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The Efficacy of ErbB Receptor-targeted Anticancer Therapeutics Is Influenced by the Availability of Epidermal Growth Factor-related Peptides[1]

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ABSTRACT

The ErbB1 and ErbB2 receptor tyrosine kinases (RTKs) play important roles in the development of numerous types of human cancer and, as such, have been pursued as anticancer targets. To understand the mechanisms contributing to the response of tumor cells to receptor-directed therapeutics, the sensitivity of the ErbB receptor-overexpressing tumor cell lines BT474 and MKN7 to specific inhibitors has been examined. The inhibitors used included monoclonal antibody (mAb) 4D5, which targets ErbB2, and the small molecular weight kinase inhibitors CGP59326 and PKI166, which block the activity of ErbB1 or both ErbB1 and ErbB2, respectively. We had reported previously that although both BT474 and MKN7 cells overexpress ErbB2, only BT474 cells show an antiproliferative response to mAb 4D5 treatment. Here, we show that MKN7 cells, which also overexpress ErbB1, are sensitive to CGP59326, displaying a 60% decrease in their proliferation after treatment with this inhibitor. Most carcinomas express multiple ErbB receptors as well as EGF-related ligands, a situation favoring activation of numerous combinations of ligand-activated receptors. Considering this, the sensitivity of MKN7 and BT474 cells to CGP59326 and mAb 4D5, respectively, was also tested in the presence of exogenous ligands. Treatment of MKN7 cells with CGP59326 in the presence of heregulin (HRG), which activates ErbB2/ErbB3, attenuated the antiproliferative effect of CGP59326 by 50%; MKN7 cells engineered to overexpress ErbB3 were completely rescued from CGP59326 by HRG. Likewise, BT474 cells treated with mAb 4D5 in the presence of epidermal growth factor, betacellulin, and HRG were rescued from its antiproliferative effects by 57, 84, and 90%, respectively. In both MKN7 and BT474 tumor cells, the degree of ligand-induced rescue from the inhibitors correlated with the potency of ErbB receptor activation and stimulation of the PI3K and MAPK intracellular signaling pathways. In comparison with the monospecific agents, treatment with the bispecific ErbB1/ErbB2 kinase inhibitor PKI166 almost completely prevented the EGF-related ligand-induced bypass of the proliferation block in the MKN7 and BT474 cells. These data suggest that the efficacy of anticancer drugs that block a single ErbB receptor may be compromised by the presence of exogenous epidermal growth factor-related ligands, a phenomenon that could be averted by simultaneously blocking multiple ErbB receptors.

INTRODUCTION

The ErbB family of type I RTKs[4] has four members, EGF receptor/ErbB1, ErbB2, ErbB3, and ErbB4. All ErbB family members share common features including an extracellular ligand-binding domain, a transmembrane portion, and an intracellular protein tyrosine kinase domain. The receptors have notable differences in their sequences, which account for differential ligand-binding, variability in enzymatic activity, and diverse affinities for downstream signaling molecules (1, 2). The ligands that bind to and activate this family of receptors are known as EGF-related peptide growth factors. They exhibit differential receptor binding specificities and are grouped into three classes: EGF, amphiregulin, and transforming growth factor-α bind ErbB1; BTC, heparin-binding EGF, and epiregulin are dual specific, binding both ErbB1 and ErbB4; finally, the neuregulins bind ErbB3 and ErbB4 (3, 4). ErbB2 remains an orphan receptor, with no soluble ligand identified to date. However, this receptor occupies a pivotal role in ErbB RTK function, being the preferred and most potent heterodimerization partner for the other ErbB receptors (5–7).

ErbB receptors not only play key roles in normal developmental processes but have also been implicated in malignant transformation. In this respect, ErbB1 and ErbB2 are involved in the growth of many human cancers. Aberrant ErbB1 expression has been reported in a number of tumor types, including breast, glioblastoma, gastric, and squamous cell carcinomas. ErbB1 overexpression in some tumors is accompanied by deletions in its extracellular domain leading to constitutive activation. Moreover, autocrine activation of ErbB1, attributable to coexpression with one or more of the EGF-related ligands, may be equally important in promoting malignancy (8, 9). Aberrant ErbB2 expression, generally attributable to gene amplification and receptor overexpression, has also been described in various types of cancer, most prominently breast and ovarian (10, 11). Abnormal expression of both ErbB1 and ErbB2 receptors has been correlated with more aggressive tumors and a poorer patient prognosis (8, 12, 13). For these reasons, ErbB1 and ErbB2 have been under scrutiny as targets for cancer therapy (9, 14).

ErbB1-specific mAbs were the earliest described therapeutics directed to this receptor. On the basis of preclinical results, the ErbB1-targeted mAb IMC-C225 is in Phase III clinical trials for the treatment of head and neck and colorectal carcinomas (9, 14). Small molecular weight ErbB1 kinase inhibitors are also in various stages of development (14, 15). Considering ErbB2 as a target, many groups have developed growth-inhibitory mAbs (16–19). In preclinical studies, mAb 4D5 has been shown to specifically inhibit the growth of ErbB2-overexpressing tumor cells (17, 19, 20), and its humanized version, Herceptin, is currently in use for breast cancer (21, 22).

ErbB1- and ErbB2-targeted inhibitors are useful not only for characterizing the receptors and intracellular pathways that drive tumor cell proliferation but also to examine mechanisms, which might contribute to inhibitor resistance. Along these lines, we have shown previously that overexpression of ErbB2 does not predict sensitivity to the inhibitory mAb 4D5. Although BT474, SKBR3, and MKN7 cells each overexpress ErbB2, the latter is insensitive to this antibody (20). This suggests that other molecular lesions prevent MKN7 cells from responding to mAb 4D5. Here, we have explored this further and, through the use of a small molecular weight ErbB1-specific inhibitor (CGP59326; Refs. 23 and 24), have found that MKN7 cells exhibit a dependency on ErbB1 signaling for proliferation.

Most tumors of epithelial origin express multiple ErbB receptors and coexpress one or more of the EGF-related ligands (8, 12), suggesting that autocrine receptor activation plays a major role in tumor

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[4] The abbreviations used are: RTK, receptor tyrosine kinase; EGF, epidermal growth factor; BTC, betacellulin; HRG, heregulin; mAb, monoclonal antibody; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride.

cell proliferation. Because the ligands activate different ErbB receptors, these findings imply that multiple ErbB receptor combinations might be active in a tumor, a characteristic that could influence its response to an ErbB-targeted therapeutic. In the work presented here we examined this by testing the ability of CGP59326 and mAb 4D5 to inhibit tumor cell proliferation in the presence of exogenous EGF-related ligands. The results show that the growth-inhibitory effects of both CGP59326 on MKN7 cells and mAb 4D5 on BT474 cells were attenuated in the presence of exogenous ligands.

PKI166 is a dual ErbB1/ErbB2 kinase inhibitor, which blocks proliferation of ErbB1- and ErbB2-driven tumor models (25–27). Using PKI166, we observed that concomitant inhibition of ErbB1 and ErbB2 caused a more stringent antiproliferative effect, preventing cells from escaping the block in the presence of exogenous EGF-related ligands. These results demonstrate an advantage of simultaneously blocking multiple ErbB receptors and have clear implications for future treatment modalities, considering the prevalence of autocrine expression of EGF-related ligands in ErbB-driven tumors.

MATERIALS AND METHODS

Growth Factors, Inhibitors, and Antibodies. HRGβ1 and BTC were from R&D Systems (Wiesbaden, Nordenstadt, Germany); EGF was from Sigma Chemical Co. (St. Louis, MO). mAb 4D5 was kindly provided by Dr. M. Sliwkowski (Genentech, Inc., South San Francisco, CA). CGP59326 and PKI166 were kindly provided by Dr. P. Traxler (Novartis Pharma AG, Basel, Switzerland). Antibodies used for Western blotting and immunoprecipitation were: ErbB1 (1:1 mix of R-1 and 528; R-1 agarose conjugated), ErbB3 (C-17), ErbB4 (C-18; agarose-conjugated-C-18), anti-c-Myc 9E10, all from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PKB, anti-phospho-PKB (serine 473), anti-ERK1/2, and anti-phospho-ERK1/2 (threonine 202/tyrosine 204), all from Cell Signaling Technology (Beverly, MA); ErbB2 (21N; Ref. 20); EGF receptor, phospho-1248 specific ErbB2, and anti-phosphotyrosine 4G10, all from Upstate Biotechnology, (Lake Placid, NY).

Cell Culture and Proliferation Assays. BT474 breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FCS (Life Technologies, Inc., Gaithersburg, MD). MKN7 gastric cancer cells were provided by Dr. C. Benz (University of California at San Francisco, San Francisco, CA) and were cultured in a 1:1 mixture of Ham's F-12 medium: DMEM supplemented with 10% FCS. MKN7 cells ectopically expressing ErbB3 (E3 cells) were isolated after infection with the pBabe-puro ErbB3 retrovirus. MKN7 cells infected with the pBabe-puro empty retrovirus served as controls. EGF and BTC were used at final concentrations of 2 and 1 nM, respectively. HRGβ1 was used at final concentrations of 0.4 and 1 nM on BT474 and MKN7 cultures, respectively. To determine the effect of different inhibitors on proliferation, cells were plated at a density of 2×10^3 cells/cm^2; 24 h later, inhibitors were added at a final concentration of 6 μg/ml for mAb 4D5, 6 μM for CGP59326, and 5 μM for PKI166. Medium with inhibitors was replenished every 24 h, and after 4 days, cells were trypsinized and counted in a hemocytometer. Each assay was done in triplicate.

Lysate Preparation, Immunoprecipitations, and Western Analyses. Cells were initially plated at a density of 3×10^4 cells/cm^2; 24 h later, inhibitors and ligands were added at the concentration described above, and treatment proceeded for the times indicated. Cells lysates were prepared by two methods; for each, cultures were first washed with ice-cold PBS containing 1 mM PMSF. Cultures were washed with ice-cold extraction buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 25 mM β-glycerol phosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 15 mM PP_i, 2 mM sodium orthovanadate, 10 mM sodium molybdate, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and 1 mM PMSF] and extracted in the same buffer containing 1% NP40. The extracts were homogenized, cleared by centrifugation, and frozen at −80°C. Protein concentrations were determined using the Bio-Rad (Munich, Germany) protein assay reagent. Alternatively, cell lysates were obtained by boiling to disrupt the antibody structure in mAb 4D5-treated BT474 cells. Cultures were scraped into 700 μl of ice-cold PBS plus 1 mM PMSF and centrifuged (4000 rpm/2 min/4°C), and the pellet was frozen in dry ice for 5 min. The pellet from a 10-cm dish was resuspended in 100 μl of buffer [100 mM Tris (pH 7.4), 2% SDS, 10 mM DTT, and 2 mM sodium vanadate], boiled for 10 min, vortexed, and cleared by centrifugation (14,000 rpm for 5 min at room temperature). The extracts were transferred to a fresh tube, frozen at −80°C, and, prior to use, the final volume was made up to 10× the original with NP40-containing extraction buffer. Immunoprecipitations and Western analyses were done by standard procedures as described previously (20, 23).

Flow Cytometry Analyses. To analyze cell cycle profiles, BT474 and MKN7 cells were plated at a density of 3×10^4 cells/cm^2 and 1.5×10^4 cells/cm^2, respectively, and 24 h later, the medium was changed, and treatment was initiated as described above. At the indicated times, cells were trypsinized, washed three times 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% NP40, 4 μg of propidium iodide/ml, and 80 μg of RNase A/ml in PBS]. After 30 min incubation in the dark on ice, cell cycle distribution was measured with a Becton Dickinson FACScan flow cytometer.

RESULTS

MKN7 Carcinoma Cells Exhibit Dependency upon ErbB1 for Proliferation. We have used the BT474 and MKN7 cell lines to examine the antiproliferative effects of ErbB-directed inhibitors. Both tumor cell lines overexpress ErbB2; however, we have shown previously that proliferation of the BT474 cells, but not the MKN7 cells, was blocked by the ErbB2-targeted growth-inhibitory mAb 4D5 (20). The observation that ErbB1 is overexpressed and strongly phosphorylated in MKN7 cells (20) suggests that this receptor is active and might be driving their proliferation. To test this hypothesis, we used an ErbB1-specific, small molecule kinase inhibitor, CGP59326 (23, 24). Treatment of MKN7 cells with CGP59326 led to a drop in ErbB1 signaling activity, as shown by a decrease in the phosphotyrosine content of the immunoprecipitated receptor (Fig. 1*A*). Importantly, CGP59326 also reduced the proliferation of MKN7 cells as shown by flow cytometry. Cells from CGP59326-treated cultures displayed a G_1 content of 74% *versus* 56% seen in control cultures (Fig. 1*B*). These results indicate that MKN7 cell proliferation is driven through ErbB1 signaling.

The Antiproliferative Effects of CGP59326 Can Be Bypassed by Treatment of MKN7 Cells with HRG. Many human tumors coexpress ErbB receptors and one or more of the EGF-related ligands (8, 9). This implies that multiple ErbB receptor combinations could be active, a characteristic that might influence the response of a tumor to a particular ErbB-targeted therapeutic agent. We, therefore, addressed how effective the ErbB1-specific inhibitor CGP59326 would be on MKN7 cells in the presence of exogenously added ErbB ligands, specifically EGF, BTC, and HRG. MKN7 cells were treated with CGP59326 in the presence of each of the three ligands. After 36 h, cells were harvested, and flow cytometry was performed (Fig. 1*C*). The cultures treated with EGF and BTC were still efficiently blocked by CGP59326, whereas HRG addition was able to partially rescue the cells from the antiproliferative effect (G_1 population in HRG-supplemented *versus* HRG-unsupplemented CGP59326-containing media: 63% *versus* 74%). Treatment of control MKN7 cultures with the ligands alone had no effect on cell cycle distribution (not shown). Thus, as expected, the ErbB1 inhibitor efficiently blocked proliferation of MKN7 cells in the presence of the ErbB1 ligands EGF and BTC. (In these experiments, BTC activated only ErbB1, because we have not detected ErbB4 protein/activity in MKN7 cells). In contrast, activation of ErbB2/ErbB3 heterodimers by HRG was able to rescue the proliferative block induced by CGP59326. This observation is particularly striking considering that MKN7 cells express very low levels of ErbB3 (20).

To further explore the role of ErbB3 in the HRG-mediated rescue, MKN7 cells expressing high levels of ectopic ErbB3 (MKN7/E3)

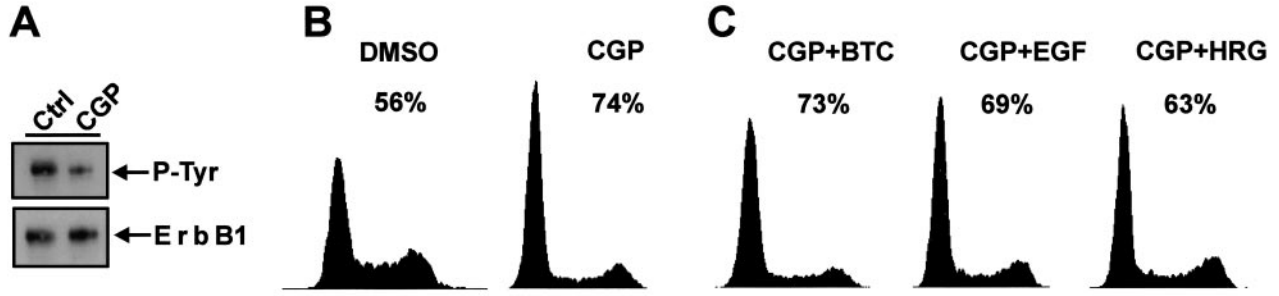

Fig. 1. Effect of the ErbB1-targeted inhibitor CGP59326 on ErbB1 phosphorylation and the cell cycle distribution of MKN7 cells. *A*, the phosphotyrosine content of ErbB1 was determined in CGP59326-treated cultures. ErbB1 was immunoprecipitated from protein lysates prepared from control DMSO vehicle-treated cultures (*Ctrl*) or cultures treated with CGP59326 (*CGP*) for 2 h. After immunoblotting with a phosphotyrosine-specific antibody (*upper panel*), the membranes were stripped and reprobed to control for ErbB1 protein levels (*lower panel*). *B*, flow cytometry analyses of control DMSO vehicle-treated cells and cells treated with CGP59326 (*CGP*). *C*, flow cytometry analyses of cells treated with a combination of CGP59326 and the indicated ligands. *C* and *D*, cells were treated for 36 h and harvested by trypsinization, and nuclei were stained with propidium iodide. The percentage of cells in the G_1 phase of the cell cycle is indicated. The experiments were performed a minimum of three times, and a typical result is shown.

were generated via retroviral infection with pBabe-puro-ErbB3 (Fig. 2*A*, *upper panels*). In control vector-infected MKN7/P cells, ErbB3 was only detectable as a phosphorylated band in immunoprecipitates from lysates of HRG-treated cultures. This phosphorylated band was dramatically increased in MKN7/E3 cells after HRG addition, consistent with higher expression of ErbB3 in these cells (Fig. 2*A*, *upper panels*). Both MKN7/P and MKN7/E3 cells displayed sensitivity to CGP59326, accumulating in the G_1 phase of the cell cycle (Fig. 2*B*). Addition of HRG partially rescued MKN7/P cells from the effects of CGP59326 (Fig. 2*B*, *upper panel*), as was observed in the parental cells (Fig. 1*C*). In contrast, the antiproliferative effect of CGP59326 on MKN7/E3 cells was completely reversed by HRG addition to the cultures (Fig. 2*B*, *lower panel*). Moreover, HRG-induced activation of ErbB2, as well as ErbB3, was much stronger in MKN7/E3 cells as compared with MKN7/P cells (Fig. 2*A*). These results suggest that the degree to which HRG overcame the CGP59326-mediated block was dependent upon the level of ErbB3 and, hence, the extent of ligand-induced activation of the ErbB2/ErbB3 heterodimer. These differences likely explain why the antiproliferative effects of CGP59326 were completely reversed in the ErbB3-overexpressing MKN7 cells.

Signaling Pathways Are Activated in HRG-treated MKN7 Cells in the Presence of CGP59326. The MAPK and PI3K pathways are two major signaling cascades downstream of the ErbB receptors, which are up-regulated in many ErbB receptor-overexpressing cancer cells (1, 2). Using antisera specific for the active, phosphorylated forms of ERK1/2 and PKB, the major kinases on the respective pathways, we have observed previously that both pathways are constitutively activated in MKN7 cells (20). Fig. 3 shows that treatment of MKN7/E3 and MKN7/P cells with CGP59326 results in a reduction in the level of phospho-ERK1/2 and phospho-PKB. However, addition of HRG to the CGP59326-treated cultures completely reversed these inhibitory effects, reactivating both pathways.

To correlate activation of the pathways with the difference in the HRG-induced rescue observed between MKN7/E3 and MKN7/P cells, a time course over 16 h was performed. In MKN7/E3 cells, ERK1/2 phosphorylation was restored to the high level seen in the untreated cultures within 10 min of HRG addition, then slowly decreased (Fig. 3, *top panel*). The effect of HRG on CGP59326-treated MKN7/P cells was slower and less pronounced. By 16 h, however, both MKN7/E3 and MKN7/P cells displayed levels of ERK1/2 phosphorylation that were significantly lower than in untreated cultures. Thus, the inhibitory effect of CGP59326 on ERK1/2 phosphorylation was more protracted than the stimulatory effect of HRG on this pathway. This suggests that ErbB1 phosphorylation is responsible for the high constitutive ERK1/2 phosphorylation observed in MKN7 cells.

PKB phosphorylation was also induced after HRG addition to CGP59326-treated cultures (Fig. 3, *third panel*). PKB phosphorylation climbed slowly in the MKN7/P cells, whereas in the MKN7/E3 cells it rapidly attained a high level. This was probably attributable to the elevated level of ErbB3 in the latter and, hence, an increased ability to couple strongly to the PI3K pathway, as demonstrated by potentiation of the association of ErbB3 with the p85 subunit of PI3K (Fig. 2*A*). Importantly, in both cell lines, at 16 h the level of active PKB was higher than in untreated cultures. Taken together, these results suggest that the inhibitory effects of CGP59326 on ErbB1-driven PKB phosphorylation could be overcome by HRG-induced

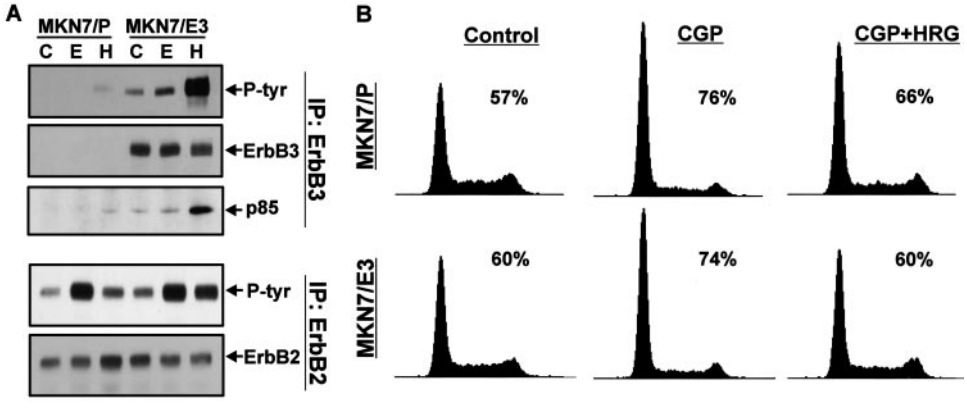

Fig. 2. Characterization of the MKN7/P and MKN7/E3 cell lines. *A*, ErbB3 and ErbB2 phosphorylation in MKN7/P and MKN7/E3 cells. ErbB3 and ErbB2 were immunoprecipitated from protein lysates prepared from cells treated with EGF (*E*), or HRG (*H*), or left untreated (*C*) for 10 min. After immunoblotting with a phosphotyrosine-specific antibody (*P-tyr*), the membranes were stripped and reprobed to control for ErbB3 and ErbB2 protein levels (*ErbB3* and *ErbB2*). Association of the p85 PI3K regulatory subunit with ErbB3 was assessed by probing the lower part of the PVDF membrane with a p85-specific antibody. *B*, analysis of cell cycle distribution after treatment of MKN7/P and MKN7/E3 cells with CGP59326 (*CGP*) and HRG. Shown is flow cytometry analysis of cells treated with DMSO vehicle control, CGP59326, and CGP59326 plus HRG. The percentage of cells in the G_1 phase of the cell cycle is indicated. The experiments were performed a minimum of three times, and a typical result is shown.

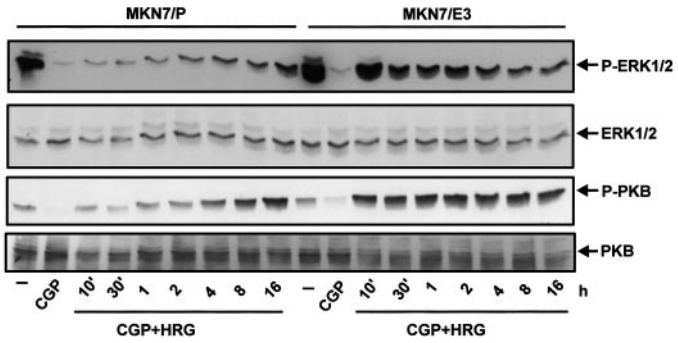

Fig. 3. Analysis of PKB and ERK1/2 phosphorylation levels in MKN7/P and MKN7/E3 cells. Cells were pretreated for 1 h with CGP59326 (*CGP*) or DMSO vehicle (−), and then HRG was added for the indicated times. Protein lysates were prepared, and 60 μg were resolved by SDS-PAGE, transferred onto a PVDF membrane, and probed for phospho-PKB (*P-PKB*) and phospho-ERK1/2 (*P-ERK1/2*). The membranes were stripped and reprobed to control for PKB and ERK1/2 protein levels (*PKB* and *ERK1/2*).

activation of ErbB2/ErbB3 heterodimers, even in MKN7/P cells, which express very low levels of ErbB3. Moreover, the more potent HRG-mediated rescue observed with the MKN7/E3 cells appears to correlate with an initial strong activation of both the MAPK and PI3K pathways.

EGF-related Ligands Bypass the G_1 Block Induced by an ErbB2-specific Inhibitor in BT474 Breast Carcinoma Cells. The ErbB2-overexpressing BT474 breast tumor cells are very sensitive to the antiproliferative effects of mAb 4D5, displaying essentially a complete block in response to treatment (20). Analogous with the above experiments on MKN7 cells, we investigated whether exogenous EGF-related ligands could also prevent BT474 cells from responding to mAb 4D5. BT474 cells were treated for 36 h with mAb 4D5 alone or in combination with BTC, EGF, or HRG, and cell cycle profiles were assessed by flow cytometry. Treatment with mAb 4D5 blocked the cells in G_1 (93% *versus* 56% in control cells; Fig. 4). Addition of any of the EGF-related ligands, however, attenuated the mAb 4D5-mediated G_1 block. The extent of attenuation varied from ~50% for EGF (G_1 population, 72%) to essentially total for BTC and HRG (G_1 population, 62 and 60%, respectively). Treatment of control BT474 cultures with the ligands alone had no effect on cell cycle distribution (Fig. 4).

Ligand-induced Bypass of the mAb 4D5-induced G_1 Block Correlates with Activation of Specific ErbB Receptors and Strong Stimulation of PKB Phosphorylation. The results in Fig. 4 show that EGF-related ligands rescue BT474 cells from the antiproliferative effects of mAb 4D5. In addition to overexpressing ErbB2, BT474 tumor cells also express the other three ErbB receptors (20). Thus, there are a number of potential ligand-receptor combinations that could contribute to the rescue. To characterize this further, we examined the effect of mAb 4D5 treatment on each ErbB receptor and asked how exogenous ligand addition impacted on this. As shown previously (20), mAb 4D5 binding to ErbB2 caused a rapid decrease in its phosphorylation (Fig. 5, *upper panel*). Addition of each ligand caused a further decrease in its phosphotyrosine content. The ligands had similar effects on ErbB2 in control BT474 cells, in the absence of mAb 4D5 (data not shown). These results suggest that mAb 4D5-bound ErbB2 was still able to interact with other ligand-bound receptors, which will be further discussed below.

We next examined the phosphotyrosine content of the other ErbB receptors. To prevent mAb-4D5-bound ErbB2 from interfering with immunoprecipitations, ErbB1 and ErbB4 were affinity isolated from cell lysates using specific antibodies conjugated to agarose beads, whereas ErbB3 was immunoprecipitated from lysates that had been boiled to disrupt the structure of mAb 4D5. Subsequent immunoblot analysis revealed barely detectable phosphotyrosine on ErbB1 and ErbB4 in control cells, as reported previously (20), and these levels were unaffected by mAb 4D5 addition to the cultures. In contrast, ErbB3 phosphotyrosine levels were reduced after mAb 4D5 treatment (Fig. 5). This result underscores the importance of ErbB2 activity in maintaining ErbB3 signaling potential in cells overexpressing ErbB2 (28). In the presence of mAb 4D5, EGF stimulated ErbB1 phosphorylation, BTC stimulated ErbB1 and ErbB4 phosphorylation, whereas HRG activated ErbB3 and ErbB4. These data reflect the specificity of each ligand (1–4) and demonstrate that BT474 cells can be rescued from the antiproliferative effects of mAb 4D5 through ligand-induced activation of ErbB1, ErbB3, and ErbB4. Moreover, a more pronounced rescue appears to correlate with the ability of a particular ligand to activate two ErbB receptors, *i.e.*, ErbB3 and ErbB4 for HRG, and ErbB1 and ErbB4 for BTC.

Next, we analyzed the contribution of the MAPK and the PI3K pathways to the ligand-induced rescue of BT474 cells. The basal constitutive phosphorylation of PKB and ERK1/2 was reduced after mAb 4D5 treatment of BT474 cultures (Fig. 6*A*), as shown previously (20). Addition of EGF, BTC, or HRG to the cultures overcame the inhibitory effect of mAb 4D5 on both signaling pathways (Fig. 6*A*). The effects of EGF, BTC, and HRG on ERK1/2 phosphorylation were similar in that each rapidly induced high ERK1/2 phosphorylation. In all cases, this activation was transient, reaching near basal levels after 6 h. In contrast, with respect to PKB phosphorylation, ligand-specific effects were observed. EGF delayed mAb 4D5-induced down-regulation of PKB phosphorylation, whereas BTC and HRG completely overcame the negative effects of mAb 4D5, strongly stimulating PKB phosphorylation (Fig. 6*A*). As observed for ERK1/2, ligand-induced PKB phosphorylation was transient. Indeed, after 6 h, PKB phosphorylation was at or below control levels in HRG-treated and EGF- and BTC-treated cultures, respectively. Taken together, these results suggest that BTC and HRG completely rescued the growth-inhibitory effects of mAb 4D5 attributable to their ability to potently stimulate the PI3K/PKB pathway, even in the presence of antibody.

We also examined expression of the transcription factor c-Myc,

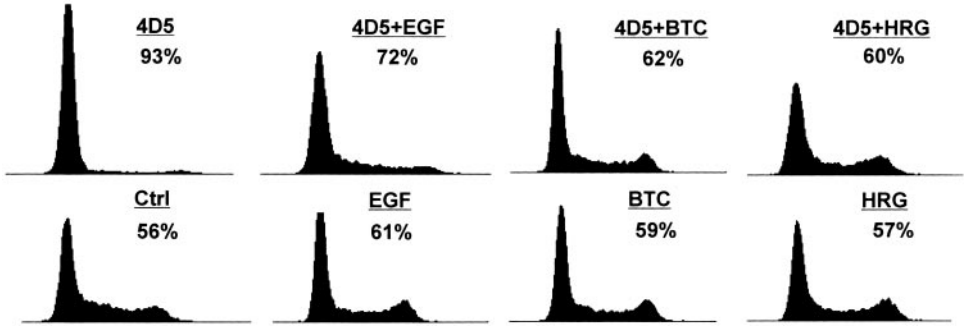

Fig. 4. Analysis of cell cycle distribution after treatment of BT474 cells with mAb 4D5 and ligands BTC, EGF, or HRG. After 36-h treatment, cells were harvested by trypsinization, and nuclei were stained with propidium iodide. Shown is the flow cytometry analysis of cells treated with: PBS vehicle control (*Ctrl*), mAb 4D5 (*4D5*), mAb 4D5 plus ligands, and ligands alone. The percentage of cells in the G_1 phase of the cell cycle is indicated. The experiments were performed a minimum of three times, and a typical result is shown.

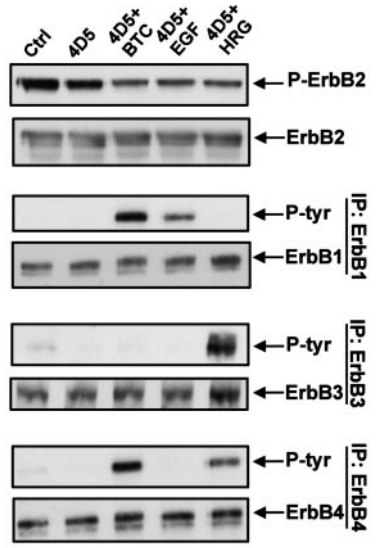

Fig. 5. ErbB receptor phosphotyrosine levels after treatment of BT474 cells with mAb 4D5 and EGF-related ligands. Protein lysates were prepared from cells treated for 30 min with mAb 4D5 plus ligands. ErbB1, ErbB3, and ErbB4 were immunoprecipitated and analyzed by immunoblotting for phosphotyrosine content using a phosphotyrosine-specific antibody (*P-tyr*), and then the blots were stripped and reprobed to control for receptor loading. Extracts were examined for ErbB2 content and phosphorylation by immunoblotting with an ErbB2 antibody and an antibody specific for phosphorylated tyrosine 1248 (*P-ErbB2*).

which has been implicated in ErbB2-dependent tumor cell proliferation (29). Treatment of BT474 cells with mAb 4D5 resulted in down-regulation of c-Myc (Fig. 6*B*), as reported previously (20). In cells treated with each ligand, the negative effect of mAb 4D5 on c-Myc expression was blocked. Indeed, c-Myc expression was initially stimulated in each ligand-treated culture, before returning to the level seen in untreated control cells (Fig. 6*B*). As expected from the above observations, the duration of c-Myc induction was more pronounced in BTC- and HRG-treated cultures than in EGF-treated cultures.

In summary, these data suggest that ErbB2-dependent BT474 cells can be rescued from the mAb 4D5-induced G_1 block through ligand-induced activation of other ErbB family members. In comparison with EGF, which rescued the cells by ~50%, a complete rescue was observed with BTC and HRG. This more complete rescue correlated with the stimulation of two ErbB receptor family members and more potent induction of PKB phosphorylation and c-Myc expression.

The Dual Specificity ErbB1/ErbB2 Kinase Inhibitor PKI166 Causes a More Stringent Block to Proliferation. The results presented above demonstrate that inhibiting a single ErbB receptor in tumor cells causes a proliferative block that can be attenuated by the presence of exogenous EGF-related ligands, which activate other ErbB family members. The dual ErbB1/ErbB2 kinase inhibitor PKI166 blocks both ErbB1 and ErbB2 enzymatic activity *in vitro*, with IC_{50}s in the low nM range (25–27). PKI166 was used to test the possibility that concomitant inhibition of ErbB1 and ErbB2 might have more dramatic antiproliferative effects than inhibiting either receptor alone. As expected, exposure of BT474 cells to PKI166 resulted in a decrease in the phosphotyrosine content of ErbB1 and ErbB2 (Fig. 7*A*). Next, MKN7 and BT474 cells were treated with PKI166 and analyzed by flow cytometry (Fig. 7*B*) and by proliferation assays (Fig. 7, *C* and *D*). PKI166 and CGP59326 had similar effects on MKN7, yielding a G_1 DNA content of 71% (Fig. 7*B*) and 74% (Fig. 1*B*), respectively. Moreover, after 4 days, there was a 60 and 90% decrease in cell number in cultures treated with CGP59326 and PKI166, respectively (Fig. 7*D*). Similarly, PKI166 was as efficient as mAb 4D5 in blocking BT474 cells, yielding cells with a G_1 DNA content of 97% (Fig. 7*B*) and 93% (Fig. 4), respectively. Furthermore, after 4 days, there was a 50 and 60% decrease in cells in cultures treated with mAb 4D5 and PKI166, respectively (Fig. 7*C*).

To test the ability of EGF-related ligands to overcome the PKI166-induced proliferative block, MKN7 and BT474 cultures were concomitantly treated with PKI166 plus EGF, BTC, or HRG and were analyzed by flow cytometry (Fig. 7*B*) and by proliferation assays (Fig. 7, *C* and *D*). The PKI166-treated MKN7 cells were insensitive to exogenous EGF and BTC, whereas HRG caused a slight decrease in G_1 content (67% *versus* 71% with PKI166 alone; Fig. 7*B*) and only increased cell number by 10% (Fig. 7*D*). This contrasts with CGP59326-treated MKN7 cultures where HRG rescued the cells by 50% (Figs. 1*C* and 7*D*). Considering BT474 cells, neither EGF nor BTC was able to bypass the effects of PKI166, whereas HRG partially overcame its inhibitory effect (G_1 content of 87% *versus* 97% with PKI166 alone; Fig. 7*B*) and increased cell number minimally (Fig. 7*C*). This contrasts with the complete proliferative rescue observed with HRG in mAb 4D5-treated BT474 cultures (Figs. 4 and 7*C*). These results clearly show that simultaneous inhibition of two ErbB receptors leads to a more stringent proliferative block than observed after inhibition of either receptor alone, data that have important implications for cancer treatment strategies.

DISCUSSION

The ErbB1 and ErbB2 RTKs have been implicated in the development of many types of human cancer. Moreover, alterations in the

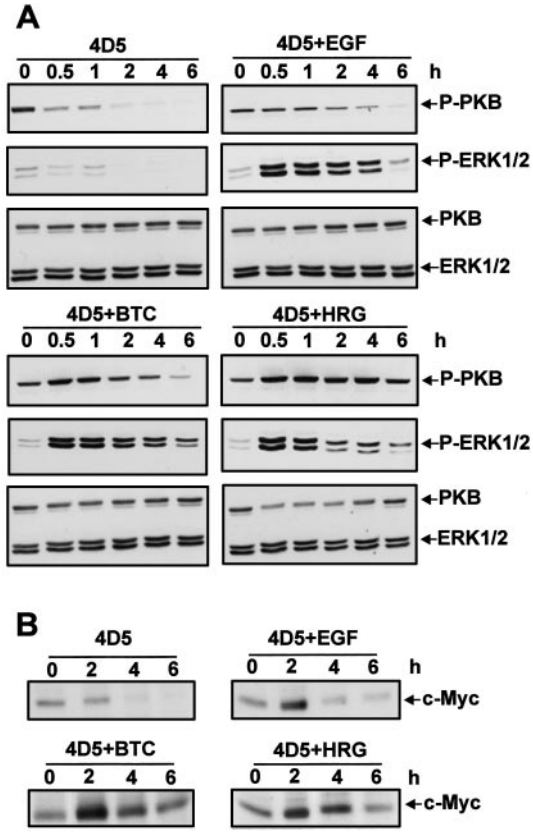

Fig. 6. Analysis of PKB and ERK1/2 phosphorylation levels and c-Myc protein levels in BT474 cells. Cells were treated for the indicated times with mAb 4D5, alone or in combination with BTC, EGF, or HRG. Protein lysates were prepared, and 60 μg were resolved by SDS-PAGE, transferred onto PVDF membranes, and probed for phospho-PKB (*P-PKB*), phospho-ERK1/2 (*P-ERK1/2*; *A*) or c-Myc protein (*c-Myc*; *B*). In *A*, the membranes were stripped and reprobed to control for PKB and ERK1/2 protein levels (*PKB* and *ERK1/2*).

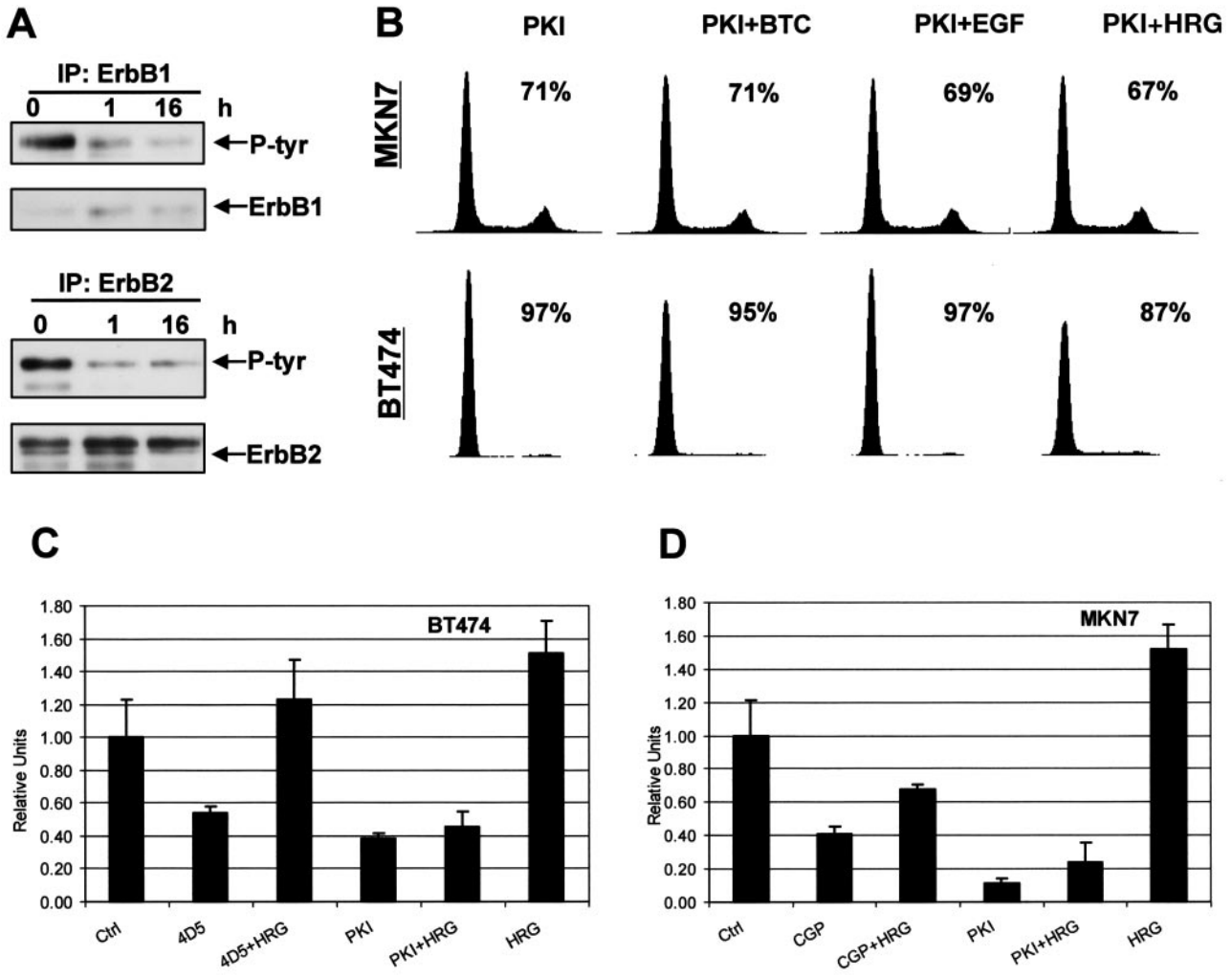

Fig. 7. Effect of ErbB inhibitors on receptor phosphorylation and cellular proliferation. *A*, the phosphotyrosine content of ErbB1 and ErbB2 was determined after 1 and 16 h of treatment of BT474 cells with PKI166. The receptors were immunoprecipitated (*IP*), transferred onto a PVDF membrane, probed for phosphotyrosine levels using a phosphotyrosine-specific antibody (*P-tyr*), and reprobed for total receptor levels. Longer exposures were required to visualize changes in ErbB1 phosphorylation because of the low amount of this receptor in comparison with ErbB2. *B*, analysis of cell cycle distribution after treatment of MKN7 and BT474 cells with PKI166 and EGF-related ligands. After 36 h of treatment with PKI166 (5 μM) alone or in combination with BTC, EGF, or HRG, cells were harvested by trypsinization, and nuclei were stained with propidium iodide. Shown is flow cytometry analysis of cultures treated as indicated, and the numbers refer to the percentage of cells in the G_1 phase of the cell cycle. The experiments were performed a minimum of three times, and a typical result is shown. *C* and *D*, proliferation was measured by counting cells after 4 days of the indicated treatment. *Bars*, SD.

expression of these receptors have been correlated with a more malignant phenotype and poor patient prognosis (8, 9, 12, 13, 30). Consequently, these receptors have been intensely scrutinized as targets for cancer therapy (10, 12, 14). There is a growing number of ErbB-directed therapeutics used for cancer treatment or in various stages of preclinical or clinical development (9, 14, 25). Indeed, the ErbB2-targeted Herceptin is used in the treatment of metastatic breast cancer patients with ErbB2-overexpressing tumors (22, 31). Despite these efforts, the parameters determining response to an ErbB-directed therapeutic require more study. Indeed, not all tumor cells, be it in cell culture (20) or in patients (22, 31), respond to inhibition of ErbB receptors, despite exhibiting aberrant ErbB1 and/or ErbB2 expression. The results presented here and previously (20) support the hypothesis that impairment of one tyrosine kinase receptor can block proliferation. However, tumor cells retain the ability to compensate for loss of receptor function. Specifically, using MKN7 and BT474 ErbB receptor-overexpressing carcinoma cell lines, we have demonstrated that a proliferative block induced as a consequence of decreased ErbB1 or ErbB2 receptor activity can be overcome by the presence of EGF-related ligands. Importantly, this phenomenon can be attenuated using the bispecific ErbB1/ErbB2 inhibitor PKI166, providing a biological rationale supporting increased efficacy through simultaneous inhibition of multiple ErbB receptors. These observations have clear implications for ErbB-directed therapeutics, considering the prevalence of autocrine expression of EGF-related peptides in ErbB receptor-driven tumors.

ErbB2-overexpressing MKN7 cells are insensitive to mAb 4D5 treatment, although mAb 4D5 induces a decrease in ErbB2 phosphorylation (20). MKN7 cells also express high levels of phosphorylated ErbB1, and detection of the ErbB1 ligands amphiregulin and transforming growth factor-α in conditioned medium from MKN7 cultures[5] suggests the presence of an autocrine loop. Consistent with a role for activated ErbB1 in MKN7 cell proliferation, treatment with the ErbB1-specific inhibitor CGP59326 resulted in G_1 accumulation. Another ErbB1-overexpressing breast tumor cell line, MDA-MB468, provides an interesting comparison. These cells are rather insensitive to the antiproliferative effects of the ErbB1/ErbB2 inhibitor Iressa, as compared with the ErbB1-overexpressing A431 carcinoma cells (32). As discussed by Moasser *et al.* (32), this relative resistance might be

[5] A. B. Motoyama and N. Torring, unpublished results.

attributable to mutation of PTEN, a negative regulator of the PI3K pathway. These observations underlie the fact that simple overexpression of ErbB1 or ErbB2 does not predict sensitivity to a targeted therapeutic. Hence, strategies designed to block more than one protein are likely to potentiate antiproliferative responses.

In MKN7 cells, inhibition of ErbB1, but not ErbB2, caused downregulation of the PI3K and MAPK pathways, indicating that the overexpressed ErbB1 has usurped signaling molecules, which couple to these pathways. This is interesting in light of a recent publication showing the importance of ErbB3 for PI3K activation in the context of ErbB2/ErbB3 heterodimers (34). The low level of ErbB3 in MKN7 cells is apparently insufficient to allow constitutive activation of PI3K downstream of ErbB2/ErbB3 heterodimers. We speculated previously that low ErbB3 expression in these cells might explain their insensitivity to mAb 4D5 (20). This possibility prompted us to engineer the ErbB3-overexpressing MKN7/E3 cells. However, even in these cells, the basal phosphotyrosine content of ErbB3 was not dramatically elevated (Fig. 2), PKB phosphorylation was unchanged (Fig. 3), and the cells remained insensitive to the antiproliferative effects of mAb 4D5.[6] These results suggest that, in MKN7 cells, a constitutive signaling platform, which maintains high activity of the MAPK and PI3K pathways, has been assembled around ErbB1.

Addition of HRG to CGP59326-treated MKN7 cultures attenuated the antiproliferative effects of the inhibitor. Even the low level of ErbB3 activity evident in HRG-treated MKN7 cultures was sufficient to partially overcome the CGP59326-mediated block in proliferation and to induce higher activation of the PI3K pathway (as measured by PKB phosphorylation) than observed in control, non-drug-treated MKN7 cells (Fig. 3). Ectopic expression of ErbB3, however, led to a more pronounced bypass of the G_1-S block. Comparison of HRG-induced PKB phosphorylation in MKN7/P and MKN7/E3 cells revealed a notable difference between the kinetics, which were more rapid in the latter. These kinetics might be explained by the immediate strong coupling of the HRG-activated ErbB2/ErbB3 heterodimer to this pathway, exemplified by the high amount of p85 complexed with ErbB3 in MKN7/E3 cells. MKN7 cells also have high constitutive phospho-ERK 1/2; however, in the presence of CGP59326, HRG did not induce long-term ERK1/2 phosphorylation, showing that ErbB1 is responsible for maintaining activation of this pathway. Taken together, these data demonstrate the potential of tumor cells to bypass the antiproliferative effects of an ErbB1-directed inhibitor via ligand induced activation of another ErbB family member, in this case ErbB3. Strikingly, this bypass does not require overexpression of that receptor. In this context, it appears that HRG-mediated activation of the PI3K/PKB pathway overcame the negative effect of CGP59326 on the MAPK pathway, allowing the cells to proliferate with lower MAPK activity than observed in untreated cells.

BT474 cells, similar to many other ErbB2-overexpressing cells, are sensitive to the antiproliferative effects of mAb 4D5, both in cell culture (17, 19, 20) and as tumor xenografts (35). There have been many studies aimed at understanding the mechanisms important for its anticancer effects. Indeed, Herceptin appears to have multiple activities, any combination of which might contribute to its *in vivo* efficacy (9, 22, 35, 36). We have shown that mAb 4D5 binding to ErbB2 causes a rapid reduction in its phosphotyrosine content (Ref. 20 and Fig. 5). Here, we demonstrate that the phosphotyrosine content of ErbB3 is also decreased after mAb 4D5 treatment of BT474 cells. Despite being an impaired kinase, ErbB3 it is an important signaling moiety in tumor cells (2) and has been implicated in ErbB2-driven breast tumor cell proliferation (28, 37). Our observations in mAb 4D5-treated BT474 cells support the role of ErbB3 as a major downstream effector of overexpressed ErbB2 and show the importance of the ErbB2/ErbB3 heterodimer in maintaining PI3K activity.

Despite the strong antiproliferative effects of mAb 4D5 on BT474 cells, exogenous addition of EGF-related ligands prevented establishment of the G_1-S block. BTC and HRG, which each stimulated the phosphorylation of two ErbB receptors, strongly activated the MAPK and PI3K pathways and potentiated a complete bypass of the block. In comparison, EGF, which stimulated only ErbB1, activated these pathways to a lesser degree and led only to a partial bypass. These observations were also reflected in the duration of c-Myc induction in response to these ligands and suggest a correlation between the ability of a ligand to reactivate downstream signaling pathways and the extent to which the ligand can overcome the antiproliferative effects of mAb 4D5.

In the context of BT474 cells, it is interesting to consider whether mAb 4D5-bound ErbB2 participated in the ligand-induced bypass or whether activation of the other ErbB receptors was sufficient. This is particularly relevant when considering the role of ErbB3, because it requires dimerization with another ErbB receptor for activation. HRG induced strong activation of ErbB3 and ErbB4 in mAb 4D5-bound BT474 cells. It has been shown that mAb 4D5 binding to ErbB2 does not exclude its participation in dimerization with other ligand-bound ErbB RTKs (19, 38). Furthermore, unlike 2C4, another ErbB2-specific mAb, 4D5 cannot block formation of HRG-induced ErbB2/ErbB3 and ErbB2/ErbB4 heterodimers (19, 38). Thus, we favor the possibility that mAb 4D5-bound ErbB2 does play a role in the ligand-induced rescue from this antiproliferative agent, although we acknowledge that additional experiments are required to confirm this hypothesis. Irrespective of the exact role of the mAb 4D5-bound ErbB2 in the ligand-induced rescue, what can be clearly correlated with the proliferative response of the cells is strong activation of downstream pathways.

It is also worth discussing the role of ErbB4 in the rescue induced by treatment of BT474 cells with HRG and BTC. In contrast with the other ErbB receptors that are thought to be important for proliferation of normal breast cells (39), ErbB4 appears to be associated with the differentiation process (40, 41). Even in breast cancer, elevated ErbB4 expression has been associated with a more differentiated phenotype and a prognostically favorable endocrine response (42, 43). The data presented here suggest that in certain instances, ErbB4 may also play a role in breast tumor cell proliferation.

Considering that tumor cells have many mutations, it is likely that therapeutic combinations targeting multiple pathways or proteins will have stronger antitumor activity as compared with monospecific agents. In this respect, it has been reported that a combination of the ErbB1-directed mAb 225 and mAb 4D5 inhibited proliferation of an ovarian tumor cell line more strongly than either mAb alone (44). In addition to ErbB-targeted mAbs, a number of different ErbB1/ErbB2-bispecific inhibitors have been described recently (14, 15, 25, 45), including PKI166 and ZD1839 (Iressa), which are currently in clinical trials. We show here that PKI166 provided a stronger antiproliferative block in the presence of EGF-related ligands in two tumor models. In comparison with CGP59326-treated cultures, PKI166-treated MKN7 cells were very resistant to exogenous HRG addition, maintaining the G_1 accumulation and showing only a slight increase in cell number. The results obtained with BT474 cells were even more dramatic. Neither EGF nor BTC treatment caused a bypass of the G_1-S block imposed by PKI166, whereas HRG only partially overcame this block. Considering that BTC and HRG completely overcame the inhibitory effects of mAb 4D5, it is evident that targeting both the ErbB1 and ErbB2 receptors was superior to targeting ErbB2 alone. In summary, when considering the prevalence of autocrine expression of

[6] H. A. Lane and A. B. Motoyama, unpublished results.

EGF-related ligands in tumors, our results suggest that it might be advantageous to simultaneously block multiple ErbB receptors. Furthermore, these observations show some of the possible limitations which ErbB-specific therapeutics might face in clinical use and could have implications for the future design of ErbB-targeted therapeutic strategies.

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The Efficacy of ErbB Receptor-targeted Anticancer Therapeutics Is Influenced by the Availability of Epidermal Growth Factor-related Peptides

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