**Rate-limiting Effects of Cyclin D1 in Transformation by ErbB2 Predicts Synergy between Herceptin and Flavopiridol**

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**Abstract**

Cyclin D1 is downstream of erbB2 and is required for erbB2 transformation. Here we report that cyclin D1 functions are essential, rate limiting for erbB2 transformation, and reciprocally increase erbB2 levels. This interaction depends on three cyclin D1 activities: cyclin-dependent kinase 4-dependent kinase activity, titration of p27, and an intrinsic transcriptional activity of cyclin D1. Drugs active against erbB2 and cyclin D1 (Herceptin and flavopiridol) were synergistically cytotoxic against erbB2-positive breast cancer cell lines. Addition of flavopiridol to Herceptin synergistically lowered erbB2 levels in these cells. Our data suggest the potential use of combinations of cyclin-dependent kinase inhibitors and Herceptin in breast cancer.

**Introduction**

Breast cancer is the most common malignancy among women, with approximately one million new cases diagnosed worldwide annually. Two genes with particular significance to this disease are erbB2 (her-2/neu) and cyclin D1. Both are frequently overexpressed in breast cancer, have prognostic significance, and are implicated in experimental models of breast cancer (1, 2). Recent work established an association between erbB2 signaling and regulation of cyclin D1-CDK complexes (3–5). Importantly, cyclin D1-deficient mice are completely resistant to erbB2-mediated mammary tumorigenesis (5). These results suggest the exciting possibility that novel CDK inhibitors may be effective in cancers containing abnormalities either in the cyclin-dependent regulatory pathway or in genes upstream of this pathway. Importantly, the anti-erbB2 monoclonal antibody trastuzumab (Herceptin) reduces cyclin D1 protein levels (4). In addition, flavopiridol inhibits CDK activity, lowers cyclin D1 levels (6), and reduces erbB2 receptor levels (7). In the current study, we demonstrate that CDK4/6 activity is specifically important for erbB2-mediated transformation and reciprocally regulates erbB2 levels. Interactions between cyclin D1 and erbB2 appear to have therapeutic relevance, because erbB2 down-regulation mediated by trastuzumab and cyclin D1-CDK inhibition mediated by flavopiridol synergistically block breast cancer cell proliferation.

**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.

**Plasmids.** Plasmids constructed included the following: pLTR-neuT (pLTR-erbB2glu) encoding the rat erbB2 mutant gene neuT from Dr. J. Marks (Duke University, Durham, NC); pCMV-cyclin D1/KD from Dr. Phil Hinds (Harvard Medical School, Boston, MA); pCMV-cyclin D1 LALA from Dr. Rene Bernard; cyclins D1 (1–267), D1 (20–295), D1 (40–295) from Dr. Rolf Muller; wild-type and kinase-dead pCMV-CDK4 and pCMV-CDK6s from Dr. S. van de Vijver; the p19ARF coding sequence from pBS-p19ARF from Dr. Charles Sherr; pCMV-HA-p27 from Drs. M. Classon and E. Harlow; and wild-type human erbB2 pDOL-erbB2 and mutant erbB2 pDOL-neuT* (Val664Glu), pDOL-V656C (TR), and pDOL-R652C from Dr. D. Stern (8, 9).

**Cell Lines.** NIH3T3 cells, human breast carcinoma cell lines MCF-7, MDA453 (MDA-MB-453), and SKBR3 cells were obtained from the American Type Culture Collection. Tetracycline-regulated MCF-7 TetOFF cells (Clontech) were transfected with tetracycline-regulated pTO-p16INK4a or pTO-cyclin D1 and were screened by immunoblotting.

**NIH3T3 Transformation Assays.** The following expression vectors were cotransfected with pLTR-neuT into NIH3T3 cells using Lipofectamine (Life Technologies, Inc.): pMMTV-p16, pMMTV-cyclin D1, pMMTV-p19ARF, pCMV-HA-p27, pCMV-cyclin D1/KD, pCMV-cyclin D1/LALA, pCMV-D1 (1–267), pCMV-D1 (20–295), or pCMV-D1 (40–295). NIH3T3 cells were cotransfected with pDOL-erbB2, pDOL-neuT*, ppDOL-V656C, or ppDOL-R652C and one of the following: pBMV-BINK, pCMV-CDK4, pCMV-CDK6, pCMV-MCDK4, pCMV-MCDK6, or pMMTV-cyclin D1. Transformed foci were identified using 1% methylene blue staining 3–4 weeks after plating.

**Immunoblotting.** For TetOFF studies, cell culture medium containing tetracycline was replaced with tetracycline-free medium, and cells were harvested 0, 6, 24, and 48 h later. For drug studies, SKBR3 and MDA453 cells were treated with flavopiridol at 10, 100, or 300 mM and/or trastuzumab at 50, 100, or 200 ng/ml. Protein lysates were obtained after 0, 1, 2, 5, or 10 h of drug exposure using 1% NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8), 1% NP40]. The following antibodies were used: p16 (Santa Cruz) at 1:100; HD-11 cyclin D1 monoclonal (Santa Cruz) used at 1:1000; cyclin D1 rabbit polyclonal (Dr. L. Zuberker, MGH Pathology, Boston, MA) at 1:1000; anti-erbB2-2 monoclonal Ab-3 (Oncogene Research Products) at 1:500; anti-phosphotyrosine-erbB-2 monoclonal at 1:500; and anti-actin goat polyclonal at 1:1000. Secondary antibodies were chosen according to species of origin and detected using enhanced chemiluminescence (Amersham).

**Dose-Response Assays.** Subconfluent MDA453 and SKBR3 cells were seeded at 1 × 10⁶ cells/well in 12-well dishes. After 24 h, cells were treated in triplicate with 2-fold serial dilutions of flavopiridol and trastuzumab (Herceptin) each, or both drugs simultaneously at the indicated fixed ratios. Cells were counted by trypan blue exclusion 48 h after drug exposure, and fractional inhibition of cell proliferation was calculated by comparison with control cultures. Combination indices were obtained using the commercial software package CalcuSyn (Biosoft, Cambridge, United Kingdom; Ref. 10).

**Clonogenic Assays.** Colony-forming assays were performed as described (11). Subconfluent MDA453 and SKBR3 cells were seeded at a density of 750 cells/well in 12-well dishes. After 24 h, cells were treated in triplicate with
flavopiridol and trastuzumab, separately and in combination, at the same doses as above. Cells were washed in drug-free medium after 48 h, maintained in drug-free medium for 7 days, and visualized with methylene blue.

Results and Discussion

Cyclin D1 Enhances erbB2 Oncogenicity and Protein Levels. Previous studies that demonstrated involvement of cyclin D1 function in transformation by erbB2 did not address the specific functions of cyclin D1 that were required for erbB2 to transform cells (3–5). We therefore sought to determine whether the interaction of cyclin D1 with the CDKs was critical to erbB2 transformation and to determine whether cyclin D1 was rate limiting for erbB2 transformation. To address these questions, we combined cyclin D1 and the specific cyclin D1-CDK4 inhibitor p16INK4a with erbB2T (neuT) in NIH3T3 transformation assays.

Transformation rates in NIH3T3 cells transfected with LTR-neuT combined with either pMMTV-p16INK4a or pMMTV-cyclin D1 were compared with transformation rates in cells transfected with neuT alone (Fig. 1A). Nontransfected cells served as a background control. Ectopic expression of p16INK4a blocked neuT-mediated transformation (Fig. 1B), whereas cyclin D1 synergistically increased erbB2-mediated transformation (Fig. 1C). A few foci isolated among the p16INK4a transfection group lacked p16INK4a expression (Fig. 1D).

Interestingly, foci transfected with neuT and cyclin D1 contained increased erbB2 levels relative to foci transfected with neuT alone (Fig. 1E). These results suggest that in addition to the established regulation of cyclin D1 by erbB2 (3), cyclin D1 may reciprocally increase erbB2 protein levels.

We confirmed reciprocal regulation of erbB2 by cyclin D1 using stable tetracycline-inducible (TetOFF) p16INK4a and cyclin D1 cell lines created from MCF-7 human breast cells. Removal of tetracycline from TetOFF p16INK4a cells increased p16INK4a levels, whereas endogenous erbB2 protein levels decreased with similar kinetics (Fig. 1F). Cyclin D1 levels increased upon tetracycline removal from TetOFF cyclin D1 cells, and erbB2 protein levels increased correspondingly (Fig. 1G). Whereas cyclin D1 mRNA levels increased in TetOFF cyclin D1 cells, erbB2 mRNA levels remained unchanged (data not shown), suggesting posttranscriptional induction. MCF-7 cells transfected with pTO-pcDNA (the empty TetOFF vector) did not display altered p16INK4a, cyclin D1 or erbB2 expression upon tetracycline removal (data not shown).

Multiple Cyclin D1 Functions Enhance erbB2-mediated Transformation. We next evaluated which additional functions of cyclin D1 contribute to increased neuT oncogenicity. Cyclin D1 acts by controlling CDK4/6 activity, by titrating inhibitory p27 away from cyclin E, and by transcriptionally repressing a G1 arrest pathway mediated by p19ARF. Our initial experiments demonstrated that cyclin D1-CDK interactions were essential for erbB2 transformation by

Fig. 1. Cyclin D1 is rate limiting for erbB2-mediated transformation. NIH3T3 cells transfected with pLTR-neuT in the presence or absence of either pMMTV-p16INK4a or pMMTV-cyclin D1 were assessed by methylene blue staining. A, representative plates from each transfection group are displayed. B, the number of foci/plate is shown for cells transfected with pLTR-neuT and/or pMMTV-p16INK4a. C, the number of foci/plate is shown for cells transfected with pLTR-neuT and/or pMMTV-cyclin D1. D, lysates were prepared from a few partially transformed foci seen in the pLTR-neuT plus pMMTV-p16INK4a transfection group to assess p16INK4a expression. NIH3T3 cells (3T3) and p16INK4a-overexpressing MCF-7 cells (p16+) were included as controls. Actin was compared as a loading control. E, NIH3T3 cells transfected with pLTR-neuT and/or pMMTV-cyclin D1 lysates were analyzed by immunoblotting for erbB2 expression. F, expression of p16INK4a was induced in stably transfected tetracycline-regulated p16INK4a MCF-7 TetOFF cells by removal of tetracycline. ErbB2, p16INK4a, and actin levels were determined by immunoblotting at the indicated times (in hours). G, lysates were analyzed by immunoblotting for erbB2, cyclin D1, and actin expression after induction of cyclin D1 in tetracycline-regulated cyclin D1 MCF-7 TetOFF cells.
Fig. 2. Multiple functions of cyclin D1 enhance erbB2-mediated transformation. NIH3T3 cells were transfected with the indicated plasmids, maintained in culture medium for 4 weeks, stained with methylene blue, and counted. A, cells were transfected with pLTR-neuT and/or pMMTV-p16INK4a, pMMTV-p19ARF, or pCMV-p27 at a 1:1 or 1:10 DNA concentration ratio. B, cells were transfected with pLTR-neuT and/or pMMTV-cyclin D1 or one of the following mutant cyclin D1 plasmids: pCMV-KD, pCMV-LALA, pCMV-D1 (1–267), pCMV-D1 (20–295), or pCMV-D1 (40–295). C, cells were transfected with pDOL-wild-type erbB2 or pDOL-neuT and/or pMMTV-BINK mutant CDK6, pCMV-CDK4, pCMV-Mutant CDK4, pCMV-CDK6, or pCMV-Mutant CDK6. D, cells were transfected with pDOL-wild-type erbB2, transforming mutant erbB2 pDOL-V656C, or nontransforming mutant erbB2 pDOL-R652C with pMMTV-BINK, pMMTV-cyclin D1, pCMV-CDK4, pCMV-Mutant CDK4, pCMV-CDK6, or pCMV-Mutant CDK6. Bars, SD.

NIH3T3 cells were transfected with a series of cyclin D1 mutants (Fig. 2B). The cyclin D1 KD point mutation, which prevents pRb phosphorylation by CDK4, inhibited neuT-mediated transformation. The LALA cyclin D1 double point mutation removes a transcription factor interaction domain, and cyclin D1 (1–267) lacks 28 COOH-terminal amino acids including the DMP1 interaction domain. Both LALA and cyclin D1 (1–267) blocked neuT-mediated transformation, confirming that cyclin D1-DMP1 interaction and subsequent inhibition of p19ARF contributes to neuT oncogenic properties. Furthermore, cyclin D1 (20–295) and cyclin D1 (40–295), which harbor deletion of the NH2-terminal 20 or 40 amino acids, respectively, are unable to interact with pRb and unable to increase neuT-mediated transformation. These results suggest that all three effector functions of cyclin D1 are important for erbB2 oncogenicity.

We used a series of CDK constructs to clarify the contribution of CDK4/6 function to erbB2 transformation. We first used a mutant CDK6 construct containing the NH2-terminal 118 amino acids of CDK6 that lacks kinase activity and lacks domains needed to bind cyclin D1. This truncated CDK6 (BINK) only contains those domains that sequester p16INK4a, p15INK4b, and p18INK4c; thereby increasing endogenous CDK4 and CDK6 function.5 When transfected into NIH3T3 cells, pMMTV-BINK enhanced transformation by neuT and wild-type erbB2 (Fig. 2C). Transformed foci with neuT and BINK contained increased levels of CDK4/6 activity, either through expression of cyclin D1 or BINK, increases erbB2 oncogenicity.

NIH3T3 transformation by wild-type erbB2 was unaltered in the presence of pCMV-CDK4 or pCMV-CDK6, encoding wild-type kinases, or pCMV-μCDK4 or pCMV-μCDK6, which encode kinase-dead CDK4 or CDK6, respectively. The lack of effect by CDK4 or CDK6 on wild-type erbB2 transformation is probably attributable to the low oncogenic potential of wild-type erbB2 in NIH3T3 cells. When cotransfected with the transforming mutant erbB2 pLTR-neuT, wild-type CDK4 and CDK6 increased the number of transformed foci. The kinase-dead CDK4 and CDK6 constructs did not suppress neuT-mediated transformation because CDK-independent functions of cyclin D1 remained intact.

Transformation of human breast epithelial cells by erbB2 can occur via homodimerization or heterodimerization with other erbB family members. Because erbB2 dimerization is critical to its oncogenic function, we evaluated whether cyclin D1 increases erbB2 transformation by influencing dimerization (Fig. 2D). erbB2 with the point mutation V656C spontaneously dimerizes and transforms NIH3T3 cells, whereas the mutant erbB2 R652C protein is nontransforming (9, 12). Coexpression of pDOL-R652C mutant erbB2, with either the BINK cdk6 mutant or cyclin D1, resulted in a 3–4-fold increase in the number of transformed foci relative to mutant erbB2 R652C alone. Similar to wild-type erbB2, mutant erbB2 R652C, which is nontransforming by itself, was unaffected by CDK4, CDK6, or the kinase-dead CDKs. Interestingly, BINK, cyclin D1, and CDK constructs did not further enhance transformation mediated by the dimerizing mutant erbB2 V656C. Because V656C is transforming caused by its ability to spontaneously dimerize, it is likely that cyclin D1 increases transformation in part by inducing erbB2 dimerization.

Trastuzumab and Flavopiridol Synergistically Inhibit Survival of Breast Cancer Cells. Our results demonstrate oncogenic synergism and reciprocal regulation between cyclin D1 and erbB2. We

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4 B. Kempkes and M. Vidal, personal communication.
therefore asked whether the synergistic relationship between these pathways could be targeted therapeutically. Trastuzumab, an anti-
erbB2 monoclonal antibody, reduces cell surface erbB2 levels (13). We explored its interactions with flavopiridol because flavopiridol both inhibits CDK4/6 activity and reduces cyclin D1 levels (6).

Serial dilutions of trastuzumab and flavopiridol were given to the erbB2-overexpressing cell lines MDA453 and SKBR3 at fixed ratios spanning therapeutically relevant doses so that data could be analyzed using the method of Chou and Talalay (10) to establish the drug combination index value. A C.I. value less than 1.0 indicates synergy, which is defined as a combination that achieves a therapeutic value greater than that expected by simple addition of the effects of the individual drugs. Dose-response assays performed on the MDA453 line (Fig. 3A) and SKBR3 cells (Fig. 3B) indicated C.I. values <1.0 at all drug combinations. MCF-7 cells, which express normal levels of erbB2, did not show drug synergism (data not shown).

We performed clonogenic assays to determine whether flavopiridol and trastuzumab synergistically inhibit survival of erbB2-overexpressing breast cancer cells. MDA453 and SKBR3 cells were treated with flavopiridol and/or trastuzumab at the same concentrations as used in the dose-response assays (Fig. 3, C and D). Data analysis indicated C.I. values <1.0 at all drug combinations in both cell lines. These results demonstrate that combined inhibition of erbB2 and cyclin D1-CDK function by trastuzumab and flavopiridol leads to synergistic inhibition of breast cancer proliferation and survival.

Upon treatment with flavopiridol and/or trastuzumab, erbB2 and cyclin D1 levels were measured in MDA453 (Fig. 4A) and SKBR3 cells (Fig. 4B). MDA453 cells demonstrated a rapid reduction of erbB2 levels (within 1 h) when trastuzumab and flavopiridol were combined, with the lowest levels being detected when higher concentrations of drugs were given. The combination of trastuzumab and flavopiridol reduced erbB2 levels more than when either drug was administered alone. SKBR3 cells displayed lower erbB2 levels within 2 h after exposure to either drug, with a markedly greater reduction evident when the drugs were coadministered, even at the lowest concentrations. In contrast, cyclin D1 levels were unaffected in these cell lines by combination drug treatment.

Flavopiridol is the first member of a novel class of CDK inhibitors to approach use in clinical practice. This class of inhibitors has a wide spectrum of potential use because loss of G1 cyclin control is thought to be a universal finding in cancer. Here we exploited recent evidence that cyclin D1 is downstream of erbB2 (3–5) to explore one potential

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**A. (MDA453)**

**Drug:** Trastuz Flavop Combined

**Dose:** A B C D A B C D

**ErbB2:**

**Cyclin D1:**

**Actin:**

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**B. (SKBR3)**

**Drug:** Flavop Combined

**Dose:** A B C D A B C D

**ErbB2:**

**Cyclin D1:**

**Actin:**

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**Fig. 3.** Trastuzumab and flavopiridol synergistically prevent breast cancer cell growth. A and B, dose-response assays. Subconfluent erbB2-overexpressing MDA453 (A) and SKBR3 (B) breast cancer cells were treated in triplicate 24 h after plating using 2-fold serial dilutions of flavopiridol, trastuzumab, or both drugs simultaneously. Drugs were combined at a fixed ratio that spanned the individual ED50 of each drug and covered a therapeutically relevant dose range. The indicated concentrations in the abscissa are for flavopiridol (nM). The corresponding trastuzumab concentrations were added at a fixed ratio of 1 flavopiridol (nM): 3.33 trastuzumab (ng/ml). 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 colonies in drug-treated cultures by the number of colonies in control cultures. Data were analyzed by the method of Chou and Talalay (10) to obtain the C.I. of the two drugs using the commercial software package CalcuSyn (Biosoft, Cambridge, United Kingdom). The ED50 is the dose required to produce the median effect. The ED values are the effective doses for the indicated inhibitory effect (e.g., ED50 is the concentration at which proliferation is 50% inhibited). C.I.s were calculated at various EDs to test for synergy that are displayed in the tables below each graph. A C.I. <1.0 indicates synergy, C and D, clonogenic assays. Subconfluent MDA453 (C) and SKBR3 (D) cells were trypsinized and seeded at a density of 750 cells/well in 12-well dishes. After 24 h, cells were treated in triplicate with 2-fold serial dilutions of flavopiridol, trastuzumab, or both drugs simultaneously at doses equal to those used in dose-response assays. Colonies were stained with methylene blue and counted by visual examination after 7 days in drug-free medium.

**Fig. 4.** Trastuzumab and flavopiridol demonstrate a greater reduction in erbB2 protein levels when administered in combination with breast cancer cells. MDA453 (A) and SKBR3 (B) cells were treated with the following drug concentrations: A, no drug; B, 50 ng/ml trastuzumab and/or 10 ng flavopiridol; C, 100 ng/ml trastuzumab and/or 100 ng flavopiridol; and D, 200 ng/ml trastuzumab and/or 300 ng flavopiridol. Protein was collected after 1 h (MDA453) or 2 h (SKBR3) of drug exposure, and 100 μg were analyzed by immunoblotting for erbB2, cyclin D1, or actin.
clinical use of CDK inhibitors. We found that all three known functions of cyclin D1 were involved in transformation by erbB2, perhaps explaining its complete dependence on cyclin D1 function for transformation (5). We further found that cyclin D1 could reciprocally up-regulate erbB2.

Flavopiridol is not a pure inhibitor of CDK 4/6 functions. It also affects other enzymes, e.g., glycogen synthase kinase 3β, which might in turn regulate cyclin D1 levels (14, 15). However, we observed synergistic cytotoxicity at 10-fold lower concentrations of flavopiridol than used in most other studies, a concentration at which glycogen synthase kinase 3β and other similar targets are not affected. Recent results suggest that flavopiridol potently inhibits CDK9/cyclin T in the low nM concentrations we used, which would decrease RNA polymerase II-mediated transcription elongation (16, 17). Thus, our results could also reflect inhibition of specific transcriptional targets of flavopiridol, the mRNAs of which may be particularly affected by the combination of trastuzumab and flavopiridol.

Our results suggest a novel rationale for a synergistic combination of flavopiridol, the first member of the developing class of CDK inhibitors, and trastuzumab, a gene-specific treatment with unique activity in breast cancer. Because both trastuzumab and flavopiridol have known activity against breast cancer cells and trastuzumab, a gene-specific treatment with unique activity in breast cancer. Because both trastuzumab and flavopiridol have known activity against breast cancer cells in vitro, our data support the initiation of clinical trials that test the efficacy of combing agents that attack both CDKs and erbB2 in breast cancer.

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References

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