

Epidermal Growth Factor Receptor Expression Is a Candidate Target of the Synergistic Combination of Trastuzumab and Flavopiridol in Breast Cancer¹

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ABSTRACT

ErbB2 and *cyclin D1* are interacting oncogenes that are particularly important in breast cancer. We demonstrated previously synergy between two drugs that separately address each oncogene, trastuzumab and flavopiridol. Here we examine the cellular basis for this interaction. Although both drugs are thought to alter cell cycle progression, the combination of trastuzumab and flavopiridol had little effect on G₁ progression or retinoblastoma protein phosphorylation. Instead, trastuzumab-flavopiridol synergistically enhanced apoptosis. Recent data have suggested that transcription elongation mediated by Cdk9 in P-TEFb is a more sensitive flavopiridol target than Cdk4. Supporting this view, we found synergy between 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole riboside and trastuzumab, but not between inhibitors of Cdk4 and trastuzumab. Similarly, a signature set of mRNAs that included the epidermal growth factor receptor (EGFR) responded to the combination of trastuzumab-flavopiridol in a gene expression array analysis. Three lines of evidence confirmed the EGFR is a potential target of flavopiridol-trastuzumab synergy: (a) EGFR protein expression was rapidly and completely lost after combination treatment; (b) a cell line that expresses amplified levels of both *erbB2* and the EGFR was resistant to the combined drugs; and (c) treatment with epidermal growth factor prevented any therapeutic effects of flavopiridol and trastuzumab, singly or in combination. Taken together, our results suggest that synergy between flavopiridol and trastuzumab can result from enhanced apoptosis, and that combination effects on EGFR expression are involved in the interaction.

INTRODUCTION

Identification of the genetic causes of cancer carries the promise that therapies can be tailored to attack its specific molecular origins. The realization that the genetic causes of cancer fit into regulatory pathways equally suggests that gene-tailored therapies can be combined in biologically meaningful combinations. The chance to combine increasingly specific modes of therapy should yield continued improvement in efficacy and decreasing toxicity.

Treatments targeted against *erbB2* (*HER-2/neu*), the major tyrosine kinase receptor molecule implicated as a contributing cause of breast cancer, have shown remarkable progress (1). The *erbB2* gene encodes a *M_r* 185,000 transmembrane glycoprotein that is a member of the EGFR³ (or *erbB*) family of receptor tyrosine kinases (2). As the preferred heterodimerization partner among ligand-bound EGFR family members, *erbB2* mediates lateral signal transduction, resulting in

mitogenesis, apoptosis, angiogenesis, and cell differentiation. The *erbB2* gene is amplified and overexpressed in approximately 20–30% of invasive breast carcinomas, and is associated with increased metastatic potential and decreased overall survival (3). Trastuzumab (Herceptin) is a recombinant humanized monoclonal antibody directed against the extracellular domain of the *erbB2* tyrosine kinase receptor (4). Clinical studies established that trastuzumab is active against *erbB2*-overexpressing metastatic breast cancers (5). Trastuzumab is currently administered in combination with chemotherapies such as paclitaxel or docetaxel (6), which increase response rates, time to progression, and survival. However, the objective response rates to trastuzumab can be quite low (5).

A clear association between *erbB2* signaling and regulation of cyclin D1-Cdk complexes was demonstrated recently by several laboratories (7–9). Importantly, cyclin D1-deficient mice are completely resistant to *erbB2*-mediated mammary tumorigenesis (9). This genetic result strongly supports the idea that a therapy aimed at cyclin D1 should equally target *erbB2* whenever their signals are indeed connected. The kinase partners of cyclins (Cdks) are especially attractive targets of cancer therapies because abnormalities in signaling through the pRb are thought to be an essential step in the development of all cancers. The Cdk inhibitor flavopiridol targets Cdk4, the partner of cyclin D1, and has already entered clinical trials (10). The therapeutic connection between *erbB2* and cyclin D1 is highlighted even more by the demonstration that trastuzumab reduces cyclin D1 mRNA and protein levels (8) at the same time that flavopiridol reduces *erbB2* receptor levels (11). The potential for reciprocal regulation between *erbB2* and cyclin D1 led us to investigate potentially synergistic interactions between trastuzumab and flavopiridol (12).

Although the known inhibition by flavopiridol of Cdk4 was an initially plausible basis for synergy with trastuzumab, recent evidence has implicated the Cdk9 component of the transcriptional elongation factor P-TEFb as a more sensitive target of flavopiridol (13). The transition from abortive to productive transcription is positively regulated by the P-TEFb complex, which contains a novel cyclin (cyclin T) in combination with Cdk9 (14). Flavopiridol markedly inhibits transcriptional elongation *in vitro* by targeting Cdk9 at an IC₅₀ 5–10-fold lower than required for its effect on any other Cdk (15). The effects of Flavopiridol on gene transcription are more global than anticipated by any potential Cdk4 antagonism as shown by gene array experiments demonstrating parallel patterns of transcriptional inhibition by flavopiridol and the pTEF-B inhibitor DRB (16). Thus, flavopiridol can be viewed as a generalized kinase inhibitor with increasingly specific effects on a spectrum of kinases ranging from tyrosine kinases, through the Cdks, and culminating in its most specific effects on Cdk9, a regulator of transcription.

Although we initially proposed that flavopiridol and trastuzumab might interact by inhibiting cell division mediated by pRb-dependent pathways, these recent data equally suggest that the two agents might combine to alter transcription of specific genetic targets. To clarify these two possibilities we sought to define the cellular basis for the activity of combined trastuzumab-flavopiridol, identify therapeutic

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³ The abbreviations used are: EGFR, epidermal growth factor receptor; Cdk, cyclin-dependent kinase; pRb, retinoblastoma protein; P-TEFb, positive transcription elongation factor b; Ant-p16, antinapedia-p16 minimal inhibitory domain-protein transduction fusion peptide; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole riboside; C.I., combination index; EGF, epidermal growth factor; BrdUrd, bromodeoxyuridine.

classes that can interact with trastuzumab, and identify candidate transcriptional targets of trastuzumab-flavopiridol combinations. Remarkably, a known heterodimeric partner of erbB2, the EGFR itself, proved to be one candidate target for their synergistic activity.

MATERIALS AND METHODS

Materials. Flavopiridol (National Cancer Institute, Bethesda, MD) was prepared in DMSO at 1 mM and stored at -20°C . Trastuzumab (Herceptin) was prepared immediately before each use at a concentration of 1 mg/ml in sterile distilled water. The 34-mer Ant-p16 minimal inhibitory domain peptide was produced by Research Genetics Invitrogen (Huntsville, AL) as described by Fahraeus *et al.* (17), and has the following sequence: GRQIKIWFQNR-RMKWKKGEGFLD TLVVLHRAGAR. The transcription inhibitor DRB and the Cdk inhibitor roscovitine were dissolved in DMSO at 10 mM (Sigma Chemical Co., St. Louis, MO). The caspase inhibitor Z-VAD-FMK was purchased from Promega Corporation (Madison, WI). EGF (Roche Molecular Biochemicals, Mannheim, Germany) was reconstituted at $4.3\ \mu\text{M}$ in PBS. The typhostins AG99 and AG1478 were purchased from Tocris Cookson, and dissolved at 5 mM each in ethanol and DMSO, respectively.

Cell Lines. The human breast carcinoma cell lines MDA453 (MDA-MB-453), BT474, and SKBR3 were obtained from the American Type Culture Collection, and maintained in RPMI 1640 (CellGro) supplemented with 10% FCS.

Dose-Response Assays. Subconfluent MDA453, BT474, or SKBR3 cells were seeded at 8×10^4 cells/well in 96-well dishes. After 24 h cells were treated in triplicate with 2-fold serial dilutions of flavopiridol, trastuzumab, roscovitine, DRB, or multiple drugs simultaneously at the indicated fixed ratios. The doses of flavopiridol and trastuzumab were chosen because they spanned the known achievable serum levels of these drugs (12). The dose of DRB was that found to have specific effects on transcription (13). Cells were also treated with combinations of flavopiridol, trastuzumab, and typhostins at serial 2-fold dilutions. Subsequently cells were treated in triplicate with 2-fold serial dilutions of flavopiridol, trastuzumab, or both drugs simultaneously at the indicated fixed ratios in the presence or absence of $20\ \mu\text{M}$ Z-VAD-FMK or 1, 10, 25, or 50 ng/ml EGF. Cells were counted by trypan blue exclusion 48 h after drug exposure as used in previous studies of both trastuzumab and flavopiridol (10, 18), and fractional inhibition of cell proliferation was calculated by comparison to control cultures. Combination indices were obtained using the commercial software package CalcuSyn (Biosoft, Cambridge, United Kingdom; method of Chou and Talalay, Ref. 19).

Flow Cytometry. MDA453 and SKBR3 cells were treated with flavopiridol at 10, 100, or 300 nM and/or trastuzumab at 50, 100, or 200 ng/ml. After 0, 6, 24, or 48 h, BrdUrd (Amersham-Pharmacia Biotech, Buckinghamshire, England) was added directly to cultures for 1 h. Cells were then fixed in 80% ethanol for at least 1 h, incubated in anti-BrdUrd antibody (Becton Dickinson, Franklin Lakes, NJ) for 30 min, and exposed to antimouse-fluorescein secondary antibody (Vector Labs, Burlingame, CA) for 30 min. Cells were resuspended in propidium iodide ($70\ \mu\text{g}/\text{ml}$) supplemented with RNase A ($25\ \mu\text{g}/\text{ml}$). DNA content was measured using a FACScan cytometer.

Microarray Analysis. MDA453 and SKBR3 cells were treated with 10 nM flavopiridol and/or 50 ng/ml trastuzumab. Total RNA was extracted after 24 h using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). RNA was also obtained from control untreated cells. Mammalian GeneFilters Microarrays and all of the relevant reagents were purchased from and used as instructed by Research Genetics Invitrogen. Briefly, arrays were prehybridized in MicroHyb solution containing Cot-1 DNA, and hybridized with ^{33}P -labeled total RNA ($8\ \mu\text{g}$) overnight. After washing, arrays were exposed to a phosphorimaging screen overnight. Images were then analyzed using Pathways software (Research Genetics Invitrogen), and Cluster analysis performed using published software from the Stanford Genome Project (20). Microarrays were stripped in 0.5% SDS and rehybridized with RNA.

Immunoblotting. MDA453, BT474, and SKBR3 cells were treated with flavopiridol at 10, 100, or 300 nM, and/or trastuzumab at 50, 100, or 200 ng/ml in the absence or presence of 25 ng/ml EGF. Protein lysates were obtained after 1 or 24 h of drug exposure using 1% NP40 lysis buffer [$150\ \text{mM}$ NaCl, $50\ \text{mM}$ Tris (pH 8.0), and 1% NP40]. The following antibodies were used at 1:1000 dilution each: pRb COOH-terminal control antibody and phospho-pRb

(Ser795; Cell Signaling, Beverly, MA); anti-EGFR polyclonal (Oncogene Research Products, Cambridge, MA); and antiactin goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA). An anti-erbB-2 monoclonal antibody-3 (Oncogene Research Products) was used at 1:500. Secondary antibodies were chosen according to species of origin and detected using enhanced chemiluminescence (Amersham-Pharmacia Biotech).

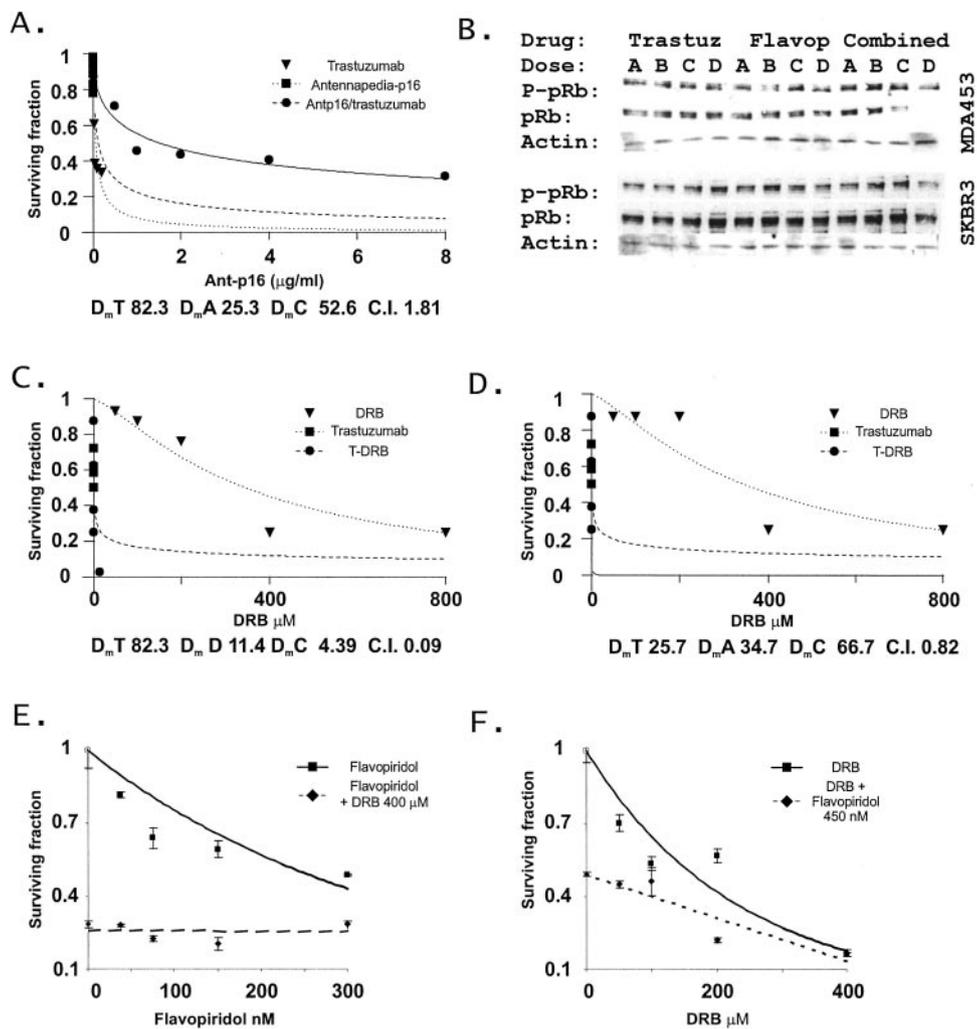
RESULTS

Trastuzumab and Flavopiridol Synergy Is Not Attributable to Cdk Inhibition. We first evaluated the potential for a pure Cdk4 inhibitor to interact synergistically with trastuzumab to determine whether this mechanism could provide a general explanation for our observed synergism between trastuzumab and flavopiridol. We used a p16-derived peptide of which the entry into cells is mediated by the Antennapedia protein transduction domain (Ant-p16) to specifically inhibit Cdk4/6 activity (17). Serial dilutions of trastuzumab and Ant-p16 were administered to MDA453 erbB2-overexpressing breast cancer cells at fixed ratios spanning their various IC_{50} s (Fig. 1A). Data were analyzed using the method of Chou and Talalay to establish drug C.I. values (19). Statistically, drug synergy, addition, and antagonism are defined by C.I. values <1.0 , $=1.0$, or >1.0 , respectively. The dose-response assay demonstrated a C.I. value of 1.81 for the combination of Ant-p16 and trastuzumab. SKBR3 cells also failed to demonstrate synergy between trastuzumab and ant-p16 (data not shown).

To additionally verify that G_1 Cdk activity may not be the relevant target of these drugs at this dose range, we examined the phosphorylation status of pRb, a target of Cdk 4 and 6. MDA453 and SKBR3 cells were treated with trastuzumab (50, 100, or 200 ng/ml) and/or flavopiridol (10, 100, or 300 nM) for 24 h. Protein was then extracted and immunoblotted using an antibody against phosphoserine 795 of pRb (Fig. 1B). Trastuzumab did not change phospho-pRb levels at these low doses. Previous studies required 100-fold more of the anti-erbB2 antibody ($10\ \mu\text{g}/\text{ml}$) to alter phospho-pRb levels (8). Flavopiridol inhibited phosphorylation only at the highest concentration tested (300 nM) in MDA453 cells but not in SKBR3 cells. Combined administration of trastuzumab and flavopiridol did not additionally alter pRb phosphorylation. These results suggested that growth suppression of erbB2-overexpressing breast cancer cells is not dependent on Cdk 4 inhibition at the lower doses of either trastuzumab or flavopiridol where we found the synergistic interaction.

Trastuzumab and Flavopiridol Are Synergistic with the Transcriptional Inhibitor DRB. Recent results suggest that flavopiridol functions as a global inhibitor of RNA polymerase II-mediated transcription because of its ability to block the activity of the transcription elongation factor P-TEFb, a complex of transcriptional cyclins and Cdk 9 (13). In contrast to its purported G_1 cyclin inhibitory effects, transcriptional inhibition has been observed at the low concentrations of flavopiridol that we found to be synergistic with trastuzumab. Furthermore, microarray analyses have demonstrated strong similarities between the effects of flavopiridol and the transcriptional inhibitor DRB on gene expression in lymphoma cells (16). To determine whether trastuzumab-flavopiridol synergy might reflect general transcriptional inhibition as an alternative mechanism, we performed dose-response assays in which MDA453 cells were treated with serial dilutions of trastuzumab and DRB at fixed ratios (Fig. 1C). The C.I. value for the trastuzumab-DRB combination in MDA453 cells was 0.09, indicating synergy between trastuzumab and DRB. Trastuzumab was similarly synergistic with DRB in SKBR3 cells showing a C.I. of 0.82 (Fig. 1D). These results suggest that an alternative inhibitor of P-TEFb, but not an alternative inhibitor of Cdk 4, can replace flavopiridol to reproduce the synergy observed between trastuzumab and

Fig. 1. Trastuzumab demonstrates synergy with the transcriptional inhibitor DRB, lack of synergy with Ant-p16, and no effect on pRb phosphorylation when combined with flavopiridol. A, MDA453 cells were treated with 2-fold serial dilutions of trastuzumab and/or the Ant-p16 fusion peptide. Drugs were combined at a fixed ratio of 1 trastuzumab (ng/ml): 511 Ant-p16 (nm), spanning the individual ED₅₀ for each drug and covering a therapeutically relevant dose range. Viable cells were counted by trypan blue exclusion 48 h after drug exposure. Fractional inhibition of cell proliferation was calculated by dividing the number of surviving cells in drug-treated cultures by the number of cells in control cultures. Data were analyzed by the method of Chou and Talalay (19) using the commercial software package Calcsyn (Biosoft). B, MDA453 and SKBR3 cells were treated with the following drug concentrations: A, no drug; B, 50 ng/ml trastuzumab and/or 10 nM flavopiridol; C, 100 ng/ml trastuzumab and/or 100 nM flavopiridol; D, 200 ng/ml trastuzumab and/or 300 nM flavopiridol. Protein was collected after 24 h of drug exposure, and 100 μg was analyzed by immunoblotting for total or phosphorylated pRb and actin. C, MDA453 cells were treated with 2-fold serial dilutions of trastuzumab and/or the transcriptional inhibitor DRB. Drugs were combined at a fixed ratio of 1 trastuzumab (ng/ml):3.19 DRB (μg/ml), spanning the individual ED₅₀ for each drug and covering a therapeutically relevant dose range. Viable cells were counted by trypan blue exclusion 48 h after drug exposure, and fractional inhibition of cell proliferation was calculated as stated above. D, SKBR3 cells were treated similarly at a fixed ratio of 1 trastuzumab (ng/ml): 3.19 DRB (μg/ml), spanning the individual ED₅₀ for each drug. Viable cells were counted by trypan blue exclusion 48 h after drug exposure, and fractional inhibition of cell proliferation was calculated as stated above. E, MDA453 cells treated with various doses of flavopiridol in the presence of a fixed high dose of DRB (400 μM) demonstrate no additional effect of the added flavopiridol. A dose-response curve of flavopiridol alone is shown for comparison. F, MDA453 cells treated with various doses of DRB in the presence of a fixed high dose of flavopiridol (450 nM) demonstrate a loss of additional effect of the added DRB in the presence of flavopiridol at high but non-lethal concentrations. A dose-response curve of DRB alone is shown for comparison.



flavopiridol. We additionally evaluated this result by adding various doses of flavopiridol to a fixed high dose of DRB (Fig. 1E) and various doses of DRB to a fixed high dose of flavopiridol (Fig. 1F). These results confirmed the idea that flavopiridol and DRB activities could substitute for one another.

Cell Cycle Effects Mediated by Trastuzumab and Flavopiridol.

Flow cytometric cell cycle analysis was performed to better understand the cellular effects of combining trastuzumab and flavopiridol. MDA453 and SKBR3 cells were treated with trastuzumab and/or flavopiridol and exposed to BrdUrd after 48 h. DNA content was measured using standard fluorescence-activated cell sorter approaches (Fig. 2). Flavopiridol and trastuzumab did not alter the G₁ population in either cell line, consistent with the observed lack of change in phospho-pRb levels. S phase cells were reduced 3–4-fold by flavopiridol at its highest concentration (300 nM); trastuzumab did not enhance this effect. The percentage of cells in G₂-M remained constant in SKBR3 cells, and increased ~2-fold in MDA453 cells. The most significant change observed in response to drug treatment was increased apoptosis. The percentage of MDA453 cells containing a subdiploid content of DNA increased up to 5-fold 48 h after individual drug treatments and 8-fold when drugs were administered together (Fig. 2, bottom left). Whereas trastuzumab alone did not induce apoptosis of SKBR3 cells, flavopiridol increased apoptosis by ~20-fold (Fig. 2, bottom right). The combination of 200 ng/ml trastuzumab and 300 nM flavopiridol additionally increased the subdiploid SKBR3

population 2-fold to achieve a 35-fold increase in apoptosis relative to the untreated population. Furthermore, addition of the caspase inhibitor Z-VAD-FMK partially blocked growth inhibition by trastuzumab and/or flavopiridol in MDA453 and SKBR3 cells (data not shown), confirming that the breast cancer cytotoxicity achieved by these drugs is attributable largely to apoptosis. Whereas numerous oncogenic pathways mediate resistance to apoptosis (21), the cyclin-dependent pathways generally enhance apoptosis (22), rather than blocking it. Therefore, we sought to identify alternative molecular targets that might be specific to the synergistic activity of trastuzumab and flavopiridol. We turned to array analyses to identify potential transcriptional targets of the trastuzumab-flavopiridol combination that might be involved in antiapoptotic pathways.

EGFR Is a Potential Target of Flavopiridol-Trastuzumab Synergy.

We analyzed general mRNA expression pattern changes in cells treated with flavopiridol-trastuzumab, because gene expression changes in flavopiridol-treated lymphoma cells implicated previously a general inhibition of transcription as the major effect of flavopiridol in the nM dose range we are studying (16). MDA453 cells were treated with 50 ng/ml trastuzumab and/or 10 nM flavopiridol, normally a subinhibitory dose of the individual drugs but inhibitory when combined. Total RNA was extracted after 24 h, and hybridized overnight onto human gene microarrays containing coding sequences for 5184 genes. Cluster analysis revealed a cluster of genes that were down-regulated to a greater extent in the combination treatment group than

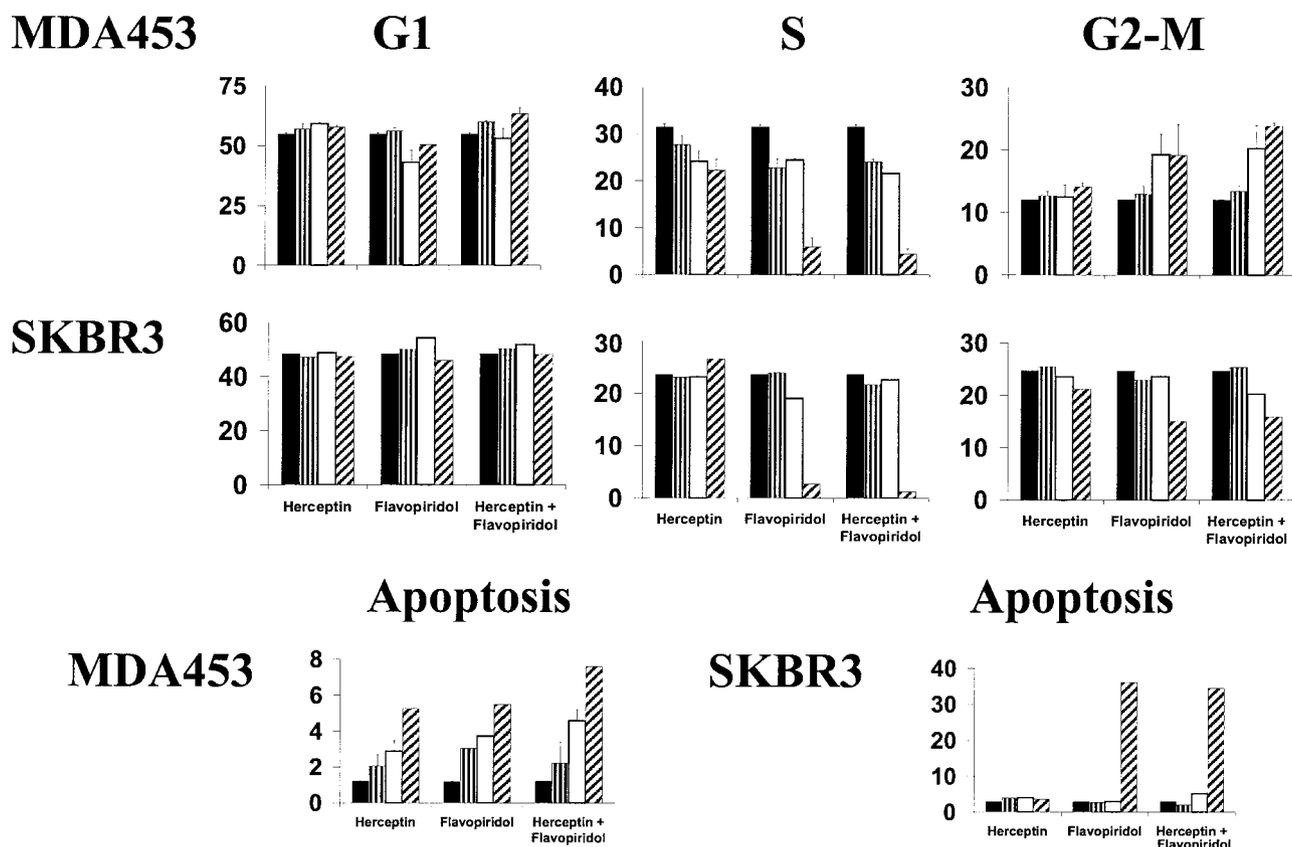


Fig. 2. Effects of trastuzumab and flavopiridol on the cell cycle. *Top*, MDA453 and SKBR3 cells were treated with trastuzumab and flavopiridol as follows: ■, untreated; □, 50 ng/ml trastuzumab and/or 10 nm flavopiridol; ▤, 100 ng/ml trastuzumab and/or 100 nm flavopiridol; ▨, 200 ng/ml trastuzumab and/or 300 nm flavopiridol. Cells were exposed to BrdUrd after 48 h of drug treatment. DNA content was measured by staining with an anti-BrdUrd antibody and propidium iodide, with subsequent flow cytometric cell cycle analysis. The percentages of G₁ (diploid), S, and G₂-M cells are shown on the Y axis. *Bottom*, subdiploid amounts of DNA were quantitated in the same samples to measure apoptosis rates (*apoptosis*); bars, \pm SD.

in untreated cells or cells that were treated with either drug individually (Fig. 3A; Ref. 20). We then examined the response of the 17 genes found in that cluster in SKBR3 cells treated with the same combination of drugs. Five genes responded similarly in SKBR3 cells including the EGFR, ribosomal protein S5, APEX nuclease, lymphoid nuclear protein related to AF4, and est AA683321 (not shown). Therefore, we chose to focus additional studies on the EGFR because it was the only member of this group of genes with an obvious potential role in inhibiting apoptosis (23).

The known interaction between the EGFR and erbB2 also led us to focus on it as a likely candidate for the combined transcriptional inhibitory effects of the trastuzumab-flavopiridol combination. Because array analysis showed a 7-fold reduction in EGFR mRNA by combination flavopiridol-trastuzumab, we treated MDA453 and SKBR3 cells with 50, 100, or 200 ng/ml trastuzumab, and/or 10, 100, or 300 nm flavopiridol for 24 h, and performed immunoblotting to confirm down-regulation of EGFR. The combination of flavopiridol and trastuzumab markedly decreased EGFR levels in both MDA453 and SKBR3 cells (Fig. 3, B and C).

Growth inhibition of breast cancer by trastuzumab and EGFR inhibitor combinations has been reported previously (24). However, the IC₅₀ for direct flavopiridol-EGFR interactions is 21–25 μ M (10). In contrast, we observe synergy between trastuzumab and flavopiridol at 1000-fold lower doses of flavopiridol. To evaluate the potential importance of this interaction we compared the effect of the trastuzumab-flavopiridol combination on a cell line with elevated expression of EGFR and erbB2 *versus* MDA453 and SKBR3 cells, which express low levels of these receptors. In contrast with SKBR 3 and

MDA 453, BT 474 cells express high levels of EGFR in addition to their known overexpression of erbB2 (Fig. 4A). The combination of trastuzumab and flavopiridol was not synergistic in BT 474 cells as seen by their C.I. of 1.46 (Fig. 4B). We then treated BT 474 with 50, 100, or 200 ng/ml trastuzumab, and/or 10, 100, or 300 nm flavopiridol for 24 h, at which time cellular protein was extracted. The combination of flavopiridol and trastuzumab had little effect on EGFR levels in BT 474 cells (Fig. 4C).

To determine whether this decreased EGFR function is required for the synergistic activity of trastuzumab and flavopiridol, drugs were administered singly or combined in the presence of 25 ng/ml EGF to reactivate remaining EGFRs (Fig. 5). Addition of EGF blocked the therapeutic effects of flavopiridol and trastuzumab in MDA453 and SKBR3 cells (Fig. 5, A and B). Immunoblotting demonstrated that addition of EGF also prevented down-regulation of both the EGFR and erbB2 in MDA453 (Fig. 5C) and SKBR3 (Fig. 5D) cells. Thus, decreased EGFR expression and activity appears to be a critical event contributing to the synergistic inhibition of breast cancer cells achieved by flavopiridol and trastuzumab.

DISCUSSION

The promise of therapies targeted to specific molecular abnormalities in breast cancer is currently confronted by the comparatively weak efficacies of the early agents being introduced into clinical practice. Although both trastuzumab and flavopiridol are promising agents with interesting activities, their clinical effects are less impressive than the basic science behind them. Progress in our use of

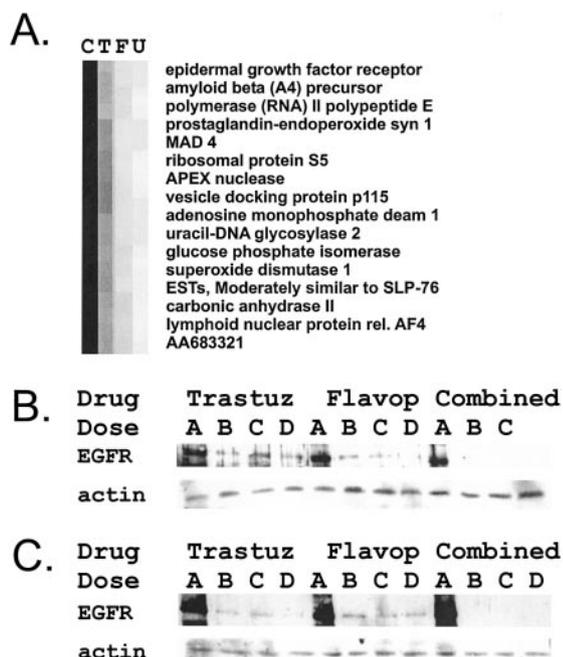


Fig. 3. Trastuzumab and flavopiridol reduce EGFR levels. A, MDA453 cells were treated with no drug (U), 50 ng/ml trastuzumab (T), 10 nm flavopiridol (F), or 50 ng/ml trastuzumab plus 10 nm flavopiridol (C) for 24 h. Total RNA (8 μ g) was collected under each condition, 32 P-labeled, and hybridized onto Mammalian GeneFilters microarrays containing 5184 coding sequences (Research Genetics Invitrogen). Phosphorimages were analyzed using Pathways software (Research Genetics Invitrogen). The ratios of gene expression in each treatment condition were then compared with untreated control cells. Hierarchical cluster analysis was performed using Cluster software from Stanford University. Shown is the cluster that most defined genes that are down-regulated in the presence of the trastuzumab/flavopiridol combination. *White* identifies induced genes and *black* identifies down-regulated genes (20). B, MDA453, and C, SKBR3 cells were treated with the following drugs: A, no drug; B, 50 ng/ml trastuzumab and/or 10 nm flavopiridol; C, 100 ng/ml trastuzumab and/or 100 nm flavopiridol; D, 200 ng/ml trastuzumab and/or 300 nm flavopiridol. Protein (100 μ g) was collected after 1 h of drug exposure and analyzed by Western blots for EGFR. Actin is shown as a loading control.

molecularly targeted therapies will come as the potency of the therapies improves, and as we learn how they are best combined. Our finding of synergy between trastuzumab and flavopiridol had an attractive scientific basis (12), but recent data cast doubt on the idea that flavopiridol is a Cdk 4/6 antagonist (10). Therefore, we sought to reassess the cellular basis for this interaction and to examine other possible mechanisms through which the two drugs might interact.

Although flavopiridol is a Cdk inhibitor that prevents pRb phosphorylation, induces G₁ arrest, and represses transcription of cyclin D1 in some systems (10), it also blocks general transcription through inhibition of the elongation factor P-TEFb (15). We examined the idea that flavopiridol inhibits G₁ progression at the dose levels where we found synergism with trastuzumab and found consistent evidence that Cdk modulation is not the primary mechanism by which flavopiridol and trastuzumab together inhibit growth of erbB2-overexpressing breast cancer cells. Trastuzumab did not interact synergistically with the Cdk inhibitor Ant-p16, nor did the combination of trastuzumab-flavopiridol alter pRb phosphorylation status in the dose range where synergism is observed. Cell cycle data demonstrated enhanced apoptosis in response to these drugs, rather than G₁ or G₂ arrest, which would have been predicted in the event of Cdk inhibition. Furthermore, our previous studies indicated that cyclin D1 levels are unaltered when cells are treated with flavopiridol and trastuzumab (12). Thus, growth inhibition mediated by this drug combination in the low doses where we observed the synergistic interaction appears to be independent of Cdk 4/6 modulation.

Other investigators demonstrated that flavopiridol changes gene

expression profiles in an identical manner to the transcriptional inhibitor DRB (16). Therefore, we sought to determine whether the transcriptional inhibitor DRB could substitute for flavopiridol in combinations with trastuzumab. Dose-response assays indicated that trastuzumab, alone or in combination with flavopiridol, interacted synergistically with DRB. Furthermore, microarray analysis demonstrated that transcription of a unique set of mRNAs is repressed by the combination of trastuzumab and flavopiridol. Thus, transcriptional inhibition, perhaps via blocking of P-TEFb, is an additional possible mechanism that may contribute to the cellular effects achieved by this drug combination. Certainly, other, as yet undiscovered, mechanisms may explain the synergy between this drug combination, but the remarkable sensitivity of P-TEFb to flavopiridol at this low dose range currently makes it the most likely target of this combination.

We identified the EGFR as a candidate target of the flavopiridol-trastuzumab combination by inspection of interesting candidate genes within our microarrays. To confirm the microarray data we demonstrated decreases in EGFR in response to combination trastuzumab-flavopiridol treatment. This was apparently an important effect of the combination because addition of EGF blocked down-regulation of EGFR and erbB2, and prevented breast cancer cell growth inhibition. This regulatory effect may be the result of complicated transcriptional and post-transcriptional effects that will require additional more detailed analyses to fully understand. The EGFR transcriptional inhibition is obvious and specific at 24 h, but additional factors are likely to be important. For example, the effect on EGFR protein levels at 1 h may be because of decreased transcription of some other protein

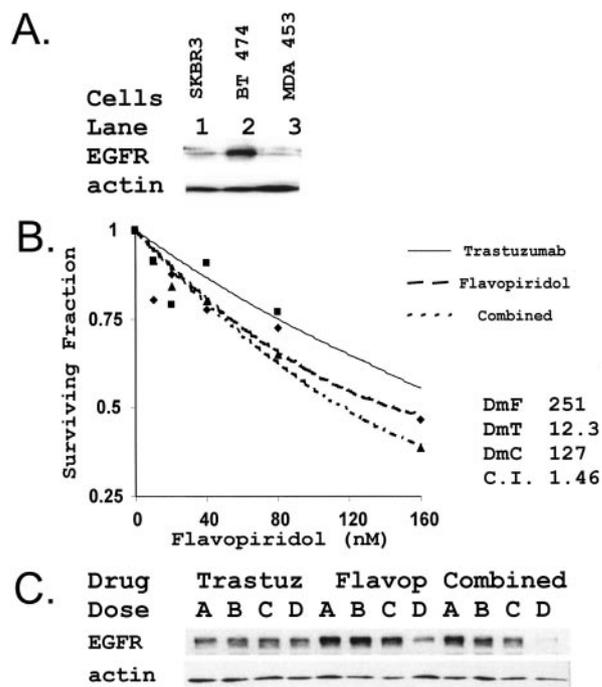


Fig. 4. Trastuzumab and flavopiridol do not synergistically inhibit cell growth nor alter EGFR expression levels in the EGFR-overexpressing BT474 breast cancer cell line. A, protein (100 μ g) was collected from exponentially growing SKBR3, BT474, and MDA453 cells, and immunoblotted for EGFR and actin. B, BT474 cells were treated with 2-fold serial dilutions of trastuzumab and/or flavopiridol. Drugs were combined at a fixed ratio of 1 flavopiridol (nm):1.67 trastuzumab (ng/ml), spanning the individual ED₅₀ for each drug and covering a therapeutically relevant dose range. Viable cells were counted by trypan blue exclusion 48 h after drug exposure, and fractional inhibition of cell proliferation was calculated as stated in Fig. 1. The concentration of flavopiridol (nm) is shown on the X axis, and the surviving fraction is displayed on the Y axis. C, BT474 cells were treated with the following drugs: A, no drug; B, 50 ng/ml trastuzumab and/or 10 nm flavopiridol; C, 100 ng/ml trastuzumab and/or 100 nm flavopiridol; D, 200 ng/ml trastuzumab and/or 300 nm flavopiridol. Protein (100 μ g) was collected after 1 h of drug exposure and analyzed by Western blots for EGFR. Actin is shown as a loading control.

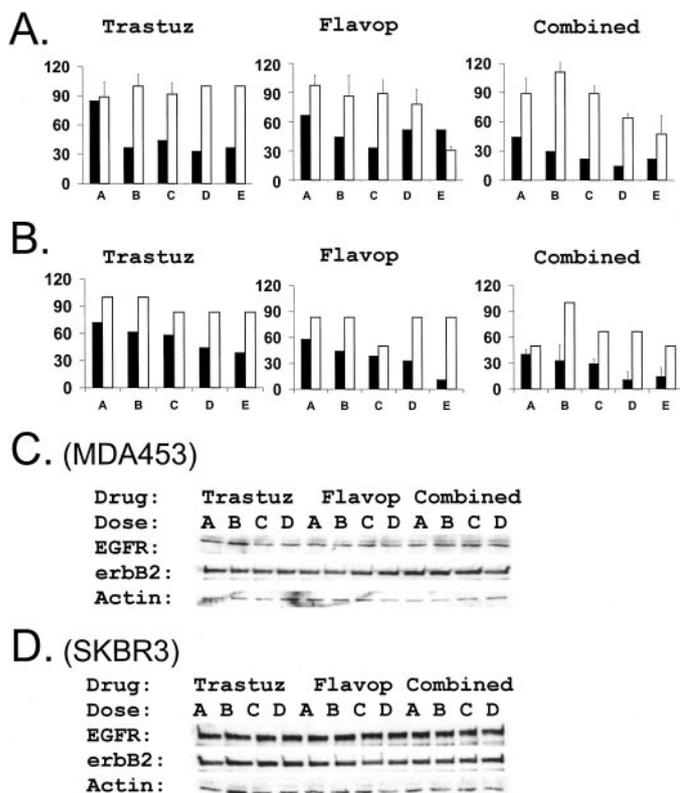


Fig. 5. EGF-mediated inhibition of EGFR, and erbB2 down-regulation correlates with loss of synergy between trastuzumab and flavopiridol. A, MDA453, and B, SKBR3 cells were treated with trastuzumab (A, 25; B, 50; C, 100; D, 150; E, 200 ng/ml) and/or flavopiridol (A, 25; B, 50; C, 100; D, 200; E, 300 nM) in the absence (■) or presence (□) of 25 ng/ml EGF. The percentage of cell viability relative to untreated cultures is shown on the Y axis. C, MDA453, and D, SKBR3 cells were treated with 25 ng/ml EGF and the following drugs: A, no drug; B, 50 ng/ml trastuzumab and/or 10 nM flavopiridol; C, 100 ng/ml trastuzumab and/or 100 nM flavopiridol; D, 200 ng/ml trastuzumab and/or 300 nM flavopiridol. Protein (100 μ g) was collected after 1 h of drug exposure and analyzed by immunoblotting for EGFR, erbB2 and actin; bars, \pm SD.

needed to stabilize the interaction between the EGFR and erbB2. Alternatively, although the IC_{50} of flavopiridol for the EGFR is 23 μ M (10), a level 5000-fold higher than those at which we see the flavopiridol-trastuzumab synergism, some weaker interaction between flavopiridol and the EGFR could destabilize potential heterodimers with erbB2. These and other mechanisms will require additional studies to clarify how the apparent transcriptional effect of flavopiridol, based on analogy to DRB and the microarray changes, can have such profound and rapid effects on EGFR protein levels.

Our results identify the EGFR as an essential target of the flavopiridol-trastuzumab combination. Simultaneous targeting of the EGFR and erbB2 is an important therapeutic strategy in breast cancer, because these receptors frequently form heterodimers (2). Previous studies established that combinatorial use of the anti-EGFR tyrosine kinase inhibitor ZD1839 (Iressa) and trastuzumab inhibited SKBR3 cell growth, and markedly enhanced apoptosis (25). Our observations suggest an alternative strategy to interrupt EGFR and erbB2 interactions by inhibiting EGFR/erbB2 expression levels, and not simply by inhibiting kinase activity. It will be important to clarify how trastuzumab-flavopiridol down-regulates the EGFR and erbB2 so that future therapies might use multiple converging inhibitors of these two key contributors to breast cancer mortality.

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