Identification of potentially useful combinations of epidermal growth factor receptor tyrosine kinase antagonists with conventional cytotoxic agents using median effect analysis

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Targeted therapy for breast carcinoma has achieved a major advance with the use of trastuzumab in Her2/*neu*-positive tumors. The epidermal growth factor receptor superfamily thus becomes an attractive target for therapeutic agents. As the epidermal growth factor receptor tyrosine kinase family has a conformational binding site, which allows small molecules to interfere with its function, we have explored the effects of a dual kinase (epidermal growth factor receptor-1 and epidermal growth factor receptor-2) inhibitor (GW282974X) with a variety of cytotoxic agents looking for synergistic effects *in vitro*. Using a median effect model in four breast cancer cell lines *in vitro*, cytotoxic agents commonly used in treatment of human malignant disease were combined with trastuzumab or one of two epidermal growth factor receptor tyrosine kinase inhibitors in a 72-h culture and then analyzed for cytotoxic effect by 3-[26]-2,5-diphenyl-tetrazolium bromide assay. Combination index values within one standard deviation of unity were considered additive, less than unity as synergistic and more than unity as antagonistic. Synergistic results were confirmed by curve shift analysis and by an enzyme-linked immunosorbent assay measuring apoptosis by cytoplasmic histone-associated DNA fragments. Quantitative real-time polymerase chain reaction analysis was used to measure the expression of three of the critical enzymes in 5′-deoxy-5-fluorouridine metabolism and activity: thymidine phosphorylase, dihydropyrimidine dehydrogenase and thymidine synthase. 5′-Deoxy-5-fluorouridine with GW282974X demonstrated global synergy, both in high and low expressing epidermal growth factor receptor breast cancer cell lines. These results were confirmed by apoptosis assay and cell counts. RNA quantification following treatment with the dual kinase inhibitor suggested reduction in thymidine synthase levels to be a potential mechanism of synergy. The triplet of trastuzumab, GW282974X and 5′-deoxy-5-fluorouridine, and the triplet of GW282974X, epirubicin and 5′-deoxy-5-fluorouridine were highly synergistic in low expression cells (MCF7/wt) and high expression cells (MCF7/adr). These experiments suggest further studies of the dual kinase inhibitor with selected cytotoxics such as 5′-deoxy-5-fluorouridine are warranted. *Anti-Cancer Drugs* 17:921–928 © 2006 Lippincott Williams & Wilkins.

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Introduction

Rapid advances in molecular biology have now identified numerous cell surface receptors and/or signal transduction pathways associated with enhanced growth. The epidermal growth factor (EGF) super family is one such control mechanism with four discrete receptors that can hetereodimerize and have tyrosine kinase binding domains that make design of small molecules to inhibit function feasible [1]. The super family is also of interest as it is involved in a variety of effects on cell function, and is intimately involved in cross-talk with convergent and divergent pathways involved in cell survival [2–7]. As a result, this super family has been under investigation as a potential target for many years [8]. A number of studies have demonstrated these potential targets to be over-expressed in numerous tumors, thus leading to the development of both monoclonal antibodies and small molecules to inhibit its function [9,10] with the hope that the target plays a vital role in human malignancy. As a single agent, however, inhibition of members of this

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receptor family has demonstrated minimal antitumor activity in humans [11] with the exception of trastuzumab interference of Her2/*neu* function in breast cancer [12], and cetuximab interference of EGF receptor (EGFR) function in colorectal and head and neck tumors [13,14].

Of particular interest in breast cancer, the EGFR super family has been demonstrated to be overexpressed in breast cancers characterized by endocrine resistance, all-*trans* retinoic acid resistance and resistance to cytotoxic agents [15,16]. All these effects are of clinical concern as the majority of patients with metastatic disease eventually succumb to their illness. Using in-vitro and in-vivo murine model systems, the combination of a classical cytotoxic agent with an inhibitor of the surface receptor has been demonstrated to enhance the antitumor effects of the former [11,17–20], possibly as a direct consequence of inhibition of the EGFR signal transduction pathway. The dual kinase lapatinib (GW572016) has also demonstrated antiproliferative effects in in-vitro and in-vivo murine systems of breast cancer [21], and may therefore in a similar manner enhance the effects of cytotoxic agents. We have therefore explored an analog of lapatinib, a dual kinase (EGFR-1 and EGFR-2) inhibitor (GW282974X) [22,23], as well as other small-molecule inhibitors with a variety of cytotoxic agents and with trastuzumab looking for synergistic effects *in vitro*. In addition, we have examined the perturbation of thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD) and thymidine synthase (TS) expression in our cell lines when they are incubated in the presence of EGFR inhibitors.

Material and methods

Reagents

Drugs, tissue culture techniques and median effect analysis were evaluated as previously described [24–26]. Docetaxel, reagent grade, was a gift of Aventis Pharmaceuticals (Bridgewater, New Jersey, USA). GW282974X was a gift of Glaxo SmithKline (Philadelphia, Pennsylvania, USA). The following agents were obtained from Sigma-Aldrich (St Louis, Missouri, USA): AG 1478, 5′-deoxy-5-fluorouridine (5′-DFUR) and 5-fluorouracil (5-FU). Epirubicin was purchased from Calbiochem (San Diego, California, USA). Gemcitabine and trastuzumab were obtained from commercial stock. AG 1478 is a tryphostin with high specificity for EGFR-1 [27], whereas GW282974X is a dual EGFR-1 and Her2/*neu* kinase inhibitor similar to lapatinib [28]. Lapatinib is also known to be active against truncated forms of Her2/*neu* [29]. Monoclonal antibodies to EGFR and Her2/*neu* were obtained from Sigma. For Western blots, an 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel was cast using the Mini-protein System from BioRad (Hercules, California, USA).

Cellular studies

Four cell lines (MCF7/wt, BT474, SK-BR3 and A-431) were obtained from the American Type Culture Collection (Rockville, Maryland, USA). A-431 is a squamous cell cancer with high expression of EGFR and thus used as a control [30,31]. MCF7/adr, a multiply resistant cell line, was a gift from Dr Kenneth Cowan, formerly at the National Cancer Institute (Bethesda, Maryland, USA).

The phenotypes of the cells studied have previously been characterized [32–38]. Recent studies, however, have noted marked variation in MCF7 cell lines as assayed by comparative genomic hybridization and cDNA expression [39]. Therefore, we reconfirmed the tissue culture cell phenotypes by immunohistochemistry and Western immunoblotting using standard techniques [36]. In brief, cells were grown to confluence in T-150 flasks and treated with 0.05% trypsin–0.53 mol/l ethylene diaminetetraacetic acid for 10 min at 37°C. Cells were pelleted, washed twice in phosphate-buffered saline (PBS) and resuspended in 0.5 ml PBS.

Isolation and quantification of receptors

Cell proteins were obtained from trypsinized cells and then denatured for 3 min at 100°C in a solution of 50 mmol/l Tris, pH 6.8, 100 mmol/l dithiothreitol, 0.1% bromophenol blue, 10% glycerol and 2% sodium dodecyl sulfate. Twenty microliters of treated solution were loaded onto the gel and run for 1 h at 100 V. The gels were subsequently stained with a 0.125% Coomassie Brilliant Blue solution, blotted onto a nitrocellulose membrane using the BioRad Mini-blot Transfer System, washed in PBS and then blocked with 5% nonfat dry milk in PBS–Tween for 1 h. The membranes were then washed, exposed to secondary goat antimouse horseradish peroxidase conjugate and detected using Opti-4CN Detection kit (BioRad). The expression of protein was scored on a semiquantitative scale using a nonspecific antibody as negative and the result with the squamous cell line A-431 as 3 + . Immunostaining for the estrogen receptor and progesterone receptor was performed by standard methods (NexES IHC Staining Module) after exposing the cells to 2 mmol/l citric acid with 9 mmol/l trisodium citrate pH6.0 vapors in a boiling water bath to permeabilize the membrane [40]. DAKO liquid DAB + substrate chromogen solution (Carpinteria, California, USA) was used for end-product staining using the manufacturer's directions.

Assessment of cytotoxicity produced by therapeutic agents

Determination of cytotoxicity of the various drugs and median effect analysis was performed by previous methods [24,26]. In brief, the cells were grown to confluence in T 150 tissue culture flasks (Corning Glass Works, Corning, New York, USA) using RPMI 1640 (Invitrogen, Carlsbad, California, USA) with 5% CO_2 and

10% heat-inactivated fetal calf serum. All cultures contained penicillin (100 μg/ml), streptomycin (0.25 μg/ml) and glutamine to a final concentration of 2 mmol/l. All cell lines were repeatedly tested for mycoplasma and had viabilities by Trypan blue exclusion greater than 95%. Harvested cells were aliquoted into 96-well dishes (Falcon 3072) at concentrations of 5000–8000 cells per well. The cells were then cultured for 24 h, cytotoxic agents or solvent controls were introduced for a 72-h incubation and the cell growth was evaluated by a 3-[26]-2,5-diphenyl-tetrazolium bromide (MTT) assay [41] using a BioRad 3550 Micro plate Reader (BioRad) [42]. IC_{50} (the dose of drug needed to cause loss of cell viability as measured by MTT in 50% of the cells) concentrations were determined by the EZ-ED50 Program (Perrella Scientific, Conyers, Georgia, USA). All reported values are the means of at least three experiments with each study having four wells per dose level. In addition, the cytotoxicity of selected combinations was measured serially over 72 h using the MTT assay at concentrations that are known to be achieved clinically.

Assessment of drug interaction

Median effect analysis, a measure of synergism or antagonism based upon the Hill equation, was determined by the method of Chou, using their computer program to determine the combination index, which reflects synergy when less than 1, additive effects when equal to 1 and antagonism when greater than 1. The advantage of this method is that up to three agents can be evaluated for synergistic effects at the same time. As the model is a linear approximation of a higher-order equation, we have previously defined additive effects to be within one standard deviation of unity, synergistic effects less than one standard deviation of unity and antagonistic effects greater than one standard deviation of unity [25,26]. Statistical differences were confirmed using the curve-shift analysis of Zhao *et al.* [43]. Curve-shift analysis was performed with the program ACT Analysis (Optimum Therapeutics LLC, Columbus, Ohio, USA) using nonlinear regression of the concentration–effect data. The results were then normalized to IC_{50} equivalents. A shift of the survival vs. IC_{50} equivalent curve to the left is an indication of synergy [43].

A total of 10 fixed drug ratios above and below the IC_{50} with a range of $0.03125N$ to $8N$, where N is a value near the IC_{50} of an individual drug, were explored by incubating the drug combinations with cells for 72 h and then determining the degree of cytotoxicity by the MTT assay. F_a is defined as the fraction of cells affected, and a plot of log dose vs. log $(F_a/1-F_a)$ gives parallel slopes if no biologic interaction is present (mutually exclusive) or converge if there is an interaction between the drugs (mutually nonexclusive), thus suggesting the appropriate model to determine the combination index.

Assessment of apoptotic effect

The commercially available Cell Death Detection ELISA$_{plus}$ kit (Roche Applied Science, Penzberg, Germany) was used to detect DNA fragmentation by an enzyme-linked immunosorbent assay as previously described [29]. This assay exploits the amount of cytoplasmic histone-associated DNA fragments produced upon cell death. The kit uses mouse monoclonal antibodies against the histone proteins. In brief cells, after the appropriate time of drug(s) exposure and after centrifugation, were lysed in 96-well plates. Twenty microliters of the supernatant were transferred to a streptavidin-coated plate that was supplied with the kit. This supernatant was incubated for 2 h in the presence of the immune reagent containing the antibodies against the histone proteins and DNA fragments. The complex was then simultaneously conjugated to form an immunocomplex on the plate, which was then subsequently read for optical density at 405 nm with a reference wavelength at 490 nm. Samples were measured in triplicate and a positive control was provided with the kit. The result is described as an enrichment factor, which was thus a relative indicator of the number of cells undergoing apoptosis as calculated by the following formula:

$$\frac{\text{mU of dying/dead cells}}{\text{mU of untreated cells}}$$

where mU is the absorbance (405–490 nm), and reflects the amount of histones and DNA fragments released into the cytoplasm from the apoptotic cells. Statistical differences were determined by *t*-test paired analysis.

Cell counts were performed using Trypan blue exclusion. Cells were exposed to drug(s) for the indicated time period, trypsinized, pelleted via centrifugation and washed twice in PBS. Cells that did not take up the dye were counted in triplicate on a hemocytometer.

Assessment of gene expression

Tissue culture cells from MCF7/wt (estrogen receptor-positive) and SK-BR-3 (estrogen receptor-negative) cell lines were grown in exponential phase in the presence of IC_{10} (drug concentrations that cause 10% cytotoxicity over 72 h) concentrations of AG 1478 and GW282974X, and samples were removed at 0, 24, 48 and 72 h. RNA was extracted from these samples according to the protocols and reagents in the RNeasy Mini kit (Qiagen, Hilden, Germany). DPD, TP and TS RNA levels were quantified by real-time reverse transcriptase-polymerase chain reaction (QRT-PCR) using the 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA) according to previously validated protocols [44]. Briefly, 40 ng RNA underwent one-step RT-PCR in a 20-μl reaction containing 1 × TaqMan One Step RT-PCR Master Mix, 1 × Multiscribe/RNAse Inhibitor Mix (Applied Biosystems), 500 μmol/l of forward primer, 500 μmol/l of reverse primer and 250 μmol/l of fluorescent

probe. Primers and probe sequences for DPD, TP, TS and β-actin have been described previously [44]. The reaction was incubated at 48°C for 30 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a single fluorescent measurement taken each cycle at 60°C. RNA levels were assessed by relative quantification using the $\Delta\Delta C_t$ method validated previously [44,45] and included in the instrument software. For the algorithm, β-actin was the reference gene used to normalize sample loading and the calibrator samples, with levels equal to 1, were respective untreated cells.

Results

Quantification of receptor expression in cell lines

Table 1 demonstrates the phenotypic results in semi-quantitative terms (0 to 3 +) that indicate that our cell lines had the same characteristics as previously described in the literature [46]. Using Western blot techniques, the expression of EGFR is below the limits of detection for MCF7/wt, BT474 and SK-BR-3, although known to be present with more sensitive techniques [47]. In the case of Her2/*neu*, the presence of this oncoprotein in MCF7/wt was not detectable and was present in the other cell lines. The epidermoid cell line A 431 serves as a well-known positive control.

Cytotoxicity of therapeutic agents

Table 2 demonstrates the concentrations of single agents needed to cause cytotoxic effects as measured by the MTT assay in a 72-h incubation. The targeted agents, AG 1478 and GW282974X, demonstrate cytotoxic effects *in vitro* with MCF7/adr most sensitive to AG 1478 (IC_{50} = 5.5 μmol/l), whereas SK-BR-3 was most sensitive to the dual kinase inhibitor GW282974X (IC_{50} = 0.6 μmol/l). The sensitivities to classical cytotoxic agents among the various cell lines during this 72-h incubation also varied as much as a log concentration. Trastuzumab was cytotoxic in all our cell lines *in vitro* under the conditions described.

Interaction of therapeutic agents–evidence for synergy

To further explore the potential interaction of an EGFR antagonist with more conventional agents, median effect analysis was performed on the four breast cancer cell lines combining either AG 1478, GW282974X or trastuzumab with five conventional cytotoxic agents in a 72-h incubation assay (Table 3). Values less than unity indicate synergy of cytotoxic effect. Docetaxel and 5′DFUR demonstrated additive to synergistic effects with AG 1478 in three of the four cell lines. 5′DFUR demonstrated global synergism with GW282974X and with trastuzumab additive to synergistic effects in three of the four cell lines. These results were confirmed by curve-shift analysis, a nonlinear regression of the concentration–effect data, which are then normalized to IC_{50} equivalents. A representative curve shift analysis confirming these synergistic findings is shown in Fig. 1. Single-agent dose–response curves are compared with the combination over the entire dose–response curve, which shift to the left of the combination indicating synergy.

Table 1 Phenotype patterns of the breast cancer cell lines compared with the epidermoid carcinoma cell line A 431 as a control

Cell line	Estrogen receptor	Progesterone receptor	EGFR	Her2/*neu*
BT474	+	+ +	–	+ + +
MCF7/wt	+	+	–	–
MCF7/adr	–	–	+ +	+ +
SK-BR-3	–	–	–	+ + + +
A 431	ND	ND	+ + + +	+ + +

+ Indicates presence of the protein by either immunohistochemical staining or by Western blot; – indicates not detected; ND indicates not done.
EGFR, epidermal growth factor receptor.

Table 2 IC_{50} of single agents incubated for 72 h with the defined cell lines and measured by MTT assay

Drug	BT474	MCF7/wt	MCF7/adr	SK-BR-3
AG 1478	32.3 ± 5.7	203.3 ± 37.9	5.5 ± 3.2	6.9 ± 2.9
Docetaxel	5.7 ± 1.6	113.5 ± 33.6	2.9 ± 0.5	80.0 ± 3.6
Epirubicin	0.4 ± 0.1	0.2 ± 0.0	7.0 ± 2.1	0.5 ± 0.2
5′DFUR	22.9 ± 4.2	6.0 ± 0.6	6.6 ± 2.5	6.4 ± 1.7
5-FU	17.0 ± 2.3	4.1 ± 0.6	6.0 ± 0.8	4.3 ± 1.1
Gemcitabine	7.2 ± 1.7	2.3 ± 0.5	2.9 ± 1.3	1.9 ± 0.1
GW282974X	4.8 ± 1.7	7.6 ± 1.8	6.8 ± 1.5	0.6 ± 0.1
Trastuzumab[a]	3.1 ± 0.9	4.7 ± 1.6	1.2 ± 0.3	7.0 ± 1.3

All values are in μmol/l as the mean ± standard deviation of two to eight experiments with four wells per point.
MTT assay, 3-[26]-2,5-diphenyl-tetrazolium bromide assay; 5′-deoxy-5-Fluorouridine; 5′DFUR, 5-FU, 5-fluorouracil.
[a]Trastuzumab is in μg/ml.

As doublets and triplets of agents are commonly used in the clinic, we also explored several potentially useful combinations of EGFR antagonists (Table 4). The triplets of (1) GW282974X, epirubicin and 5′DFUR; (2) GW282974X, AG 1478 and 5′DFUR; and (3) GW282974X, trastuzumab and 5′DFUR all demonstrated synergy in a low expressing EGFR/Her2/*neu* cell line (MCF7) and a high expression cell line (MCF7/adr).

Determination that the interaction causes apoptosis

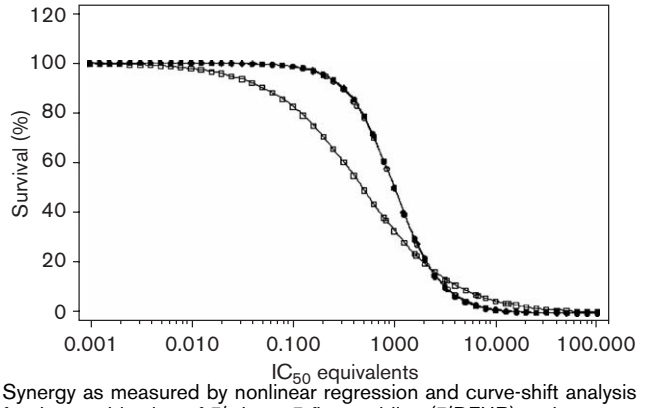
The combination of 5′DFUR and GW282974X was incubated at the F_{a50} (50% cytotoxic effect) for 72 h with cells analyzed for apoptosis by the enzyme-linked immunosorbent assay and for total cell count of viable cells by Trypan blue exclusion. Differences were determined by standard deviations and paired *t*-tests. Both the EGFR-expressing MCF7/adr cell line and the low expressing SK-BR-3 demonstrated enhanced apoptosis in the presence of 5′DFUR and GW282974X (Figs 2 and 3), which were confirmed by serial cell counts (data not shown). These results were time dependent and required at least a 48-h exposure of drugs (Fig. 3).

Table 3 Doublet combinations of agents incubated for 72 h and analyzed by median effect analysis

Drug combination		Cell line			
		BT 474	MCF7/wt	MCF7/adr	SK-BR-3
AG 1478	docetaxel	1.0 + 0.2	0.8 + 0.0	1.1 + 0.3	1.3 + 0.1
AG 1478	epirubicin	2.1 + 0.2	2.9 + 0.2	0.1 + 0.1	0.2 + 0.1
AG 1478	5′DFUR	0.6 + 0.0	1.5 + 0.1	0.2 + 0.0	0.2 + 0.1
AG 1478	5-FU	2.2 + 0.2	1.1 + 0.2	0.3 + 0.1	2.3 + 0.6
AG 1478	gemcitabine	1.5 + 0.2	1.2 + 0.2	0.6 + 0.1	0.4 + 0.1
GW282974X	docetaxel	3.6 + 0.3	2.5 + 0.3	0.5 + 0.1	2.1 + 0.3
GW282974X	epirubicin	1.1 + 0.1	1.7 + 0.3	0.8 + 0.1	0.5 + 0.1
GW282974X	5′DFUR	0.6 + 0.1	0.7 + 0.0	0.2 + 0.0	0.7 + 0.0
GW282974X	5-FU	3.4 + 0.7	0.6 + 0.1	0.3 + 0.0	0.6 + 0.1
GW282974X	trastuzumab	4.6 + 0.5	1.0 + 0.1	1.2 + 0.2	1.8 + 0.3
GW282974X	gemcitabine	1.9 + 0.3	1.5 + 0.2	0.6 + 0.1	3.1 + 0.4
Trastuzumab	docetaxel	1.0 + 0.0	0.4 + 0.0	3.7 + 0.7	0.4 + 0.0
Trastuzumab	epirubicin	1.3 + 0.1	1.1 + 0.3	0.1 + 0.0	1.1 + 0.1
Trastuzumab	5′DFUR	1.6 + 0.1	0.8 + 0.0	0.1 + 0.0	1.0 + 0.1
Trastuzumab	5-FU	1.8 + 0.3	1.2 + 0.1	0.9 + 0.2	1.3 + 0.2
Trastuzumab	gemcitabine	1.6 + 0.3	2.5 + 0.2	0.2 + 0.0	1.6 + 0.2

Values are CI_{50} shown at F_{a50} (50% cytotoxic effect) ± standard deviation with numbers less than unity as synergistic.
5′DFUR, 5′-deoxy-5-Fluorouridine; 5-FU, 5-fluorouracil.

Fig. 1



Synergy as measured by nonlinear regression and curve-shift analysis for the combination of 5′-deoxy-5-fluorouridine (5′DFUR) and GW282974X in SK-BR-3 cells. Effect is normalized. The open squares are the combination, closed squares GW282974X and open circles 5′DFUR.

Table 4 Triplet combinations in a sensitive and a multiply resistant cell line

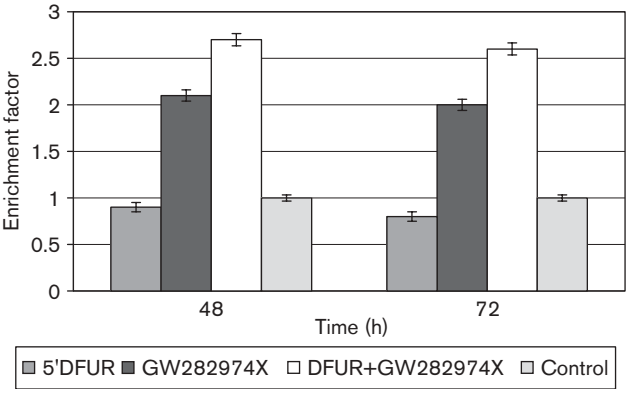
Drug combination			Cell line	
			MCF7/wt	MCF7/adr
AG 1478	docetaxel	epirubicin	0.6 ± 0.0	1.6 ± 0.3
AG 1478	docetaxel	5-FU	1.4 ± 0.2	0.4 ± 0.0
AG 1478	epirubicin	5-FU	0.5 ± 0.0	5.3 ± 0.4
GW282974X	epirubicin	5′DFUR	0.6 ± 0.1	0.4 ± 0.1
GW282974X	ag 1478	5′DFUR	0.9 ± 0.2	0.7 ± 0.1
GW282974X	trastuzumab	5′DFUR	0.8 ± 0.1	0.3 ± 0.0

Values shown are the CI at F_{a50} ± standard deviation.
5′DFUR, 5′-deoxy-5-Fluorouridine; 5-FU, 5-fluorouracil.

Molecular basis of cytotoxic synergism

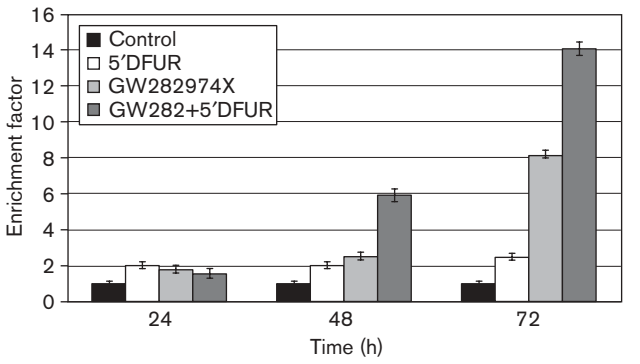
The basis of cytotoxic synergism in the low expression Her2/*neu* MCF7/wt cell line and the high expression SK-BR3 cell line was evaluated by incubating these cells with either AG 1478 or GW282974X and assessing changes in TP, DPD and TS expression at 24, 48 and 72 h. To be consistent with synergy, increases in the TP/DPD ratio and reduction in TS levels from treatment would be expected. Preclinical studies have shown associations between increased capecitabine (and its metabolite, 5′DFUR) response and high TP/DPD levels [48], consistent with the roles of TP and DPD as major enzymes in the activation of 5′DFUR to 5-FU, and clearance of 5-FU, respectively [49,50]. Other studies have shown associations between improved 5-FU response and low TS levels, consistent with TS inhibition being a major mechanism of 5-FU cytotoxicity [51].

Fig. 2



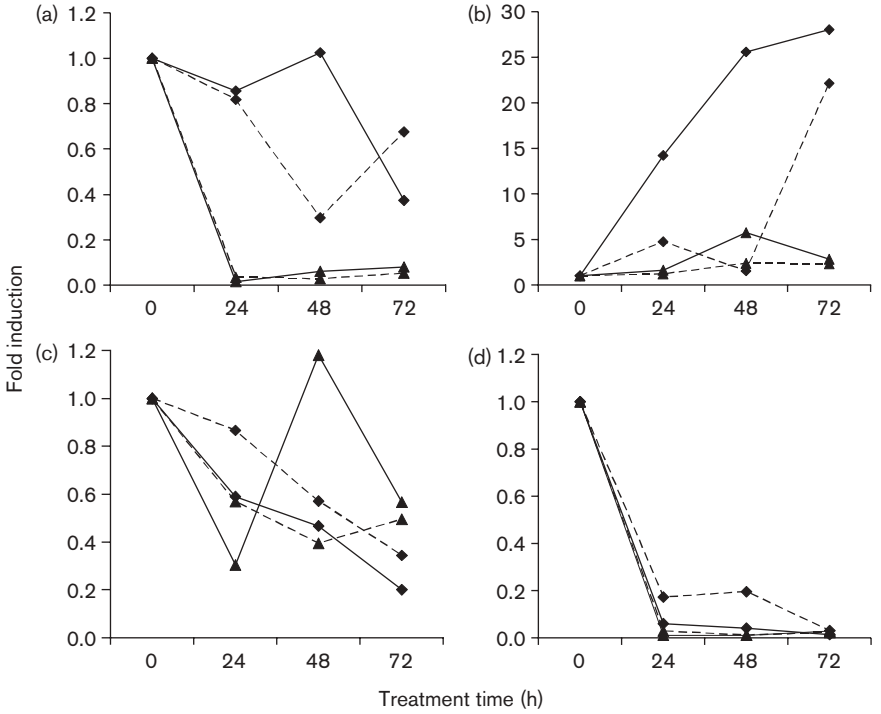
Apoptosis in the epidermal growth factor receptor-expressing MCF7/adr cell line is enhanced by the combination of the 5′-deoxy-5-fluorouridine (5′DFUR) and GW282974X. Values are ± standard deviation. All differences between combination and single agent are significant ($P < 0.01$).

Fig. 3



Apoptosis generated in the cell line SK-BR-3 with 5′-deoxy-5-fluorouridine (5′DFUR), GW282974X and the combination. All values are ± standard deviation. Results are significant by paired *t*-test for combination vs. single agent after prolonged exposure to the drug. $P < 0.01$.

Fig. 4



Levels of thymidine phosphorylase (TP) (a), dihydropyrimidine dehydrogenase (DPD) (b), thymidine synthase (c) RNA and TP/DPD (d) in MCF-7/wt (diamond) and SK-BR3 (triangle) treated with AG 1478 (solid lines) and GW282974X (dashed lines) for 24, 48 and 72 h. All levels are shown relative to those in untreated cells (time = 0, level = 1).

TP/DPD levels, however, markedly decreased in both MCF7 and SK-BR3 cell lines following treatment with either AG 1478 or GW282974X (Fig. 4). In SK-BR3 cells, the TP/DPD reduction appeared primarily to result from a sharp reduction in TP levels (Fig. 4a), whereas in MCF7 cells, a marked DPD induction appeared the major component (Fig. 4b). The major difference with respect to the agents was the more rapid induction of DPD in MCF7 cells by AG 1478 compared with GW282974X (Fig. 4b). TS levels, however, did reduce in both cell types using both agents, with the greatest reduction been approximately five-fold in SK-BR3 cells after 72 h treatment with GW282974X (Fig. 4c).

Discussion

Cell lines have been used as model systems to reflect human malignant disease as they have genotypic and phenotypic changes that may reflect cancer cells *in vivo* [46]. Other workers have, however, noted that cells may undergo further genotypic and phenotypic changes when cultured, and thus represent a model system that may only in part mimic human malignancy [39]. Therefore, we phenotyped our cells to ensure that the expression of Her2/*neu* and EGFR varied across cell lines. In addition, it should be emphasized that these models do not represent the heterogeneity both in growth and in phenotypic expression of human malignancy, do not take into account the three-dimensional structure of the tumor, do not account for tumor–host interactions, and furthermore provide no measure of therapeutic index.

In an attempt to make our findings more relevant to the clinical condition, we have analyzed a variety of breast cancer cell lines with differing phenotypic expressions. With this short-term in-vitro assay, the dual kinase inhibitor GW282974X was cytotoxic to cells expressing high levels of either Her2/*neu* and/or EGFR, and in cells expressing low levels of these surface receptors. These results thus parallel the findings of Konecny *et al.* [21] with lapatinib in which antiproliferative effects were noted in all their breast cancer cell lines. In contrast, the more specific EGFR kinase inhibitor AG 1478 demonstrated two orders of magnitude differences in the concentration needed to kill cells with low levels of receptor expression compared with cells with high levels of receptor expression. These differences suggest that the dual kinase inhibitor may act through a variety of mechanisms beside receptor inhibition. Trastuzumab also demonstrated cytotoxic effects in all cell lines in this model system, which may reflect enhanced sensitivity of the method. Docetaxel, which is highly active in many

cell lines [42], was not particularly cytotoxic in two of our cell lines.

Using median analysis and short-term culture, we have previously identified potentially clinically useful combinations of drugs, and in the present studies identify global synergy between the metabolite of capecitabine, 5′DFUR, and the dual EGFR kinase inhibitor, GW282974X. These results were confirmed using curve-shift analysis, which corrects for the nonlinear nature of the dose–response curve. Trastuzumab with 5-FU has previously been reported to be antagonistic in tissue culture [38], which was not confirmed using the capecitabine metabolite 5′DFUR in a xenograft model [52]. Our assay technique also suggests that the EGFR kinase inhibitor AG 1478 is antagonistic with 5-FU *in vitro* and the combination of trastuzumab with 5-FU demonstrates at least antagonistic effects in three of the four cell lines tested. Hence, the discordant in-vitro results from the in-vivo findings may in part be cell line specific and may also reflect the assay technique used to demonstrate cytotoxic effect.

We have also identified in these studies that the triplet combination of GW282974X with epirubicin and 5′DFUR and the triplet GW282974X, trastuzumab and 5′DFUR are markedly synergistic both in a tumor cell line with low expression of EGFR and Her2/*neu*, and in a cell line with high expression. Of note, the MCF7/adr cell line expresses multiple mechanisms of resistance but is sensitive to these triplets.

The mechanism of synergy was further explored with RT-PCR as Magne *et al.* [53] have identified the upregulation of TP *in vitro* by ZD1839 (Iressa) in the head and neck cell line CAL33 as one mechanism of enhancing cytotoxicity of 5′DFUR. Examining TP levels after 72-h IC_{10} incubation of AG 1478 and GW282974X in low (MCF-7) and high (SK-BR3) Her-2/*neu* expressing cell lines, however, our results have shown that induction of TP may not be a mechanism of the observed synergism in breast cancer cell lines with these agents and 5′DFUR, and in principle with other 5-FU prodrugs. Reduced DPD levels or increased TP/DPD levels, also previously associated with increased fluoropyrimidine response [48], were not observed. Rather, our results point towards reduction of TS levels following treatment with AG 1478 and GW282974X as a potential mechanism, with TS levels reduced over 72 h of treatment with the EGFR inhibitors in both cell lines. Inhibition of TS is considered a major mechanism of fluoropyrimidine cytotoxicity [51], so reduction in TS levels by the agents is consistent with the enhancement of 5′DFUR sensitivity observed. Further study of the mechanisms underlying the reduction of TS levels by the tyrosine kinase inhibitors would appear warranted.

In summary, we have identified several synergistic doublets and triplets of drugs that include 5′DFUR, the metabolite of capecitabine, which may have clinical application, as their modes of toxicity do not overlap. Dual kinase inhibition with GW282974X gave more effective synergy than the putative EGFR antagonist AG 1478 and suggests further studies may be warranted.

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