for HF activity is raised to more than 6 fibres, at least two-thirds of the xenograft active compounds become false negatives. Interestingly, activity in subcutaneously placed fibres did not correlate with likely activity in either peritoneal or subcutaneous xenografts (data not shown). Since HF response did not account for all of the xenograft active agents, the nature of the compounds and test conditions for these agents were examined. In all of these compounds, the xenograft activity was obtained using a route, schedule or other experimental condition not available or not routinely used in the HF assay (data not shown); in the case of some slower growing solid tumours, for example, activity was obtained using Q4Dx3 or Q7Dx3 schedules which are not available in the standard 4-day HF assay.

A second general indicator of likely xenograft activity actually emerges from consideration of in vitro screening data. Tables 3A

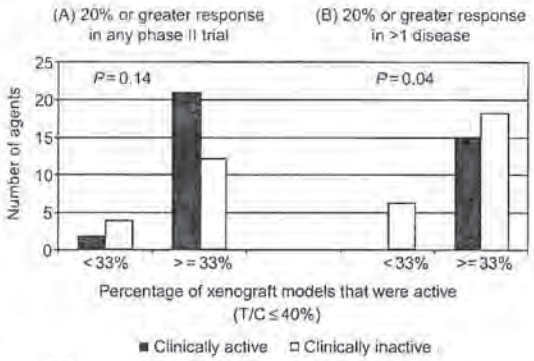

Figure 2 Overall xenograft activity & clinical activity. Agents were tested in a median of 12 xenografts (range 2–45) and a median of 4 clinical diseases (range 1–24). The 33% cutoff was chosen retrospectively to provide a useful benchmark which minimized false negatives

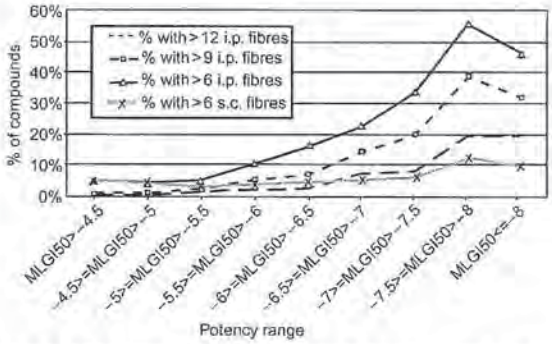

Figure 3 Mean log of GI_{50} versus percentage of compounds with hollow fiber response in at least *n* fibers

and 3B demonstrate that compounds with evidence of selective activity for successively greater numbers of lung or breast carcinoma cell lines had successively increased likelihood of demonstrating activity in lung or breast xenograft models. Compounds with evidence of in vitro selectivity in 6 or more lung cell lines exhibited activity in 33% (compared to 17.5% overall) of corresponding xenografts. Compounds with evidence of in vitro selectivity in breast cell lines were even more likely (44%) to be active in the corresponding xenografts (compared to 21% overall). When this effort was extended to other histologies, no other significant correlations emerged (data not shown).

Indicators of HF activity

Given that HF response can, to a certain extent, predict some level of xenograft activity, it becomes of interest to enquire if predictors of activity in the HF model can be gleaned from in vitro screening data or structural properties of the molecules. Table 4 demonstrates that in the case of breast, lung, ovarian, CNS and melanoma cell lines, selective activity in the particular in vitro panel does show a trend toward correlating with emergence of activity in the corresponding cell types in the HF panel. For example, 48% of the agents that were selective for breast cell line activity in vitro also showed activity (growth inhibition of 50%) in the breast carcinoma HF, compared to 39% of compounds selected for study in hollow fibres for any reason. Colon carcinoma cell line activity did not correlate with HF colon cancer cell line activity.

Greater potency in the 60 cell line assay, as indicated by the GI_{50}, correlates exceptionally well with increasing activity in the HF model with peritoneal fibres (Figure 3) ($P < 0.0001$). For example, 37% of compounds with a $\log_{10}$ GI_{50} of at least -7.5 demonstrate activity in at least 10 out of 24 i.p. fibres, whereas the fraction of all compounds with this level of activity in the HF assay is only 6%. Similar analyses were carried out utilizing activity in s.c. fibres and i.p. plus s.c. fibres. As with the comparison of HF-to-xenografts, these analyses showed that activity in the s.c. fibres did not correlate with in vitro 60 cell line potency, and that activity in the i.p. plus s.c. fibres was less correlative than the i.p. fibre response alone (data not shown).

The mean GI_{50} for only the 12 cell lines used in the HF assay was also examined to determine if this measure was more correlative than the average potency in all 60 cell lines. Although 12 cell line in vitro potency still correlates positively with HF activity, the correlation is not as striking as that obtained using the 60 cell line GI_{50} (data not shown).

'Differential' activity of a compound between distinct cell types in the in vitro screen may be an indication of a basis for expecting selective anti-proliferative effect in vivo and ultimately in the

Table 3A Analysis of in vitro lung histology response vs lung xenograft response

No. lung lines with GI_{50} < mean GI_{50}	Lung xenograft inactive	Lung xenograft active	Total tested	% active	χ^2	*P*
3	299	79	378	21%	9.99	0.00158
4	206	70	276	25%	24.1	<0.0001
5	133	56	189	30%	29.6	<0.0001
6	78	38	116	33%	23.8	<0.0001

Table 3B Analysis of in vitro breast histology response vs breast xenograft response

No. breast lines with GI_{50} < mean GI_{50}	Breast xenograft inactive	Breast xenograft active	Total tested	% active	χ^2	*P*
3	189	53	242	22%	0.665	0.415
4	159	46	205	22%	0.884	0.347
5	103	38	141	27%	5.62	0.0180
6	34	27	61	44%	25.0	<0.0001

Table 4 Histological comparison between 60 cell line screen and hollow fibre assay

HF disease type	I.	II.	χ^2	*P*
Breast	48%	39%	14.9	1.11E–04
Colon	26%	27%	0.182	0.670
Lung	65%	59%	10.7	0.00106
Ovarian	55%	40%	14.5	1.42E–04
CNS	58%	47%	15.9	<0.0001
Melanoma	30%	25%	8.85	0.00293

I. This column shows the activity rate for the HF tumour type where 4 or more cell lines in the same 60 cell line disease panel were responsive.
II. This column shows the overall activity rate for the HF tumour type for all compounds tested.

Table 5 Differential activity and HF activity

	Responsive IP fibres						
Δ	0	1–3	4–6	7–9	≥10	Total	% with 4 or more fibres
Δ≤.5	34	76	33	20	36	199	45%
0.5<Δ≤1	138	427	154	46	50	815	31%
1<Δ≤1.5	179	389	138	38	42	786	28%
1.5<Δ≤2	77	150	55	21	18	321	29%
Δ>2	39	97	38	6	3	183	26%
Total	467	1139	418	131	149	2304	30%
						$\chi^2 = 79.4$, $P < 0.0001$	

Table 6 Molecular weight and HF activity

	Responsive IP fibres						
MW	0	1–3	4–6	7–9	≥10	Total	% with 4 or more fibres
MW≤250	47	102	27	11	2	189	21%
250<MW≤500	330	815	311	78	103	1637	30%
500<MW≤750	68	175	65	27	30	365	33%
750<MW≤1000	14	25	10	13	7	69	43%
MW>1000	8	22	4	1	7	42	29%
Total	467	1139	417	130	149	2302	30%
						$\chi^2 = 55.3$, $P < 0.0001$	

Table 7 Hydrogen bonding sites and HF activity

	Responsive IP fibres						
Potential H bond sites	0	1–3	4–6	7–9	≥10	Total	% with 4 or more fibres
0 to 3	110	233	70	21	8	442	22%
4 to 6	226	577	223	53	58	1137	29%
7 to 10	105	250	97	33	59	544	35%
11 to 15	16	51	21	17	13	118	43%
≥16	10	28	7	7	11	63	40%
Total	467	1139	418	131	149	2304	30%
						$\chi^2 = 88.3$, $P < 0.0001$	

Table 8 Molecular weight and HF activity (independent of hydrogen bonding sites)

	Responsive IP fibres (4–6 hydrogen bonding sites)				
MW	0	1–3	4–6	≥7	Total
MW≤250	24	41	15	4	84
250<MW≤500	178	488	186	101	953
MW>500	27	48	19	6	100
Total	229	577	220	111	1137
				$\chi^2 = 11.0$, $P = 0.0895$	

Table 9 Hydrogen bonding and HF activity (independent of MW)

	Responsive IP fibres (250 <MW ≤500)					
Potential H-bond sites	0	1–3	4–6	7–9	≥ 10	Total
0–3	86	163	55	14	8	326
4–6	175	488	188	45	56	952
≥7	69	164	68	19	39	359
Total	330	815	311	78	103	1534
					$\chi^2 = 35.8$, $P < 0.0001$	

clinic. Indeed, the potential for this to emerge was a key rationale for embarking on the 60 cell line screening paradigm (Alley et al, 1988). The difference in GI_{50} for any cell line and the average GI_{50} for all 60 cell lines was computed for the compounds studied in the peritoneal fibres. The largest differential or 'Δ' over the 60 cell lines is recorded by cell type and value for each compound. Table 5 shows that increasing Δ actually correlates negatively with increasing likelihood of observance of activity in the HF model ($P < 0.0001$). For example, the percentage of compounds which respond in at least 4 i.p. fibres is greater with a Δ of ≤0.5 (45%) than the percentage with Δ > 2 (26%). The same trend is displayed when comparing Δ with xenograft activity, but the correlation is not statistically significant (data not shown).

The effect of molecular weight and the number of potential hydrogen bonds in the compound structure on activity in the HF assay were also examined. Compounds in low MW ranges (160 to 480) are generally considered to be more 'drug-like' than those in higher ranges (Ghose et al, 1999). Interestingly, the number of responsive i.p. fibres generally increases with increasing MW up to a MW of 1000 (Table 6). For MW greater than 1000 activity appears to decrease, but there are too few compounds of this size level to generalize. In molecules with 7 to 10 potential hydrogen bonds, 35% of compounds responded in a least 6 i.p. fibres versus 30% overall ($P < 0.0001$) (Table 7). This percentage increased to 43% in compounds with 11 or more potential hydrogen bonds. Since, however, the number of heteroatoms contributes to both the number of hydrogen bonds and MW, interdependence of MW and hydrogen bond counts was examined and the results shown in Table 8. Using the constant hydrogen bond range of 4 to 6, the correlation between MW and HF activity was no longer statistically significant ($P = 0.0895$). However, using the constant MW range of 250 to 500, the hydrogen bond parameter, now independent of the effect of MW, still correlated strongly with HF response ($P < 0.0001$) (Table 9). Neither MW or the number of hydrogen bonds had any influence on activity in the s.c. fibres. The

relationship between these physiological properties and xenograft activity had also been examined; although the same conclusions could be drawn, the correlations were not as strong (data not shown).

According to the Lipinski Rule to Five (Lipinski et al, 1997), compounds with more than 5 hydrogen bond donors (HBD) or more than 10 hydrogen bond acceptors (HBA) are more likely to have poor oral absorption. Among the compounds tested in the HF assay, 95% had less than 5 HBD and 95% less than 10 HBA; since the percentages of compounds with favourable HBD and HBA counts was so great, an analysis of the effect of these parameters was not performed. In fact, these parameters were identical to the compounds entering the in vitro screening system.

Unfortunately, only about 75% of compounds selected for the HF assay are tested in that system, primarily due to problems with insufficient supply of compound. The 25% of selected but not tested compounds represent a potential source of bias in these analyses, particularly as the compounds for which it is not possible to obtain the quantities necessary for in vitro testing might represent greater structural complexity. In fact, the mean MW of non-tested compounds was 485 (vs 420 for tested compounds), with only approximately 1.3 additional H bond sites per molecule (data not shown).

DISCUSSION

The analysis of xenograft versus clinical results illustrates that a histology to histology comparison of these models to activity in the clinic cannot be reliably discerned for these 'empirically' selected compounds acting against non-molecularly characterized tumours. Although, with the exception of lung, histological matches were not found between in vivo models and clinical response, activity in multiple xenograft models does appear to predict for some degree of clinical activity. Interestingly, increasing the activity threshold to a T/C of ≤10 did not increase the likelihood of a positive clinical outcome, indicating that this activity threshold is probably too stringent. Requiring greater clinical activity (activity in 2 or more diseases) does improve the correlation between xenograft activity and clinical activity. Although the more stringent definition of clinical activity may not be suitable for making decisions on whether to advance agents from phase II to phase III trials, as a purely statistical exercise, it does support the conclusion that activity in multiple xenograft models is a useful predictor of clinical activity.

In contrast to results achieved with the DTP xenograft system, Fiebig (Scholz et al, 1990) has developed xenograft tumours which retain characteristics similar to the clinical specimens, having been grown from slow-growing and well-differentiated cell lines. Fiebig reports that when treating these xenograft tumours with the same standard agents as were utilized in the clinic for the corresponding patient tumours, the response of these xenografts in comparison to patient tumours was 90% (19/21) for sensitive and 97% (57/59) for non-responding tumours respectively. While this degree of correspondence between clinically used agent activity in xenografts and the clinic is gratifying, it is uncertain how to translate that experience to the evaluation of new agents with no prior defined clinical activity. As the clinically used standard agents have in all cases proven active in many xenograft models, this result at one level might be in accord with our finding that the number of xenografts in which an agent is active correlates with likely activity in the clinical setting.

The results presented in Figures 1 and 2 may be taken to argue against the use of activity in an empirically selected xenograft model to predict activity in the same histologic type of cancer in the clinic, and indeed that result has influenced the current philosophy underlying NCI's drug discovery and development programme (Sausville and Feigal, 1999). Nonetheless, definition of an active agent in xenografts would allow optimization of schedule, assessment of molecular target endpoints, and increase the probability of clinical activity. It is therefore beneficial to have the ability to select agents with a likelihood of xenograft activity using a rapid and inexpensive test. The HF assay was developed to serve as a discriminator for compounds emerging from an empirical in vitro cell line screen. Our analyses indicate that greater levels of response in the i.p. fibres correlate with greater likelihood of xenograft activity. The same cannot be stated for responses in the s.c. fibres. Double (Phillips et al, 1998) reports that the NCI protocol for HF does not allow sufficient time for angiogenesis to occur, and hypothesizes that s.c. response may be underestimated with NCI's protocol. Folkman (Hahnfeldt et al, 1999), however, claims that a tumour does not require vessels until it is 3–4 mm in diameter; the polyvinylidene fibres are only 1 mm. Subcutaneous response is dependent on extravascular drug concentration, so the lack of correlation between s.c. fibres response and xenograft activity may be speculatively related to factors such as dose, route, schedule, hydrostatic pressure and hydration state of the host.

As with xenograft to clinical comparisons, activity against a single specific HF histology was generally not useful for predicting xenograft activity in the same histology. The lack of histologic correlations in this case can perhaps be explained by the minimal number (2 of each histology) in the HF assay. In contrast, most panels in the 60 cell line in vitro assay consist of a minimum of 6 different cell types, so cell panel specificity is more readily defined. Note that the correlations between in vitro and either HF or xenografts are only apparent when 4 or more cell lines respond.

The examination of the in vitro screening data indicate strong histologic correlations with HF activity for all panels but colon, while indications of differential activity (high 'Δ'), did not correlate with HF activity. This observation combined with that of the correlation of HF response with increasing in vitro potency may indicate that the standard HF assay is sensitive to strongly cytotoxic as opposed to 'differentially' acting agents. It can also be taken to indicate that if differential activity is truly suggested from in vitro results, a HF experiment addressing that particular set of cell types may need to be designed or specific target manipulation should be considered.

Our analysis of MW and hydrogen-bonding factors revealed a correlation between increasing MW, as well as greater hydrogen-bonding sites and HF activity. This suggests that in choosing test agents for further development, a compromise should be made between low MW for drug development purposes and the number of hydrogen-bonding sites for efficacy. HBA and HBD counts were not revealing, as 95% of the input to the in vitro screen was in the favourable range for these factors.

These results may be considered to define a 'road-map' for charting the transition of a compound from an in vitro screening result to a clinical candidate. If the development strategy of a compound is to remain empiric, consideration of the heterogeneous nature of in vivo model to clinical correlation might require strategies to define the likelihood of clinical activity, and again overall potency and activity in a large number of HF fibres might allow the best delineation of compounds to consider for further

development. It could be argued from this experience that compounds with an anti-proliferative effect at 10^{-6} M have a relatively higher likelihood of affecting all growth in a responsive tumour. Compounds of the future will likely be advanced to clinical testing with an eye toward addressing a defined molecular abnormality or target in the tumour cell. Greater numbers ($n \geq 4$) of redundant cell types expressing the target, whether in an in vitro assay such as the 60 cell line screen, or an in vivo assay such as the HF assay, might be expected to have concordant effects in xenografts bearing the same target. Finally, as efforts are consolidated to derive engineered animal strains to provide models of tumour biology and pathophysiology (http://www.nci.nih.gov/dcb/odhome.htm#MOUSE), consideration of the 'baseline' experience of an empirically oriented drug discovery program might usefully benchmark the types of compounds suitable for advancement to such models.

ACKNOWLEDGEMENTS

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Electronic Patent Application Fee Transmittal

Application Number:	10356824
Filing Date:	03-Feb-2003
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Filer:	Ginger R. Dreger/Sherrie Dufault
Attorney Docket Number:	GNE-0329-D1

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	130	130

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for continued examination	1801	1	810	810
		Total in USD ($)		**940**

Electronic Acknowledgement Receipt	
EFS ID:	6271294
Application Number:	10356824
International Application Number:	
Confirmation Number:	4326
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Customer Number:	35489
Filer:	Ginger R. Dreger/Sherrie Dufault
Filer Authorized By:	Ginger R. Dreger
Attorney Docket Number:	GNE-0329-D1
Receipt Date:	15-OCT-2009
Filing Date:	03-FEB-2003
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RAM confirmation Number	3172
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The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	10-15-09-RCE.pdf	43854 a88392465b1abedb9ef24888b271fc5cc8e3653ce	no	1
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
2		10-15-09-Response.pdf	77075 8f8955cc1d0b7e884da2416eb6f4496b01c45dbb	yes	12

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	12

Warnings:					
Information:					
3	Affidavit/Dec/Exhibit after Notice of Appeal	10-15-09-Declaration.pdf	311000 144de075a4fe48ad26f5e9808122ae515d460185	no	5
Warnings:					
Information:					
4	Affidavit/Dec/Exhibit after Notice of Appeal	10-15-09-ExhibitsA-H.pdf	11557824 3639415569573108b048f084824c9b959b967768	no	93
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5	Fee Worksheet (PTO-875)	fee-info.pdf	32091 ecc8fb1762980317490a4fe39438ee50a608077	no	2
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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
10/356,824	02/03/2003	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
FOR	NUMBER FILED	NUMBER EXTRA					
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	10/15/2009	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 17	Minus	** 33	= 0	X $ =		OR	X $52=	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***5	= 0	X $ =		OR	X $220=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/TINA J. BARDEN/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/03/2003	Virginia E. Paton	GNE-0329-D1	4326

35489 7590 12/28/2009

Arnold & Porter LLP (24126)
Attn: IP Docketing Dept.
555 Twelfth Street, N.W.
Washington, DC 20004-1206

EXAMINER	
HOLLERAN, ANNE L	

ART UNIT	PAPER NUMBER
1643	

MAIL DATE	DELIVERY MODE
12/28/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	10/356,824	PATON ET AL.	
	Examiner	Art Unit	
	ANNE L. HOLLERAN	1643	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *15 October 2009*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *34-39,42-44 and 47-54* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *34-39,42-44 and 47-54* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 10/15/2009 has been entered.

Claims 34-39, 42-44, 47-54 are pending.

The election of species requirement for the third therapeutic agent (see Office action mailed 2/16/2006) is WITHDRAWN.

Claims 34-39, 42-44, 47-54 are examined on the merits.

Claim Rejections Withdrawn:

Claim Rejections - 35 USC § 103

The rejection of claims 34-39, 42-44, 47-49, 52 and 53 under 35 U.S.C. 103(a) as being unpatentable over Baselga-1996 (Baselga, et al., Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in the IDS) in view of Perez (Perez et al., Seminars in Oncology, 23(5, suppl 11): 41-45, 1996, October) and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994) is

WIHDRAWN in view of the declaration of Mark X. Sliwkowski, PhD, and the applicants' persuasive arguments..

The rejection of claims 38 and 43 under 35 U.S.C. 103(a) as being unpatentable over Baselga-1996 (Baselga, et al., Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in the IDS) in view of Perez (Perez et al., Seminars in Oncology, 23(5, suppl 11): 41-45, 1996, October) in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994; cited in the IDS), and further in view of van Oosterom (van Oosterom, et al. Anti-Cancer Drugs, 6(3): 356-368, 1995, June, Abstract only; cited in the IDS) for the reasons of record is WIHDRAWN in view of the declaration of Mark X. Sliwkowski, PhD, and the applicants' persuasive arguments.

Double Patenting

The rejection of claims 34-39, 42-44, 47-49, and 52-54 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4, and 8-29 of U.S. Patent No. 5,720,954 (cited in the IDS) in view of Baslega-1996 (Baselga, J. et al. Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in IDS), in view of Perez (Perez et al., Seminars in Oncology, 23(5, suppl 11): 41-45, 1996, October; cited above) and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994) is WIHDRAWN in view of the declaration of Mark X. Sliwkowski, PhD, and the applicants' persuasive arguments.

The rejection of claims 34-39, 42-44, 47-49, and 52-54 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 14, 23, 27-33 and 36 of U.S. Patent No. 5,770,195 (cited in IDS) in view of Baslega-1996 (Baselga, J. et al. Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in IDS), in view of Perez; cited previously) and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994) for the reasons of record is WIHDRAWN in view of the declaration of Mark X. Sliwkowski, PhD, and the applicants' persuasive arguments.

The rejection of claims 34-39, 42-44, 47-49, and 52-54 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-7 and 9-13 of U.S. Patent No. 6,387,371 in view of Baslega-1996 (Baselga, J. et al. Journal of Clinical Oncology, 14(3): 737-744, 1996, March; cited in IDS), in view of Perez; cited previously), and further in view of Baselga-1994a (Baselga, J., et al, Proceedings of the American Association for Cancer Research, 35: 380, Abstract #2262, 1994) is WIHDRAWN in view of the declaration of Mark X. Sliwkowski, PhD, and the applicants' persuasive arguments.

New Grounds of Rejection:

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection

is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 34-39, 42-44, 47-54 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over allowed claims 1-6, 9, 10, 12, 13, 24, 26, 32 and 33 of copending Application No. 09/208,649. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of 09/208,649 encompass the inventions of the instant application. Both applications are filed on the same day and the claims in the instant case claim an improvement over the claims in 09/208,649(see MPEP 804). Although the application data sheet for this application indicates that 10/356,824 is a "divisional" of 09/208,649, the instant case is in fact a "continuation" of 09/208,649, because 10/356,824 was not filed as a result of a restriction requirement made in 09/208,649.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anne Holleran, whose telephone number is (571) 272-0833. The examiner can normally be reached on Monday through Friday from 9:30 am to 5:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Larry Helms, can be reached on (571) 272-0832. Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (571) 272-1600.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. The faxing of such papers must conform to the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The Official Fax number for Group 1600 is (571) 273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll free).

Anne L. Holleran
Patent Examiner
December 19, 2009

/Alana M. Harris, Ph.D./

Primary Examiner, Art Unit 1643

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10356824	PATON ET AL.
	Examiner	Art Unit
	ANNE L HOLLERAN	1643

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
updated search of US Patents, EAST, history in file	6/23/2008	alh
updated search of US Patents, EAST, history in file	3/16/2009	alh
udated search of US Patents, EAST, history in file	12/19/2009	alh

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	3248	424/130.1,133.1,138.1,141.1,143.1,155.1,174.1.ccls.	USPAT	OR	OFF	2009/12/19 14:09
L2	296	(her2 or erbb2 or cerbb2 or erbb2her2 or cerbb2her2 or p185 or p185her2 or p185erbb2 or p185cerbb2 or p185her2cerbb2 or p185her2erbb2 or p185erbb2her2).clm.	USPAT	OR	OFF	2009/12/19 14:09
L3	56	1 and 2	USPAT	OR	OFF	2009/12/19 14:09

12/ 19/ 2009 2:12:53 PM
C:\ Documents and Settings\ aholleran\ My Documents\ EAST\ Workspaces\ 10356824.wsp

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
10/356,824	02/03/2003	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	750
☒ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	0
☒ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	0
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	750

APPLICATION AS AMENDED – PART II

AMENDMENT		(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
	03/25/2010	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 17	Minus	** 33	= 0	X $ =		OR	X $52=	0
	Independent (37 CFR 1.16(h))	* 3	Minus	***5	= 0	X $ =		OR	X $220=	0
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT		(Column 1)		(Column 2)	(Column 3)	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA					
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/KATINA TOBIN/

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
10/356,824	02/03/2003	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
FOR	NUMBER FILED	NUMBER EXTRA					
☒ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	750
☒ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	0
☒ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	0
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	750

APPLICATION AS AMENDED – PART II

AMENDMENT 03/25/2010	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 17	Minus	** 33	= 0	X $ =		OR	X $52=	0
Independent (37 CFR 1.16(h))	* 3	Minus	***5	= 0	X $ =		OR	X $220=	0
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/KATINA TOBIN/

THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Virginia E. Paton	Attorney Docket #:	GNE-0329R1D1
Serial No.	10/356,824	Group Art Unit	1643
Filing Date	02/03/2003	Examiner:	Holleran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
Title:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS – MARCH 26, 2010

MAIL STOP – AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO OFFICE ACTION

Sir:

This is in response to the Office Action mailed on December 28, 2009 in connection with the above-identified patent application. A Terminal Disclaimer and Information Disclosure Statement accompany the present Amendment.

Amendments to the Specification begin on page **2** of this paper.

Amendments to the Claims begin on page **3** of this paper.

Remarks/Arguments begin on page **6** of this paper.

Amendments to the Specification:

Paragraph [0001] of the specification has been amended as follows:

[0001] This is a ~~divisional~~ continuation of non-provisional application No. 09/208,649, filed Dec. 10, 1998, which claims priority under 35 USC §119 to provisional application No. 60/069,346, filed Dec. 12, 1997, the entire disclosures of which are hereby incorporated by reference.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

1-33. (Canceled)

34. (Previously presented) A method for the treatment of a human patient with breast cancer that overexpresses ErbB2 receptor, comprising administering a combination of an antibody that binds ErbB2, a taxoid, and a further growth inhibitory agent to the human patient in an amount effective to extend the time to disease progression in the human patient, wherein the antibody binds to epitope 4D5 within the ErbB2 extracellular domain sequence.

35. (Previously presented) The method of claim 34 wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

36. (Previously presented) The method of claim 34 wherein the antibody cross-blocks binding of 4D5 to the ErbB2 extracellular domain sequence.

37. (Previously presented) The method of claim 34 wherein the antibody binds to amino acid residues in the region from about residue 529 to about residue 625 of the ErbB2 extracellular domain sequence.

38. (Previously presented) A method for the treatment of a human patient with breast cancer characterized by overexpression of ErbB2 receptor, comprising administering an effective amount of a combination of an anti-ErbB2 antibody which binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid, and a further therapeutic agent, to the human patient.

39. (Previously presented) The method of claim 38 wherein the breast cancer is metastatic breast carcinoma.

40-41. (Canceled)

42. (Previously presented) The method of claim 38 wherein the antibody is a humanized 4D5 anti-ErbB2 antibody.

43. (Currently amended) The method of claim 38 wherein the taxoid is paclitaxel ~~or docetaxel~~.

44. (Previously presented) The method of claim 38 wherein efficacy is measured by determining the time to disease progression or the response rate.

45-46. (Canceled)

47. (Previously presented) The method of claim 38, wherein the further therapeutic agent is selected from the group consisting of: another ErbB2 antibody, EGFR antibody, ErbB3 antibody, ErbB4 antibody, vascular endothelial growth factor (VEGF) antibody, cytokine, and growth inhibitory agent.

48. (Previously presented) A method for the treatment of a human patient with ErbB2 overexpressing breast cancer, comprising administering a combination of an antibody that binds epitope 4D5 within the ErbB2 extracellular domain sequence, a taxoid and a further growth inhibitory agent, in the absence of an anthracycline derivative, to the human patient in an amount effective to extend the time to disease progression in the human patient.

49. (Previously presented) The method of claim 48 wherein the breast cancer is metastatic breast carcinoma.

50. (Currently amended) The method of claim 38 wherein the ~~third~~ further therapeutic agent is another ErbB2 antibody.

51. (Currently amended) The method of claim 38 wherein the ~~third~~ further therapeutic agent is a vascular endothelial growth factor (VEGF) antibody.

52. (Previously presented) The method of claim 38 wherein the further therapeutic agent is a growth inhibitory agent.

53. (Previously presented) The method of claim 52 wherein the growth inhibitory agent is a DNA alkylating agent.

54. (Previously presented) The method of claim 42 wherein the antibody is administered as a 4mg/kg dose and then weekly administration of 2mg/kg.

Remarks/Arguments

The specification has been amended to reflect the correct relationship of the present application to its parent, application Serial No. 09/208,649.

Claims 34-39, 42-44 and 47-54 are under examination and stand rejected. Claims 43, 50 and 51 have been amended. The amendments are fully supported by the specification as originally filed and do not add new matter. All amendments were made without prejudice or disclaimer. Applicants explicitly reserve the right to pursue any deleted subject matter in one of more continuing applications.

Claim Rejections Withdrawn

Applicants note and appreciate the withdrawal of the following earlier rejections:

rejection of claims 34-39, 42-44, 47-49, 52 and 53 under 35 U.S.C. 103(a) as allegedly being unpatentable over Baselga-1996 (Baselga et al., Journal of Clinical Oncology, 14(3):737-744, 1996) in view of Perez (Perez et al., Seminars in Oncology, 34(5, suppl 11):41-45, 1996) and further in view of Baselga-1994a (Baselga et al., Proceedings of the American Association for Cancer Research, 35:380, Abstract #2262, 1994);

rejection of claims 38 and 43 under 35 U.S.C. 103(a) as allegedly being unpatentable over Baselga-1996 (Baselga et al., Journal of Clinical Oncology, 14(3):737-744, 1996) in view of Perez (Perez et al., Seminars in Oncology, 34(5, suppl 11):41-45, 1996) and further in view of Baselga-1994a (Baselga et al., Proceedings of the American Association for Cancer Research, 35:380, Abstract #2262, 1994), and further in view of van Oosterom (Van Oosterom et al., Anti-Cancer Drugs, 6(3):356-368 (1995) – Abstract only);

rejection of claims 34-43, 42-44, 47-49, and 52-54 on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1, 4, and 8-29 of U.S. Patent No. 5,720,954 in view of Baselga-1996 (Baselga et al., Journal of Clinical Oncology, 14(3):737-744, 1996) in view of Perez (Perez et al., Seminars in Oncology, 34(5, suppl 11):41-

45, 1996) and further in view of Baselga-1994a (Baselga et al., Proceedings of the American Association for Cancer Research, 35:380, Abstract #2262, 1994);

the rejection of claims 34-39, 42-44, 47-49, and 52-54 on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 14, 23, 27-33 and 36 of US 5,770,195, in view of Beselga-1996, in view of Perez and further in view of Baselga-1994a; and

the rejection of claims 34-39, 42-44, 47-49, and 52-54 on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1-7 and 9-13 of US 6,387,371 in view of Baselga-1996, in view of Perez, and further in view of Baselga-1994a.

New Grounds of Rejection

Claims 34-39, 42-44, 47-54 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over allowed claims 1-6, 9, 10, 12, 13, 24, 26, 32 and 33 of copending Application No. 09/208,649. According to the rejection, although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of 09/209,649 encompass the inventions of the instant application. The Examiner notes that although the application data sheet for this application indicates that 10/356,824 is a "divisional" of 09/208,649, the instant case is in fact a "continuation" of 09/208,649, because it was not filed as a result of a restriction requirement.

The attached Terminal Disclaimer is believed to obviate the present rejection.

Conclusion

In conclusion, all claims pending in this application are believed to be in *prima facie* condition for allowance, and an early action to that effect is respectfully solicited.

The Commissioner is authorized to charge any additional fees, including any fees for additional extension of time, or credit overpayment to **Deposit Account 50-2387** (Attorney Docket No. **GNE-0329R1D1 (24126.549)**).

Respectfully submitted,

Date: March 26, 2010

By Electronic Signature: /GINGER R. DREGER/
Ginger R. Dreger, Esq.
Reg. No. 33,055

ARNOLD & PORTER LLP
555 Twelfth Street, NW
Washington, D.C. 20004-1206
Telephone: +1 415.356.3000
Facsimile: +1 415.356.3099

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Examiner: Holleran, Anne L.
	)	
Virginia E. Paton	)	Art Unit: 1643
	)	
Application Serial No.: 10/356,824	)	Confirmation No. 4326
	)	
Filed: February 3, 2003	)	Attorney's Docket No. GNE-0329-R1D1
	)	
For: **TREATMENT WITH ANTI-ErbB2 ANTIBODIES**	)	**Customer No. 35489**

TERMINAL DISCLAIMER

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

1. The owner, Genentech, Inc., having a principal place of business at 1 DNA Way, South San Francisco, California 94080, represents that it is the owner of the entire right, title and interest in the invention disclosed and claimed in the above-identified patent application, and is also the owner of the entire right, title and interest in the invention disclosed and claimed in U.S. Patent Application No. 09/208,649. The assignment concerning both the above-identified application and U.S. Patent Application No. 09/208,649 was recorded in the Assignment Database of the United States Patent and Trademark Office on March 22, 1999, at Reel 9854 and Frame 0866.

2. Genentech, Inc. hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. §§ 154 to 156 and 173, as presently shortened by any terminal disclaimer, of U.S. Patent Application No. 09/208,649. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application, and is binding upon the grantee, its successors or assigns.

3. For submission on behalf of an organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the organization.

4. I hereby declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

5. The undersigned is an attorney of record.

6. The Commissioner is authorized to charge any fees, including any fees for extension of time, or credit overpayment to **Deposit Account No. 50-2387** (Attorney Docket No. **GNE-0329-R1D1 (24126.549)**).

Respectfully submitted,

Date: March 26, 2010

By Electronic Signature:/GINGER R. DREGER/
Ginger R. Dreger, Reg. No. 33,055

ARNOLD & PORTER LLP
555 Twelfth Street, NW
Washington, D.C. 20004-1206
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application	)	PATENT APPLICATION
Inventor(s): Virginia E. Paton	)	Art Unit: 1643
Application No.: 10/356,824	)	Examiner: Holleran, Anne L.
Filed: February 3, 2003	)	
Title: Treatment with anti-ErbB2 antibodies	)	

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. §1.97

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Listed below or on an attached Form PTO-1449 is information known to applicant(s). A copy of each listed publication and foreign patent, each cited pending unpublished U.S. application, and all additional publications other than U.S. patents and U.S. parent application publications or is identified, with an asterisk (*), as having been previously cited in related **U.S. Parent Application No.: 09/208,649, filed December 10, 1998,** being submitted herewith, along with a concise explanation of information in a foreign language, if any, pursuant to 37 C.F.R. §1.97-1.98.

1.98 (d) A copy of any patent, publication or other information listed in an information disclosure statement is not required to be provided if it was previously cited by or submitted to the office in a prior application, provided that the prior application is properly identified in the statement and relied upon for an earlier filing date under 35 U.S.C. 120.

Applicants respectfully request that the listed information be considered by the Examiner and be made of record in the above-identified application. If form PTO-1449 is enclosed, the Examiner is requested to initial and return it in accordance with MPEP §609. Each of these references listed on the attached form PTO-1449 **without an asterisk (*)**, was cited by the examiner in the parent **U.S. Application No.: 09/208,649, filed December 10, 1998.**

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, material to patentability as defined in §1.56.

☐ This statement qualifies under *37 C.F.R. §1.97*, subsection (b) because (check all that apply):

- ☐ (1) It is being filed within 3 months of the application filing date and is other than a continued prosecution application under § 1.53(d)
  -- OR --
- ☐ (2) It is being filed within 3 months of entry of a national stage
  -- OR --
- ☐ (3) It is being filed before the mail date of the first Office Action on the merits
  -- OR --
- ☐ (4) It is being filed before the mailing of a first Office Action after the filing of a request for continued examination under § 1.114.

☒ *37 C.F.R. §1.97(c)*. If this statement is being filed after the latest of: (1) three months beyond the filing date of a national application; (2) three months beyond the date of entry of the national stage as set forth in §1.491 in an international application; or (3) the mailing date of a first Office action on the merits, but before the mailing date of the earlier of a final office action under §1.113 or a notice of allowance under §1.311, then:

- ☐ a certification as specified in §1.97(e) is provided below; **or**
- ☒ a fee of $180.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this statement.

☐ *37 C.F.R. §1.97(d)*. If this statement is being filed after the mailing date of the earlier of a final office action under §1.113 or a notice of allowance under §1.311, but before payment of the issue fee, then:

A. a certification as specified in §1.97(e) is completed below; and

B. a petition under 37 C.F.R. §1.97(d) requesting consideration of this statement is submitted herewith; **and**

C. a fee of $130.00 as set forth in §1.17(i)(1) is authorized below, enclosed, or included with the payment of other papers filed together with this statement.

☒ *Fee Authorization*. The Commissioner is hereby authorized to charge the above-referenced fees of **$180.00** and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. **50-2387 (Docket No. GNE-0329 D1 (24126.549))**.

Respectfully submitted,
Arnold & Porter LLP

Dated: March 26, 2010

By Electronic Signature: /GINGER R. DREGER/
Ginger R. Dreger, Reg. No. 33,055

Arnold & Porter LLP
555 Twelfth Street, NW
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Tel (415) 356-3000
Fax (415) 356-3099
Customer No. 35489

INFORMATION DISCLOSURE STATEMENT PTO-1449	ATTY. DOCKET NO. GNE-0329 D1 US	SERIAL NO. 10/356,824
	APPLICANT Virginia E. Paton	
	FILING DATE 02/03/2003	GROUP: 1643

U.S. PATENT DOCUMENTS

EXAMINER'S INITIALS	PATENT NO.	DATE	NAME	CLASS	SUBCLASS	FILING DATE
	US 6,387,371	05/2002	Hudziak, et al.			

FOREIGN PATENT DOCUMENTS

EXAMINER'S INITIALS	PATENT NO.	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
						☐	☒

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Cottin, et al., "Impairment of diastolic function during short-term anthracycline chemotherapy", Br. Heart J., 73: 61-64, (1995)
*	Johnson, et al., "Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials", British Journal of Cancer, 84(10): 1424-1431, (2001)
*	Lane, et al., "ErbB2 potentiates breast tumor proliferation through modulation of $p27^{Kip1}$-Cdk2 complex formation: Receptor overexpression does not determine growth dependency", Molecular and Cellular Biology, Vol. 20, No. 9, pages 3210-3223, (2000)
*	Osborne, et al., "Antagonism of chemotherapy-induced cytotoxicity for human breast cancer cells by antiestrogens", Journal of Clinical Oncology, Vol. 7, No. 6, pages 710-717, (1989)
*	Woods, C. et al., "Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway", Molecular Medicine, Vol. 1, No. 5, pages 506-526, (1995)
*	Woods, K, et al., "Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line", Biochemical Pharmacology, Vol. 47, No. 8, pages 1449-1452, (1994)
EXAMINER	DATE CONSIDERED

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Notice of References Cited	Application/Control No. 09/208,649	Applicant(s)/Patent Under Reexamination HELLMANN, SUSAN D.	
	Examiner ANNE L. HOLLERAN	Art Unit 1643	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,387,371	05-2002	Hudziak et al.	424/138.1
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Cottin, Y, et al. Br. Heart Journal, 73: 61-64, 1995
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Electronic Patent Application Fee Transmittal	
Application Number:	10356824
Filing Date:	03-Feb-2003
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Filer:	Ginger R. Dreger/Sherrie Dufault
Attorney Docket Number:	GNE-0329-D1

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
Statutory disclaimer	1814	1	140	140
		Total in USD ($)		**320**

Electronic Acknowledgement Receipt	
EFS ID:	7292328
Application Number:	10356824
International Application Number:	
Confirmation Number:	4326
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Customer Number:	35489
Filer:	Ginger R. Dreger/Sherrie Dufault
Filer Authorized By:	Ginger R. Dreger
Attorney Docket Number:	GNE-0329-D1
Receipt Date:	26-MAR-2010
Filing Date:	03-FEB-2003
Time Stamp:	12:00:34
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$320
RAM confirmation Number	11466
Deposit Account	502387
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees) Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	GNE-0329-D1-03-26-10-TXMLAmend.pdf	175026 74e5d1607b0901f13d7b261193dd28afe1a db896	no	1
Warnings:					
Information:					
2		GNE-0329-D1-03-26-10-Amendment.pdf	151555 e8610c64b3e42f0f5804987 5625a7bdeb1e 1f86e	yes	8

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Specification	2	2
Claims	3	5
Applicant Arguments/Remarks Made in an Amendment	6	8

Warnings:					
Information:					
3	Terminal Disclaimer Filed	GNE-0329-D1-03-26-10-TermDisclaimer.pdf	88049 91a71ed4ad8be8fc2b27647 5b02c8bb1f8 a58b3	no	2
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Filed (SB/08)	GNE-0329-D1-03-26-10-IDS.pdf	202960 391e492f35582fd0df7605 28dd8c101a3d8 3aa3	no	4
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
5	NPL Documents	Cottin-Impairment.pdf	346781 5b8b2e1da2b29ee655720972560d8c7c3f 41acc	no	4
Warnings:					
Information:					
6	Fee Worksheet (PTO-875)	fee-info.pdf	31481 1088db94c0e0d7795a9befcfa1f3592db63 0e7e	no	2
Warnings:					

Information:	
Total Files Size (in bytes):	995852

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/21 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	15

Application Number	10/356,824
Filing Date	February 3, 2003
First Named Inventor	Virginia E. Paton
Art Unit	1643
Examiner Name	Holleran, Anne L.
Attorney Docket Number	GNE-0329-D1

ENCLOSURES *(Check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☒ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☒ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☒ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☐ Other Enclosure(s) (please identify below):

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Arnold & Porter LLP		
Signature	/GINGER R. DREGER/		
Printed name	Ginger R. Dreger, Esq.		
Date	March 26, 2010	Reg. No.	33,055

FILED VIA EFS ON MARCH 26, 2010

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Virginia E. Paton	Docket No.:	GNE-0329R1D1
Serial No.:	10/356,824	Group Art Unit:	1643
Filing Date:	February 3, 2003	Examiner:	Holleran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
For:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS—MARCH 26, 2010

REQUEST FOR REFUND

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

Please refund Deposit Account No. 50-2387 in the sum of $940.00 for the fees for Request for Continued Examination that was inadvertently filed via EFS on March 25, 2010 in the present application.

The United States Patent and Trademark Office is hereby authorized to please charge any additional fees, including any fees for additional extension of time, or credit any overpayments to our Deposit Account No. **50-2387** (referencing Attorney's Docket No. **GNE-0329-D1 (24126-549)**.

Respectfully submitted,

Date: March 26, 2010

By Electronic Signature: /BARRIE D. BELL/
Barrie D. Bell, Reg. No. 46,740

ARNOLD & PORTER LLP
555 Twelfth Street, NW
Washington, D.C. 20004-1206
Telephone: +1 415.356.3000
Facsimile: +1 415.356.3099

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Virginia E. Paton	Docket No.:	GNE-0329R1D1
Serial No.:	10/356,824	Group Art Unit:	1643
Filing Date:	February 3, 2003	Examiner:	Holleran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
For:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS—MARCH 26, 2010

COMMUNICATION RE RCE INADVERTENTLY FILED IN PRESENT APPLICATION

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

Applicants inadvertently filed an RCE on March 25, 2010 via EFS, in the present application (as can be seen by the application number on the RCE Transmittal filed on March 25, 2010, attached hereto for your convenience). We have filed the RCE in the correct application today via EFS filing. Therefore, Applicants hereby request that the RCE not be entered in the present application.

The United States Patent and Trademark Office is hereby authorized to please charge any additional fees, including any fees for additional extension of time, or credit any overpayments to our Deposit Account No. **50-2387** (referencing Attorney's Docket No. **GNE-0329-D1 (24126-549)**.

Respectfully submitted,

Date: March 26, 2010

By Electronic Signature: /BARRIE D. BELL/
Barrie D. Bell, Reg. No., 46,740

ARNOLD & PORTER LLP
555 Twelfth Street, NW
Washington, D.C. 20004-1206
Telephone: +1 415.356.3000
Facsimile: +1 415.356.3099

PTO/SB/30 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	11/512,525
Filing Date	August 29, 2006
First Named Inventor	Audrey Goddard
Art Unit	1646
Examiner Name	Li, Ruixang
Attorney Docket Number	GNE-0158-D2

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. ☒ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. ☒ Other Amendment after Final Office Action filed on January 25, 2010

   b. ☐ Enclosed

      i. ☐ Amendment/Reply

      ii. ☐ Affidavit(s)/ Declaration(s)

      iii. ☐ Information Disclosure Statement (IDS)

      iv. ☐ Other _____

2. Miscellaneous

   Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a

   a. ☐ period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. ☐ Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.
   The Director is hereby authorized to charge the following fees any underpayment of fees or credit any overpayments to

   a. ☒ Deposit Account No. 50-2387.

      i. ☒ RCE fee required under 37 CFR 1.17(e)

      ii ☒ Extension of time fee (37 CFR 1.136 and 1.17)

      iii ☐ Other _____

   b. ☐ Check in the amount of $ _____ enclosed

   c. ☐ Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/GINGER R. DREGER/	Date	March 25, 2010
Name (Print/Type)	Ginger R. Dreger, Esq.	Registration No.	33,055

FILED VIA EFS FILING ON MARCH 25, 2010

Signature			
Name (Print/Type)		Date	

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt

EFS ID:	7296429
Application Number:	10356824
International Application Number:	
Confirmation Number:	4326
Title of Invention:	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Customer Number:	35489
Filer:	Barrie D. Greene/Sherrie Dufault
Filer Authorized By:	Barrie D. Greene
Attorney Docket Number:	GNE-0329-D1
Receipt Date:	26-MAR-2010
Filing Date:	03-FEB-2003
Time Stamp:	16:07:27
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Refund Request	GNE-0329D1-03-26-10-RequestRefund.pdf	82356 ec135fabf8e8bfba8588603645ce3c5a9134e5a6	no	1

Warnings:	
Information:	

2	Miscellaneous Incoming Letter	GNE-0329D1-03-26-10-CommreRCE.pdf	127798 ec2c1fa91f586c2a46e0e14a5870b31e096dc2160	no	2

Warnings:	
Information:	
Total Files Size (in bytes):	210154

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Virginia E. Paton	Docket No.:	GNE-0329R1D1
Serial No.:	10/356,824	Group Art Unit:	1643
Filing Date:	February 3, 2003	Examiner:	Hollcran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
For:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS—MARCH 26, 2010

REQUEST FOR REFUND

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

Please refund Deposit Account No. 50-2387 in the sum of $940.00 for the fees for Request for Continued Examination that was inadvertently filed via EFS on March 25, 2010 in the present application.

The United States Patent and Trademark Office is hereby authorized to please charge any additional fees, including any fees for additional extension of time, or credit any overpayments to our Deposit Account No. **50-2387** (referencing Attorney's Docket No. **GNE-0329-D1 (24126-549)**.

Respectfully submitted,

Date: March 26, 2010

By Electronic Signature: /BARRIE D. BELL/
Barrie D. Bell, Reg. No. 46,740

ARNOLD & PORTER LLP
555 Twelfth Street, NW
Washington, D.C. 20004-1206
Telephone: +1 415.356.3000
Facsimile: +1 415.356.3099

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Applicant:	Virginia E. Paton	Docket No.:	GNE-0329R1D1
Serial No.:	10/356,824	Group Art Unit:	1643
Filing Date:	February 3, 2003	Examiner:	Holleran, Anne L.
Customer No.:	35489	Confirmation No.:	4326
For:	**TREATMENT WITH ANTI-ErbB2 ANTIBODIES**		

FILED VIA EFS—MARCH 26, 2010

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Alexandria, Virginia 22313-1450

Dear Sir:

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Respectfully submitted,

Date: March 26, 2010

By Electronic Signature: /BARRIE D. BELL/
Barrie D. Bell, Reg. No., 46,740

ARNOLD & PORTER LLP
555 Twelfth Street, NW
Washington, D.C. 20004-1206
Telephone: +1 415.356.3000
Facsimile: +1 415.356.3099

Electronic Patent Application Fee Transmittal

Application Number:	10356824
Filing Date:	03-Feb-2003
Adjustment date: 04/06/2010 SDIRETA1 03/26/2010 INTEFSW 00000854 502387 10356824 01 FC:1251 130.00 CR 02 FC:1801 810.00 CR **Title of Invention:**	Treatment with anti-ErbB2 antibodies
First Named Inventor/Applicant Name:	Virginia E. Paton
Filer:	Ginger R. Dreger/Sherrie Dufault
Attorney Docket Number:	GNE-0329-D1

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	130	130

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for continued examination	1801	1	810	810
		Total in USD ($)		**940**

Application Number	Application/Control No.	Applicant(s)/Patent under Reexamination	
	10/356,824	PATON ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed : 3/26/10	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:

Felicia D. Roberts

09/208,649

U.S. Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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NOTICE OF ALLOWANCE AND FEE(S) DUE

35489 7590 10/08/2010

Arnold & Porter LLP (24126)
Attn: SV Docketing Dept.
1400 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
HOLLERAN, ANNE L	
ART UNIT	PAPER NUMBER
1643	

DATE MAILED: 10/08/2010

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/03/2003	Virginia E. Paton	GNE-0329-D1	4326

TITLE OF INVENTION: TREATMENT WITH ANTI-ERBB2 ANTIBODIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	01/10/2011

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

35489 7590 10/08/2010

Arnold & Porter LLP (24126)
Attn: SV Docketing Dept.
1400 Page Mill Road
Palo Alto, CA 94304

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/03/2003	Virginia E. Paton	GNE-0329-D1	4326

TITLE OF INVENTION: TREATMENT WITH ANTI-ERBB2 ANTIBODIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	01/10/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
HOLLERAN, ANNE L.	1643	424-143100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☐ Issue Fee

☐ Publication Fee (No small entity discount permitted)

☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

☐ A check is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/03/2003	Virginia E. Paton	GNE-0329-D1	4326

35489 7590 10/08/2010

Arnold & Porter LLP (24126)
Attn: SV Docketing Dept.
1400 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
HOLLERAN, ANNE L	
ART UNIT	PAPER NUMBER
1643	

DATE MAILED: 10/08/2010

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 587 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 587 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 08/07) Approved for use through 08/31/2010.

Notice of Allowability	Application No.	Applicant(s)	
	10/356,824	PATON ET AL.	
	Examiner	Art Unit	
	ANNE L. HOLLERAN	1643	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *amendment filed 3/26/2010*.

2. ☒ The allowed claim(s) is/are *34-39, 42-44, and 47-54*.

3. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 3/26/2010
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☒ Interview Summary (PTO-413), Paper No./Mail Date *20100924* .
7. ☒ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

/Alana M. Harris, Ph.D./
Primary Examiner, Art Unit 1643

Examiner-Initiated Interview Summary	Application No.	Applicant(s)	
	10/356,824	PATON ET AL.	
	Examiner	Art Unit	
	ANNE L. HOLLERAN	1643	

All Participants:

(1) *ANNE L. HOLLERAN*.

(2) *Ginger Dreger*.

Status of Application: *allowed*

(3) _____.

(4) _____.

Date of Interview: *24 September 2010*

Time: _____

Type of Interview:

☒ Telephonic
☐ Video Conference
☐ Personal (Copy given to: ☐ Applicant ☐ Applicant's representative)

Exhibit Shown or Demonstrated: ☐ Yes ☐ No
If Yes, provide a brief description:

Part I.

Rejection(s) discussed:

Claims discussed:

Prior art documents discussed:

Part II.

SUBSTANCE OF INTERVIEW DESCRIBING THE GENERAL NATURE OF WHAT WAS DISCUSSED:

Left message for applicants' representative that application is in condition for allowance.

Part III.

☒ It is not necessary for applicant to provide a separate record of the substance of the interview, since the interview directly resulted in the allowance of the application. The examiner will provide a written summary of the substance of the interview in the Notice of Allowability.

☐ It is not necessary for applicant to provide a separate record of the substance of the interview, since the interview did not result in resolution of all issues. A brief summary by the examiner appears in Part II above.

(Applicant/Applicant's Representative Signature – if appropriate)

Examiner's Comment

Terminal Disclaimer

The terminal disclaimer filed on 3/26/2010 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of a US Patent granted on application 09/208,649 has been reviewed and is accepted. The terminal disclaimer has been recorded.

The provisional rejection of claims 34-39, 42-44, 47-54 over allowed claims 1-6, 9, 10, 12, 13, 24, 26, 32 and 33 of copending application no. 09/208,649 is WITHDRAWN in view of the terminal disclaimer filed 3/26/2010.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anne Holleran, whose telephone number is (571) 272-0833. The examiner can normally be reached on Monday through Friday from 9:30 am to 5:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Misook Yu, can be reached on (571) 272-0839. Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (571) 272-1600.

Papers related to this application may be submitted to Group 1600 by facsimile transmission. The faxing of such papers must conform to the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The Official Fax number for Group 1600 is (571) 273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll free).

Anne L. Holleran
Patent Examiner
/Alana M. Harris, Ph.D./
Primary Examiner, Art Unit 1643

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10356824	PATON ET AL.
	Examiner	Art Unit
	ANNE L HOLLERAN	1643

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47

CLAIM		DATE									
Final	Original	09/24/2010									
	1	-									
	2	-									
	3	-									
	4	-									
	5	-									
	6	-									
	7	-									
	8	-									
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	28	-									
	29	-									
	30	-									
	31	-									
	32	-									
	33	-									
1	34	=									
2	35	=									
3	36	=									

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10356824	PATON ET AL.
	Examiner	Art Unit
	ANNE L HOLLERAN	1643

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47

CLAIM		DATE									
Final	Original	09/24/2010									
4	37	=									
5	38	=									
6	39	=									
	40	-									
	41	-									
7	42	=									
9	43	=									
10	44	=									
	45	-									
	46	-									
11	47	=									
16	48	=									
17	49	=									
12	50	=									
13	51	=									
14	52	=									
15	53	=									
8	54	=									

Issue Classification

Application/Control No.	Applicant(s)/Patent Under Reexamination
10356824	PATON ET AL.
Examiner	**Art Unit**
ANNE L HOLLERAN	1643

ORIGINAL		INTERNATIONAL CLASSIFICATION	
CLASS	SUBCLASS	CLAIMED	NON-CLAIMED
424	143.1	A 6 1 K 39 / 395 (2006.01.01)	C 0 7 K 16 / 28 (2006.01.01)
			C 0 7 K 16 / 30 (2006.01.01)

CROSS REFERENCE(S)

CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				
424	130.1	133.1	134.1	135.1	136.1
424	138.1	141.1	143.1	152.1	155.1
424	156.1	172.1	174.1		

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
1	34	8	54												
2	35														
3	36														
4	37														
5	38														
6	39														
7	42														
9	43														
10	44														
11	47														
16	48														
17	49														
12	50														
13	51														
14	52														
15	53														

/ANNE L HOLLERAN/ Examiner.Art Unit 1643 (Assistant Examiner)	9/24/2010 (Date)	**Total Claims Allowed:** 17
/Alana M. Harris, Ph.D./ Primary Examiner, Art Unit 1643 (Primary Examiner)	(Date)	O.G. Print Claim(s): 1 O.G. Print Figure: None

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	10356824	PATON ET AL.
	Examiner	Art Unit
	ANNE L HOLLERAN	1643

SEARCHED			
Class	Subclass	Date	Examiner
424	130.1,133.1,134.1, 135.1,136.1,138.1,141.1,143.1,152.1,155.1,156.1,172.1, 174.1	09/24/2010	alh

SEARCH NOTES		
Search Notes	Date	Examiner
updated search of US Patents, EAST, history in file	6/23/2008	alh
updated search of US Patents, EAST, history in file	3/16/2009	alh
udated search of US Patents, EAST, history in file	12/19/2009	alh
updated search of US Patents, interference and PGPubs, EAST, history in file	9/24/2010	alh

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner
424	130.1,133.1,134.1, 135.1,136.1,138.1,141.1,143.1,152.1,155.1,156.1,172.1, 174.1	9/24/2010	alh

/ANNE L HOLLERAN/ Examiner.Art Unit 1643	

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

BIB DATA SHEET

CONFIRMATION NO. 4326

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
10/356,824	02/03/2003	424	1643	GNE-0329-D1

APPLICANTS

Virginia E. Paton, Oakland, CA;
Steven Shak, Burlingame, CA;
Susan D. Hellmann, San Carlos, CA;

**** CONTINUING DATA ***************************

This application is a DIV of 09/208,649 12/10/1998
which claims benefit of 60/069,346 12/12/1997

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

04/24/2003

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☑ No Verified and Acknowledged /ANNE L HOLLERAN/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		CA	1	33	5

ADDRESS

Arnold & Porter LLP (24126)
Attn: SV Docketing Dept.
1400 Page Mill Road
Palo Alto, CA 94304
UNITED STATES

TITLE

Treatment with anti-ErbB2 antibodies

FILING FEE RECEIVED		
1152	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

BIB (Rev. 05/07).

INFORMATION DISCLOSURE STATEMENT PTO-1449	ATTY. DOCKET NO. GNE-0329 D1 US	SERIAL NO. 10/356,824
	APPLICANT Virginia E. Paton	
	FILING DATE 02/03/2003	GROUP: 1643

U.S. PATENT DOCUMENTS

EXAMINER'S INITIALS	PATENT NO.	DATE	NAME	CLASS	SUBCLASS	FILING DATE
	US 6,387,371	05/2002	Hudziak, et al.			

FOREIGN PATENT DOCUMENTS

EXAMINER'S INITIALS	PATENT NO.	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION	
						YES	NO
						☐	☒

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

	Cottin, et al., "Impairment of diastolic function during short-term anthracycline chemotherapy", Br. Heart J., 73: 61-64, (1995)
*	Johnson, et al., "Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials", British Journal of Cancer, 84(10): 1424-1431, (2001)
*	Lane, et al., "ErbB2 potentiates breast tumor proliferation through modulation of $p27^{Kip1}$-Cdk2 complex formation: Receptor overexpression does not determine growth dependency", Molecular and Cellular Biology, Vol. 20, No. 9, pages 3210-3223, (2000)
*	Osborne, et al., "Antagonism of chemotherapy-induced cytotoxicity for human breast cancer cells by antiestrogens", Journal of Clinical Oncology, Vol. 7, No. 6, pages 710-717, (1989)
*	Woods, C. et al., "Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway", Molecular Medicine, Vol. 1, No. 5, pages 506-526, (1995)
*	Woods, K, et al., "Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line", Biochemical Pharmacology, Vol. 47, No. 8, pages 1449-1452, (1994)
EXAMINER	DATE CONSIDERED

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

/Anne Holleran/ 09/24/2010

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /A.H./

Notice of References Cited	Application/Control No. 09/208,649	Applicant(s)/Patent Under Reexamination HELLMANN, SUSAN D.	
	Examiner ANNE L. HOLLERAN	Art Unit 1643	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,387,371	05-2002	Hudziak et al.	424/138.1
	B	US-		duplicate citation	
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	duplicate citation Cottin, Y. et al. Br. Heart Journal, 73: 61-64, 1995
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

09/24/2010

U.S. Patent and Trademark Office
PTO-892 (Rev. 01-2001)

/Anne Holleran/

Notice of References Cited

Part of Paper No. 20080307

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /A.H./

PART B - FEE(S) TRANSMITTAL

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CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

35489 7590 10/08/2010

Arnold & Porter LLP (24126)
Attn: SV DOCKETING
1400 Page Mill Road
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Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited VIA ELECTRONIC FILING addressed to the Mail Stop ISSUE FEE address above, in the USPTO on the date indicated below.

Ginger R. Dreger	(Depositor's name)
/GINGER R. DREGER/	(Signature)
January 3, 2011	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/03/2003	Virginia E. Paton	GNE-0329-D1	4326

TITLE OF INVENTION:

TREATMENT WITH ANTI-ERBB2 ANTIBODIES

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510.00	$300.00	$0	$1810.00	01/10/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
HOLLERAN, ANNE L	1643	424-143100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Arnold & Porter LLP
2 Diane Marschang
3 Ginger R. Dreger

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: GENENTECH, INC.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): SOUTH SAN FRANCISCO, CA

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
☒ Issue Fee
☒ Publication Fee (No small entity discount permitted)
☒ Advance Order - # of Copies 2

4b. Payment of Fee(s):
☐ A check in the amount of the fee(s) is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☒ The Director is hereby authorized to charge the required fee(s), or credit any overpayment, to Deposit Account Number **50-2387**

5. **Change in Entity Status** (from status indicated above)
☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27. ☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

The Director of the USPTO is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.
NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /GINGER R. DREGER/ Date January 3, 2011

Typed or printed name Ginger R. Dreger Registration No. 33,055

This collection of information is required by 37 CFR 1.311 . The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.
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Electronic Patent Application Fee Transmittal

Application Number:	10356824
Filing Date:	03-Feb-2003
Title of Invention:	TREATMENT WITH ANTI-ERBB2 ANTIBODIES
First Named Inventor/Applicant Name:	Virginia E. Paton
Filer:	Ginger R. Dreger/Susan Tamada
Attorney Docket Number:	GNE-0329-D1

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	1501	1	1510	1510
Publ. Fee- early, voluntary, or normal	1504	1	300	300

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	1810

Electronic Acknowledgement Receipt	
EFS ID:	9155260
Application Number:	10356824
International Application Number:	
Confirmation Number:	4326
Title of Invention:	TREATMENT WITH ANTI-ERBB2 ANTIBODIES
First Named Inventor/Applicant Name:	Virginia E. Paton
Customer Number:	35489
Filer:	Ginger R. Dreger/Susan Tamada
Filer Authorized By:	Ginger R. Dreger
Attorney Docket Number:	GNE-0329-D1
Receipt Date:	03-JAN-2011
Filing Date:	03-FEB-2003
Time Stamp:	19:16:51
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$1810
RAM confirmation Number	6904
Deposit Account	502387
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	GNE_0329_IssueFeeTrans.pdf	104924 81a90420044bbace732e882bda7b90327ffa c5c75	no	1
Warnings:					
Information:					
2	Fee Worksheet (PTO-875)	fee-info.pdf	31953 87d0c45d3e55c3d641ecd0bffa7852d9a0fa 2a53	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	136877		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/356,824	02/22/2011	7892549	GNE-0329-D1	4326

35489 7590 02/02/2011

Arnold & Porter LLP (24126)
Attn: SV Docketing Dept.
1400 Page Mill Road
Palo Alto, CA 94304

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 1827 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

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