Synergistic inhibition of breast cancer cell lines with a dual inhibitor of EGFR-HER-2/neu and a Bcl-2 inhibitor

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Abstract. The epidermal growth factor receptor (EGFR) (ErbB1) and HER-2/neu (ErbB2) are members of the ErbB family of receptor tyrosine kinases. These receptors are overexpressed in a variety of human tumors and overexpression generally correlates with poor prognosis and decreased survival. Lapatinib, a reversible inhibitor of both EGFR and HER-2/neu, has shown some success in achieving clinical responses in heavily pretreated advanced cancer patients. GW2974 is a reversible dual inhibitor similar to lapatinib, but GW2974 was not progressed to clinical trials due to pharmacokinetic issues. Bcl-2, an anti-apoptotic protein, is also overexpressed in a number of human tumors. Bcl-2 inhibitors induce apoptosis and sensitize cancer cells to other therapies. The purpose of this study was to assess the effects of combining ErbB and Bcl-2 inhibitors on the growth of human breast cancer cell lines. EGFR/HER-2/neu tyrosine kinase inhibitors (lapatinib and GW2974) were combined with Bcl-2 inhibitors (HA14-1 or GX15-070) and the anti-proliferative effects were determined by the MTT tetrazolium dye assay. Combinations were tested in MCF-7 human breast cancer cells, a HER-2/neu transfected MCF-7 cell line (MCF/18), and a tamoxifen-resistant MCF-7 cell line (MTR-3). A synergistic inhibitory effect was observed with the combination of inhibitors of EGFR-HER-2/neu (lapatinib or GW2974) and Bcl-2 (GX15-070 or HA14-1) on the growth of the MCF-7, MCF/18, and MTR-3 human breast cancer cell lines. This study suggests that simultaneously blocking the ErbB family of receptor tyrosine kinases and Bcl-2 family of proteins may be a benefit to breast cancer patients.

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Introduction

EGFR (epidermal growth factor receptor, ErbB1) and HER-2/neu (ErbB2) are members of the ErbB family of receptor kinases (1-3). When EGFR ligands, e.g. TGF-α, bind to the EGF receptor, heterodimerization with HER-2/neu activates these tyrosine kinases to trigger a cascade of events which leads to cell proliferation and survival (1). The ligands are often produced by the same tumors that overexpress the EGFR and/or HER-2/neu causing activation of survival pathways via autocrine loops (1).

The occurrence of EGFR and HER-2/neu overexpression in a variety of human tumors, and the correlation of overexpression with poor prognosis and decreased survival (1-10), suggests that these receptors would be likely targets in cancer therapy. Use of inhibitors of these receptors as monotherapies, e.g. trastuzumab, gefitinib, and erlotinib, has led to advances in clinical treatment; however, many patients do not respond to these therapies or instead develop resistance. Several mechanisms have been advanced to explain resistance to these therapies. Although HER-2/neu has no known ligand, in addition to being overexpressed, it is often constitutively activated in many tumors (1,11,12). Ligand-independent activation of the EGFR has also been suggested as a mechanism for resistance to EGFR antibody therapy (1). uPA (urokinase plasminogen activator), a regulator of extracellular matrix degradation and tissue remodeling, has been implicated in this ligand-independent activation. Cetuximab, an EGFR monoclonal antibody, is unable to inhibit this pathway (13) limiting its inhibitory effect.

Lapatinib is a potent reversible inhibitor of both EGFR and HER-2/neu tyrosine kinases (14). It is relatively specific for these kinases and results in cytostatic or cytotoxic effects, depending on tumor cell type (14,15). Lapatinib exposure causes decreased phosphorylation of EGFR and HER-2/neu *in vitro* and *in vivo* [(14,15); Burris H, *et al*, Breast Cancer Res Treat 82 (Suppl): S18, abs. 39, 2003]. It also causes decreased phosphorylation of MAPK-ERK1/2 and AKT and decreased expression of cyclin D, downstream effectors of cell proliferation and survival pathways (14,15).

Synergistic effects of lapatinib with trastuzumab have been observed *in vitro* in the growth of HER-2/neu overexpressing breast cancer cells (Konecny GE, *et al*, Proc Am Assoc Cancer Res, abs. 4974, 2002) and lapatinib can overcome trastuzumab

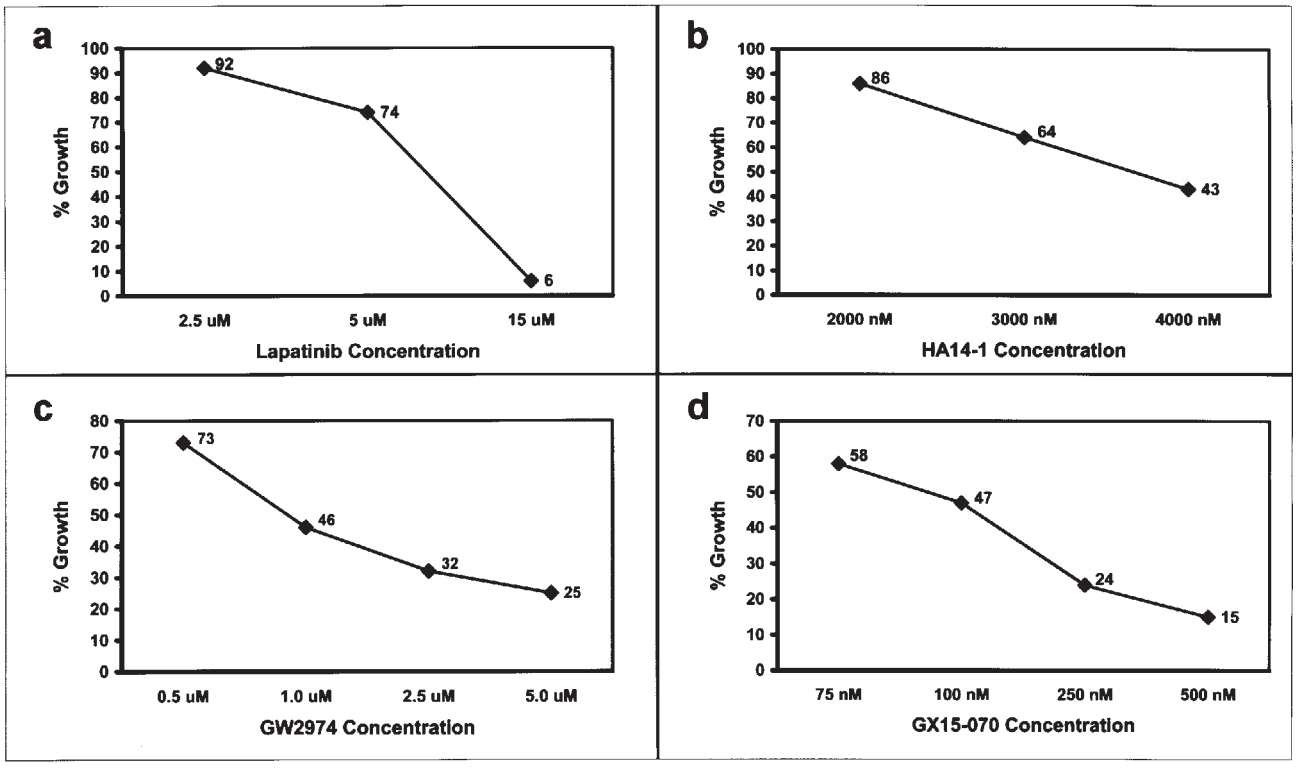

Figure 1. Percentage of growth in MCF-7 human breast cancer cells after exposure to (a) lapatinib, (b) HA14-1 Bcl-2 inhibitor, (c) GW2974 commercial lapatinib-like compound, or (d) GX15-070 pan Bcl-2 inhibitor. Cell number determined by the MTT tetrazolium dye assay after treatment with the solvent of each agent (DMSO) was used as the control.

resistance in breast cancer cells [Konecny G, *et al*, Breast Cancer Res Treat 82 (Suppl): S171, abs. 1010, 2003]. Lapatinib in combination with tamoxifen effectively inhibits the growth of tamoxifen-resistant ErbB2 overexpressing MCF-7 mammary tumor-xenografts (16).

Lapatinib is an oral agent that achieves peak serum concentration at 3-4 h with an effective half-life of 24 h (17). Phase I clinical trials have shown that the most common adverse effects are grade-1 to -2 diarrhea, rash, and GI symptoms (De Simone PA, *et al*, Proc ASCO, abs. 375, 2002; Adams VR, *et al*, Proc ASCO, abs. 374, 2002; Burris HA, *et al*, Proc ASCO, abs. 994, 2003). A phase Ib study of 66 heavily pretreated advanced cancer patients resulted in 3 partial responses and 6 stabilizations among the 20 breast cancer patients, the majority of which failed trastuzumab therapy.

Lapatinib has the advantage of having dual targets as well as being a small molecule inhibitor. Unlike single target inhibitors, it is potent in inhibiting ErbB1/ErbB2 heterodimers. It also may inactivate ErbB2/ErbB3 heterodimers which are activators of the PI3K-AKT pathway (18). Compared to monoclonal antibodies (moAb) which target epitopes on the extracellular domain (ECD), small molecules exert effects intracellularly. MoAbs also have limited effects in the presence of mutated and/or truncated forms of the receptors. In addition, constitutively activated receptors cannot be affected by moAbs.

The anti-apoptotic protein, Bcl-2, is found in a number of human tumors (19-21). The presence of Bcl-2 facilitates the protection of tumor cells from apoptosis and blocking Bcl-2 can either induce apoptosis in cancer cells or sensitize them to other therapies. Bcl-2 antisense oligonucleotides have been shown to induce apoptosis in cells of myeloid leukemia, and lung, breast and colorectal carcinomas and have been shown to sensitize breast cancer cells and myeloid leukemia cells to chemotherapeutic drugs (19,22-27). The combination of a Bcl-2 phosphorothioate antisense and radiation has been shown to be a highly potent therapy for nasopharyngeal cancer in an animal model system (28). This dual action of Bcl-2 inhibitors, inducing apoptosis and sensitizing cells to other therapies, suggests that treatment regimens using both a growth factor receptor inhibitor and a Bcl-2 inhibitor may have greater efficacy in treating human tumors. This study shows that combining an inhibitor of both ErbB1 and ErbB2 with a Bcl-2 inhibitor produces synergistic antitumor effects in several breast cancer cell lines that model hormone therapy sensitive, hormone therapy insensitive, and HER-2/neu overexpressing breast cancers.

Materials and methods

Materials. The MCF-7 human breast cancer cell line transfected with a control vector, MCF/neo, and the HER-2/neu transfected MCF-7 cell line, MCF/18, were supplied by Genentech, Inc., South San Francisco, CA. The tamoxifen-resistant MCF-7 variant (MTR-3) was developed from MCF-7 cells after long-term selection in 1 μM tamoxifen with 5% charcoal-stripped fetal bovine serum (29). Lapatinib was a gift from GlaxoSmithKline, Collegeville, PA. The GX15-070 pan Bcl-2 inhibitor was a gift from Gemin X Biotechnologies, Inc., Montreal, Canada. HA14-1, a commercially available Bcl-2 inhibitor, and GW2974 were purchased from Sigma Aldrich, St. Louis, MO.

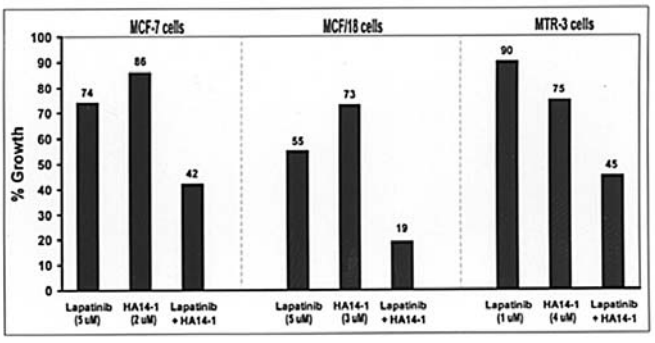

Figure 2. Percentage of growth in MCF-7 human breast cancer cells, MCF/18 HER-2/neu transfected MCF-7 cells, and a tamoxifen-resistant variant from MCF-7 cells (MTR-3) with the combination of lapatinib (GlaxoSmithKline) and HA14-1. The results are presented as medians from individual assays using combination concentrations which gave optimal growth inhibition for each cell line.

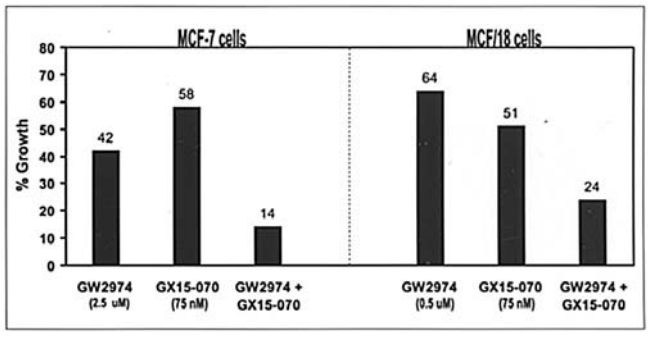

Figure 3. Percentage of growth in control vector transfected MCF-7 human breast cancer cells and HER-2/neu transfected MCF-7 cells (MCF/18) with the combination of the lapatinib-like agent (GW2974) and GX15-070 (pan Bcl-2 inhibitor: Gemin X Biotechnologies, Inc.). The results are presented as medians from individual assays using combination concentrations which gave optimal growth inhibition for each cell line.

Cell growth experiments. The MCF/neo and MCF/18 cells were maintained in a 50:50 mix of Ham's media and high glucose DMEM media supplemented with 10% fetal bovine serum, L-glutamine, and G418 in 5% CO_2 at 37˚C. MTR-3 cells were maintained in phenol red-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum. Cells were plated in 24-well plates at 20,000 cells/well and incubated overnight to allow attachment. Cells were treated with various concentrations of lapatinib or GW2974 (0.25-10 μM) and/or HA14-1 (2,000-4,000 nM) or GX15-070 (50-500 nM). As a control, cells were treated with corresponding concentrations of DMSO, the solvent for all 4 agents. The cells were incubated for an additional 3 days, and the cell number was determined using the MTT tetrazolium dye assay. Each single dose and combination was done in triplicate in each assay. Percent of control was normalized to control (untreated) cells (100% growth).

Statistical analysis. Synergism of the growth inhibitory effects was determined using Biosoft CalcuSyn software. This program calculated the combination index (CI) equation based on the equation of Chou-Talalay (30,31).

Results

In preliminary experiments, a wide dose range of each agent (lapatinib, 0.05-30 μM; GW2974, 0.25-10 μM; HA14-1, 2,000-80,000 nM; GX15-070, 50-1000 nM) was tested on the control vector transfected MCF-7 cell line (MCF/neo). From these experiments, the optimal doses of each agent were established for the combination studies. Dose-dependent growth inhibition was seen in the MCF/neo and HER-2/neu transfected MCF/18 lines with exposure to each of the four agents and in the tamoxifen-resistant variant from MCF-7 cells (MTR-3) in which only lapatinib and HA14-1 were tested. Resulting dose response curves from the treatment of the MCF-7 cells with each agent are shown in Fig. 1.

The combination of lapatinib and HA14-1 was tested on the MCF-7 and MCF/18 cells (Fig. 2). Synergistic inhibition was observed with this combination in the MCF-7 cells, as confirmed by isobologram analysis. Although an enhanced anti-proliferative effect was suggested by the graphic representation of this combination in the MCF/18 cells (Fig. 2), isobologram analysis did not confirm synergism (Table I). This combination (lapatinib and HA14-1) was also shown to have a synergistic anti-proliferative effect in the tamoxifen-resistant variant MTR-3 cells (Fig. 2, Table I).

Table I. Anti-proliferative activity of lapatinib and HA14-1 in human breast cancer cells using the multiple drug-effect equation of Chou-Talalay method of isobologram analysis.[a]

Cell line	HA14-1 concentration (nM)	Lapatinib concentration (μM)	CI
MCF-7	2,000	5	0.494
	3,000	5	0.623
	4,000	5	0.651
MCF/18	2,000	5	1.275
	3,000	5	1.300
	4,000	5	1.499
MTR3	1,000	1	1.044
	2,000	1	0.677
	4,000	1	0.350

[a]CI (combination index) <1, synergistic effect; CI=1, additive effect; CI>1, antagonistic effect. Concentrations shown are those which gave optimal inhibitory effects in each cell line.

The combination of GW2974 and the GX15-070 pan Bcl-2 inhibitor produced synergistic growth inhibition in the MCF-7 and MCF/18 cell lines (Fig. 3). Results shown are from the combination concentrations which gave optimal inhibition in each cell line (results presented as the median from all experiments). Isobologram analysis confirmed synergism in both cell lines (Table II) by resulting in a combination index (CI) <1.0.

Table II. Anti-proliferative activity of GW2974 and GX15-070 in human breast cancer cells using the multiple drug-effect equation of Chou-Talalay method of isobologram analysis.[a]

Cell line	GW2974 concentration (μM)	GX15-070 concentration (nM)	CI
MCF-7	0.50	100	1.019
	1.00	100	0.788
	2.50	100	0.594
	5.00	100	0.330
MCF/18	0.25	75	0.760
	0.50	75	0.593
	0.75	75	0.459
	1.00	75	0.320

[a]CI (combination index) <1, synergistic effect; CI=1, additive effect; CI>1, antagonistic effect. Concentrations shown are those which gave optimal inhibitory effects in each cell line.

Discussion

Dimerization of the EGFR (ErbB1) and HER-2/neu (ErbB2) receptors triggers a cascade of events resulting in cell proliferation and survival (1). Overexpression of these receptors in human tumors correlates with poor prognosis and decreased survival in cancer patients (1-10). Treatment with inhibitors of EGFR or HER-2/neu as single agents has had a significant impact on treatment paradigms, but the tumors of many patients do not respond to these agents, and most tumors that do respond eventually develop resistance.

Lapatinib, a potent reversible inhibitor of both EGFR and HER-2/neu, when combined with trastuzumab produces synergistic antitumor effects in HER-2/neu overexpressing breast cancer cells. Treatment with single-agent lapatinib as initial therapy for patients with breast cancers positive for HER-2/neu gene amplification by FISH resulted in partial responses in 35% of the patients in an interim analysis of a phase II study (Gomez HL, *et al*, Proc ASCO, abs. 3046, 2005). This inhibitor has the advantage of targeting both the EGFR and HER-2/neu receptors. It also targets the ErbB2/ErbB3 heterodimers which are activators of the PI3K/AKT pathway (18). As a small molecule inhibitor, lapatinib may have advantages over monoclonal antibodies in that it may inhibit constitutively activated receptors as well as mutated or truncated receptors.

The anti-apoptotic protein, Bcl-2, has been shown to be overexpressed in a number of human tumors (19-21). Overexpression of Bcl-2 may lead to protection of the tumor cells from apoptosis, thereby enabling their proliferation. Bcl-2 inhibitors can induce apoptosis and sensitize human cancer cells to chemotherapeutic agents (19,22-27) and radiation (28). GX15-070 (Gemin X Biotechnologies) is designed to induce apoptosis by inhibiting the Bcl-2 family of proteins. It is the first drug of its kind to be tested in humans in a phase I trial for solid tumors. Clinical breast cancer studies have shown an inverse relationship between levels of HER-2/neu and Bcl-2 expression (32-34). Mechanistic studies, however, have shown that HER-2/neu gene transfer into MCF-7 breast cancer cells strongly up-regulates Bcl-2 and Bcl-X_L expression (32,35,36) and that antisense-mediated HER-2/ neu down-regulation also suppresses Bcl-2 expression (32,37). Milella has reported that exposure of breast cancer cells to trastuzumab (the humanized monoclonal antibody that binds to the ectodomain of the HER-2/neu protein) attenuates Bcl-2/Bcl-X_L expression and increases susceptibility to apoptosis induction (32). These findings would suggest that simultaneously targeting Bcl-2 and the EGFR and HER-2/neu receptors would be a more effective approach to breast cancer therapy.

Given that multiple pathways are often implicated in cancer cell growth and that there is crosstalk between the components of these pathways, single-target inhibition may be insufficient to induce durable antitumor effects. Targeted agents can be combined to inhibit multiple pathways and/or components within the same pathway, e.g. receptor tyrosine kinases and downstream proteins. These combinations could potentially be more efficient in affecting various signaling pathways as well as pathway crosstalk involved in tumor growth or survival.

This study has demonstrated synergy with the combination of dual inhibitors of EGFR and HER-2/neu (using either lapatinib or GW2974) and inhibitors of Bcl-2 (using either GX15-070 or HA14-1) in MCF-7 human breast cancer cells and in a tamoxifen-resistant variant (MTR-3). Although synergism was not statistically evident in HER-2/neu transfected MCF-7 cells with HA14-1, an enhanced effect was suggested (Fig. 2).

This data confirms clinical observations that treatment with inhibitors of the ErbB family of receptors (Herceptin, Iressa, Erbitux, Tarceva) as single agents does not produce significant response rates. Our data would suggest that addition of inhibitors of components in pathways downstream from the ErbB receptors may be necessary due to the possibility of constitutively activated pathways in human tumors. Bcl-2 is a downstream component that may be an even more attractive target because of its role as an anti-apoptotic protein. One would assume that inhibiting its effects would make the tumor more accessable to the effects of other therapies. The results from this study provide affirmation that addition of a Bcl-2 inhibitor to an inhibitor of ErbB receptors, whether through its ability to work more effectively downstream of the receptors or by enhancing apoptosis and possibly reducing resistance to other therapies, greatly enhances the effects of ErbB inhibitors. This study warrants an evaluation of the combination of a dual EGFR/HER-2/neu inhibitor and a Bcl-2 inhibitor in clinical trials for the treatment of patients with metastatic breast cancer.

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