Flavopiridol Blocks Integrin-Mediated Survival in Dormant Breast Cancer Cells

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ABSTRACT

Purpose: Breast cancer micrometastases in the bone marrow are resistant to chemotherapy. They can remain dormant for years before some begin to proliferate. We seek to understand survival mechanisms and develop targeted approaches to eliminating these cells.

Experimental Design: In an in vitro model of dormancy, basic fibroblast growth factor 2 (FGF-2), abundant in the bone marrow, inhibits the growth of well-differentiated cells in the 2- to 10-cell stage and up-regulates integrin α5β1. Through this integrin, cells bind fibronectin, spread out, and acquire a survival advantage, partly through activation of the phosphatidylinositol 3-kinase/Akt pathway. We investigated the effects of Taxotere, flavopiridol, and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase and p38 inhibitors on survival of dormant clones and that of flavopiridol on expression of integrins, adhesion strength, and phosphorylation of Akt, ERK 1/2, and p38.

Results: Dormant MCF-7 and T-47D cell clones were resistant to Taxotere concentrations 10-fold higher than needed to eliminate growing clones but were almost completely eradicated by 200 nmol/L flavopiridol. Flavopiridol decreased phosphatidylinositol 3-kinase/Akt pathway activity that has been described classically as being due to its activity that has been described classically as being due to its inhibition of cyclin-dependent kinases (9, 10). However, flavopiridol also inhibits RNA polymerase II (11), resulting in down-regulation of a broad array of mRNAs (12). Because of this characteristic effect, we investigated the activity of flavopiridol in dormant breast cancer cells that rely on the up-regulated expression of integrin α5β1 to receive survival signaling from fibronectin.

Conclusions: Flavopiridol has pleiotropic effects on key targets involved with survival of dormant breast cancer cells and may represent a useful approach to eliminating cells dependent on multiple signal pathways for survival.

INTRODUCTION

Although cancer is characterized as a proliferative disease, most solid tumors primarily consist of cells that are not dividing at any given time point (1–3). This renders them less than optimally susceptible to chemotherapy agents used particularly for their ability to kill cycling cells. One of the scenarios wherein cancer cells are not proliferating and are least vulnerable to chemotherapy is the case of microscopic breast cancer metastases in the bone marrow (4). Breast cancer cells metastasize to the bone marrow early in the course of the disease wherein they have the potential to remain dormant for years (5). They survive adjuvant chemotherapy treatment given to eradicate them (4) and can eventually begin to proliferate again, resulting in relapsed metastatic disease lethal to the patient (6). The cells that remain dormant are generally well differentiated, are estrogen receptor positive, and do not have amplified HER-2/neu (7).

We investigated molecules that potentially modulate dormancy in an in vitro model that proposes that fibroblast growth factor 2 (FGF-2), present in the bone marrow stroma, inhibits proliferation of well-differentiated breast cancer cells and induces expression of integrins α5 and β1. Through α5β1, the cells specifically ligate fibronectin present in the stroma and receive survival signaling that includes activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (8). Disrupting this specific interaction with blocking peptides or antibodies or blocking the Akt pathway with specific inhibitors reverses the specific survival of these dormant cells on fibronectin-coated plates or in a human bone marrow coculture (8).

A potential therapeutic approach to eradicating dormant cells could be to disrupt the expression of integrins α5 and β1 or the activation of Akt initiated by the interaction of this integrin pair with fibronectin by using small molecules that are easily administered systemically, have good pharmacokinetic profiles, and are well tolerated. One such compound is flavopiridol. Flavopiridol is a synthetic flavone with antitumor profiles, and are well tolerated. One such compound is flavopiridol. Flavopiridol is a synthetic flavone with antitumor activity that has been described classically as being due to its inhibition of cyclin-dependent kinases (9, 10). However, flavopiridol also inhibits RNA polymerase II (11), resulting in down-regulation of a broad array of mRNAs (12).

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MATERIALS AND METHODS

Cells and Culture. MCF-7 and T-47D cells were obtained from American Type Culture Collection (Frederick, MD). Proliferation assays were carried out as before (13). Briefly, cells were incubated in tissue culture plates in DMEM/10% FCS (standard medium) with variable concentrations of flavopiridol and cultured for a variable number of days. Medium was refreshed after 3 days in culture. On the day of assay, viable floating and adherent cells were counted in 0.2% trypan blue. In clonogenic assays, cells were cultured in DMEM/10% FCS with or without 10 ng/mL rhFGF-2 (R&D Systems, Minneapolis, MN) on fibronectin-coated plates (BioCoat, Becton Dickinson, Lincoln Park, NJ), with medium changed every 3 days. Cells were seeded at clonogenic densities of 2,000 cells per well for MCF-7 cells and 1,000 cells per well for T-47D cells, empirically determined for optimal clone formation in 24-well plates. Medium and FGF-2 were replaced after 3 days when Taxotere, flavopiridol (Aventis Inc., Bridgewater, NJ), mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitors UO126 or PD98059 or p38 inhibitors SB203580 or PD169316 (Calbiochem) were also added in the various experiments. After 6 days, floating and loosely attached cells were washed away and attached cells were stained with 0.1% crystal violet in 2% ethanol/10 mmol/L sodium borate (pH 9.0). Growing (≥30 cells) or dormant (≤10 cells) clones were counted.

Western Blots. Cells were incubated in standard medium on fibronectin-coated 100-mm plates at 300,000 cells per plate in medium to which FGF-2 was not to be added and 600,000 cells per plate in medium to which 10 ng/mL FGF-2 was added. FGF-2 was replaced whenever fresh medium or medium supplemented with flavopiridol or Taxotere was added. Lysates were prepared and assayed by Western blot with rabbit polyclonal antibodies to integrins α2, α5, β1, β3, and β4, phospho-Ser473 of Akt and total Akt, total ERK, and total p38 (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-Ser21/9 of glycogen synthase kinase (GSK) 3 α/β isofoms (Cell Signaling, Beverly, MA), and mouse monoclonal antibodies to phospho-ERK, phospho-p38 (Santa Cruz Biotechnology) and His (Invitrogen) and detected by enhanced chemiluminescence as before (14). Coomasie blue–stained membranes were used to verify equal loading. Western band densities were divided by densities of nonspecific protein bands from corresponding lanes of the stained membranes that were least affected by flavopiridol treatment. Phosphoprotein band densities were divided by corresponding total protein band densities, and ratios were normalized against untreated controls. All experiments were carried out at least three times.

Cell Detachment Assays. Cells were cultured in quadruplicate on plastic or fibronectin-coated 24-well plates at 20,000 MCF-7 cells per well and 15,000 T-47D cells per well + 10 ng/mL FGF-2 for 3 days. On day 3, the cells were incubated with fresh medium and FGF-2, with or without 200 nmol/L flavopiridol. On day 4, cells were washed once with PBS and incubated with 0.25 mL Cell Dissociation Solution (Sigma, St. Louis, MO) at 37°C and 5% CO2 for variable times. The Cell Dissociation Solution was aspirated, the cells were washed once gently with PBS and stained with 0.1% crystal violet in 2% ethanol/10 mmol/L sodium borate (pH 9.0) for 20 minutes at room temperature, washed exhaustively in distilled water, dried, extracted with 10% acetic acid, and the A600 was measured.

Akt Adenoviral Transductions. Adenoviral vectors expressing His-tagged wild-type Akt, a dominant-negative Akt mutant, and a constitutively active Akt mutant or β-gal gene were constructed, cultured, and titered as described previously (15). Dormant cells incubated for 3 days with FGF-2 (10 ng/mL) on fibronectin were infected with adenoviral supernatant on day 3 at variable multiplicities of infection (MOI). Transduction and expression efficiencies, assayed using immunohistochemical detection of β-gal at 24 and 72 hours, were approximately 30% at MOI of 2 and did not increase with higher MOIs. Lysates were prepared at 24 and 72 hours for Western blots. Dormant colonies were counted 72 hours after transduction.

RESULTS

We have previously described an in vitro model of dormancy in well-differentiated breast cancer cell lines (8). In this model, MCF-7 and T-47D breast cancer cells incubated with 10 ng/mL FGF-2 on fibronectin form clones consisting of cells with a spread-out appearance and large cytoplasm to nucleus ratios on fibronectin (8). These clones are restricted to 2 to 10 cells after 6 days in culture, having undergone no more than three cell divisions (4 ± 2 and 8 ± 2, in contrast to growing clones that are at least 30 cells in size in the absence of FGF-2. The dormant clones have an altered integrin repertoire, including increased expression of integrins α5 and β1, through which they bind fibronectin and gain a specific survival advantage. The PI3K/Akt pathway is activated and is responsible at least in part for survival of these dormant clones (8).

Clinical studies have showed that bone marrow micrometastases in patients with breast cancer are resistant to chemotherapy (4). To determine the susceptibility of dormant breast cancer cells in our in vitro model to chemotherapy relative to that of growing cells, we compared their response to the chemotherapeutic agent Taxotere. We used the two well-differentiated breast cancer cell lines in our previous study (8) as a paradigm for well-differentiated breast cancer cells that are typically capable of remaining dormant as micrometastases in the bone marrow of patients (5, 7). In a clonogenic assay, Taxotere had an ED50 of <1 nmol/L and completely eliminated growing MCF-7 clones at 5 nmol/L and T-47D clones at 10 nmol/L concentrations (Fig. 1). In contrast, Taxotere concentrations as high as 100 nmol/L were not capable of eliminating dormant cell clones cultured on fibronectin.

In our previous studies, FGF-2 induced the up-regulation of mRNA and protein levels of a number of cellular integrins (8). Blocking experiments showed that interaction between integrins α5β1 and fibronectin endowed specific survival effects in dormant clones. We hypothesized that flavopiridol, a transcription inhibitor, would interfere with the up-regulated expression of these integrins and inhibit the survival advantage provided by their ligation. Figure 2 shows that flavopiridol did indeed inhibit the up-regulated expression of integrins α5 and β1, as well as that of α2, β3, and β4, the other integrins that were induced by FGF-2 in MCF-7 and T-47D cells as determined by Western blot. The effect became evident at 150 nmol/L for most integrins and prevalent at 200 nmol/L flavopiridol in both cell lines.
The intensity of the inhibition varied among the integrins from ~50% to nearly 100%. In control experiments with Taxotere treatment instead of flavopiridol, there were no effects on the levels of any of the integrins up-regulated by FGF-2 (not shown).

The altered integrin expression modified the adhesion properties of these cells. In a detachment assay, FGF-2 treatment resulted in a sustained increase in adherence to fibronectin without significantly affecting adherence to tissue culture–coated plastic or collagen I (Fig. 3A, shown are raw data for MCF-7 cells). Treatment with flavopiridol reversed the specific increase in adhesion to fibronectin but had minimal effects on adhesion to plastic or collagen I. This became evident in cells treated with FGF-2 when the percent detachment in cells

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**Fig. 1** Taxotere eradicates growing but not dormant breast cancer cell clones. A total of 2,000 MCF-7 cells per well or 1,000 T-47D cells per well were incubated on fibronectin-coated 24-well plates without (growing clones) or with (dormant clones) 10 ng/mL FGF-2 (day 0). The medium and FGF-2 were changed and Taxotere was added on day 3. Cells were fixed and stained on day 6 and growing and dormant clones were counted as described in Materials and Methods. The data were normalized to the untreated controls. Bars, SD.

**Fig. 2** Flavopiridol inhibits expression of integrins α2, α5, β1, and β3 up-regulated by FGF-2 on fibronectin. MCF-7 and T-47D cells were incubated at low density on fibronectin-coated tissue culture plates in standard medium (day 0). On day 1, the medium was replaced with fresh medium and FGF-2. On day 4, lysates were prepared and analyzed by Western blot with antibodies to the integrins shown. Nonspecific bands from Coomasie blue–stained membranes were used to verify equal loading (Ctrl.). Band intensities were measured using a densitometer. The ratios of the integrin band density to that of the control band density were normalized to the FGF-2–treated, no-flavopiridol treatment control ratios (relative ratios).
treated with flavopiridol was compared with that in cells not treated with flavopiridol. Percent detachment was calculated as 1 - \( A_{t0} / A_{00} \), where \( t \) is the time of incubation with Cell Dissociation Solution, \( t_0 \) is the zero time point, and \( A_{00} \) is the absorbance corrected by subtracting the background from the stained wells containing no cells. Figure 3B shows this fibronectin-specific effect of flavopiridol by plotting the detachment ratio, or percent detachment, + flavopiridol/percent detachment, no flavopiridol, as a function of time for the experiment shown in Fig. 3A with MCF-7 cells and a comparable experiment with T-47D cells. The detachment ratios on fibronectin were significantly greater at all time points of detachment for FGF-2—treated than for untreated cells, whereas those on plastic and collagen overlapped for the most part or showed small differences inconsistently. These experiments show that flavopiridol reverses the specific adherence to fibronectin in dormant cells endowed by incubation with FGF-2.

Based on these results, we hypothesized that reversing the specific adherence to fibronectin in cells treated with FGF-2 would permit flavopiridol to inhibit the survival of dormant clones. We first determined the effects of flavopiridol on proliferation in these two cell lines on tissue culture—coated plates to determine their sensitivity to this compound. Figure 4A shows that flavopiridol inhibited the proliferation of both MCF-7 and T-47D cells on tissue culture—coated plates in a dose-dependent manner with an ED50 of 25 to 40 nmol/L. The ED50 for inhibition of growing clones on fibronectin was 50 to 100 nmol/L (Fig. 4B). When assessing the effects on dormant clones, in contrast to the effects observed with Taxotere, flavopiridol almost completely eliminated dormant MCF-7 and T-47D clones at 200 to 300 nmol/L. The ED50 was between 100 and 150 nmol/L for MCF-7 cells and 50 and 100 nmol/L for T-47D cells.

Several signal pathways are likely to be active in dormant cells adhering to fibronectin. Our previous studies have showed that blocking ligation of integrin \( \alpha_5\beta_1 \) by fibronectin can partially inhibit dormant clone survival (8). Although sustained Akt phosphorylation was present, inhibiting Akt activation or that of P13K, its upstream activator only partially abrogated dormant clone survival as well. The current data demonstrating that flavopiridol was more effective than blocking peptides or antibodies in eliminating dormant clones suggested that flavopiridol had additional effects on survival signaling besides the ones on decreasing integrin expression. Because Akt signaling was shown to play a role, it was important to determine if it was a target of flavopiridol. To determine if flavopiridol interfered with Akt phosphorylation, we did Western blots with anti—phospho-Akt antibody on lysates from dormant cells treated with variable doses of flavopiridol. Figure 5 shows that flavopiridol decreased the Ser473 phosphorylation of Akt 24 hours after treatment of dormant cells incubated with FGF-2 on fibronectin. The ED50 for inhibition was between 100 and 150 nmol/L flavopiridol for both cell lines. Total Akt levels remained mostly undiminished. The dephosphorylation of Akt was accompanied by a dose-dependent dephosphorylation of Ser21/9 of GSK3 \( \alpha/\beta \), demonstrating a functional significance to the dephosphorylation of Akt. GSK3 phosphorylation was very sensitive to flavopiridol and was significantly inhibited at 50 to 100 nmol/L concentrations, suggesting effects on additional upstream activators of GSK3.

Taken together, these data suggest that the inhibitory effects of flavopiridol on survival of dormant clones are due to its effects on a number of targets that individually contribute to survival. Restoring any one is unlikely to reverse the effects of flavopiridol. To test this hypothesis, we transduced the cells with adenoviral vectors to determine if a constitutively active Akt mutant can reverse dormant clone inhibition by flavopiridol. As controls, we also transduced the cells with wild-type and a dominant-negative Akt mutant and a control vector expressing \( \beta\)-gal. Transduction efficiencies were in the 30% range at MOIs from 5 to 50 as determined by transductions with the \( \beta\)-gal vector and immunohistochemical detection (data not shown). Cells were incubated with FGF-2 on fibronectin-coated plates. On day 3, fresh medium was added containing FGF-2 and adenoviral vectors, and lysates were collected 24 and 72 hours later. Figure 6 is a Western blot stained with antibody to His demonstrating expression of His-tagged Akt isoforms in MCF-7 cells transduced with the adenoviral vectors at MOIs of 2 and 5, and the resultant inhibition of GSK phosphorylation by expression of dominant negative Akt 72 hours after transduction. Similar results were obtained with T-47D cells (data not shown). To determine the contribution of Akt inactivation on flavopiridol-induced dormant clone survival, MCF-7 and T-47D cells were incubated with 10 ng/mL FGF-2 on fibronectin and transduced 2 days later with the constitutively active Akt adenoviral vectors at variable MOIs up to 50. Wild-type and dominant-negative Akt and \( \beta\)-gal vectors were used as controls. On day 3, the medium was changed, and included FGF-2 (10 ng/mL), flavopiridol (100 nmol/L), and viral supernatants. Colonies were stained on day 6 and counted. Flavopiridol consistently inhibited clonal survival, as before. However, neither constitutively active nor wild-type Akt transduction had any significant effects on reversing this inhibition (data not shown). This suggested that although flavopiridol reversed the activation of Akt signaling in dormant clones, this effect was neither the only nor the primary mechanism of flavopiridol-mediated abrogation of dormant clone survival in these well-differentiated breast cancer cells.

We investigated the effects of flavopiridol on two other pathways with reported effects on cell survival, ERK and p38. Figure 7A shows that ERK1 and ERK2 activation was sustained at 3 days after incubation with FGF-2 on fibronectin. Flavopiridol up to 300 nmol/L had little or no effect on the phosphorylation state of either ERK, but it did effectively diminish by a maximum of about half the total ERK protein levels as determined by Western blot. Another member of the ERK signaling family, p38, was phosphorylated at baseline and did not have a significant increase in phosphorylation with FGF-2 treatment (Fig. 7B). Its level of phosphorylation was also minimally affected by flavopiridol, although total protein levels of p38 did decrease with treatment. This effectively decreased somewhat the phospho-p38 in the cell. To determine whether either ERK or p38 contribute to survival of dormant clones, we incubated them with MEK inhibitors UO126 (0.1 and 1 \( \mu \)mol/L; ED50 72 nmol/L for MEK1, 58 nmol/L for MEK2) and PD98059 (2 and 20 \( \mu \)mol/L; ED50 2 \( \mu \)mol/L for MEK) and p38 inhibitors SB 203580 (0.6 and 6 \( \mu \)mol/L; ED50 600 nmol/L for p38) and PD169316 (0.1 and 1 \( \mu \)mol/L; ED50 89 nmol/L for p38). Figure 7C shows that inhibition of both MEK and p38 inhibited dormant clone survival.
survival by 45% to 60% in both MCF-7 and T-47D cells. This suggested that these pathways also contribute individually to the survival of dormant clones. The data presented here support a role for a drug such as flavopiridol, with effects on multiple targets, in eradicating dormant breast cancer cells that are resistant to conventional chemotherapy and that depend on multiple signaling pathways for survival.

**DISCUSSION**

Data presented here show that well-differentiated breast cancer cells rendered dormant by FGF-2 on a fibronectin substratum in an *in vitro* model (8) were resistant to chemotherapy with Taxotere but sensitive to physiologically relevant concentrations of flavopiridol (200 nmol/L; refs. 16–18). In this model, cells acquired an altered integrin repertoire that included increased expression of integrins α5 and β1, likely as a part of a general differentiation process imparted by their interaction with FGF-2. Blocking integrin α5β1 binding by fibronectin or inhibiting PI3K or Akt activation partially inhibited survival of dormant breast cancer clones but could not eliminate them (8). In contrast, flavopiridol almost completely eliminated the survival of dormant clones.

![Fig. 4 Flavopiridol inhibits survival of growing and dormant breast cancer cells. A, a total of 50,000 MCF-7 cells per plate or 5,000 T-47D cells per plate were incubated on triplicate tissue culture–coated plates with variable concentrations of flavopiridol and proliferation was measured by counting cells in 0.2% trypan blue at the variable times indicated. Experiments are representative of at least three carried out at different times. B, clonogenic assays of MCF-7 and T-47D cells on fibronectin without (growing clones) or with (dormant clones) 10 ng/mL FGF-2 incubated with variable concentrations of flavopiridol. MCF-7 cells (2,000 cells per well) and T-47D cells (1,000 cells per well) were incubated with and without FGF-2 in 24-well fibronectin-coated plates (day 0). The medium and FGF-2 were replaced and flavopiridol was added on day 3. Cells were fixed and stained on day 6. Growing and dormant clones were counted as described in Materials and Methods. The data were normalized to the untreated controls. Bars, SD.](http://clincancerres.aacrjournals.org)
Previous studies have shown that the extracellular matrix influences survival through adhesion receptors (19) that include integrin \( \alpha_5 \beta_1 \) (20) and initiate survival signaling when bound to their ligands through the PI3K/Akt pathway (19, 20). Interaction with the extracellular matrix is one of the mechanisms for resistance to chemotherapy (21). Activation of the PI3K/Akt pathway promotes resistance to chemotherapy in breast cancer (22), whereas inhibition of this pathway enhances the chemosensitivity of lung cancer cells (23).

Our experiments showed that flavopiridol caused a decrease in the expression of integrins up-regulated by FGF-2, including integrins \( \alpha_5 \) and \( \beta_1 \). This resulted in a reversal of an increased fibronectin-specific adhesion in FGF-2–treated cells. Flavopiridol also inhibited Akt phosphorylation induced by FGF-2 on fibronectin. Evidently, this dual effect proved more efficacious than individual effects of blocking integrin ligation or inhibition of Akt signaling and suggested that a multitargeted approach with a pleiotropic agent such as flavopiridol may be ultimately more useful.

Indeed, as expected, enforced reexpression of constitutively active Akt did not reverse the inhibitory effects of flavopiridol on dormant clone survival, demonstrating that additional signal pathways that contributed to the survival of these dormant cells were also abrogated by incubation with flavopiridol. In our previous study, inhibiting PI3K directly had a more pronounced effect on dormant clones than inhibiting Akt and also modified their spread appearance (8). This suggested additional signaling downstream of PI3K not mediated through Akt that contributed to the survival of dormant clones.

Flavopiridol has been shown to have many effects. It has been described classically as an inhibitor of cyclin-dependent kinases (9, 10), but has other functions as well. Primarily, it can induce apoptosis in noncycling cells (24, 25). Mechanisms of cell death in noncycling cells stem from another major cellular effect of flavopiridol, that of a broad-spectrum inhibitor of transcription (12). Flavopiridol inhibits RNA polymerase II (11, 26) by binding and inhibiting its coactivator, elongation factor P-TEFb, at a stoichiometry of 1:1 (11). The resulting effects that contribute to apoptosis include down-modulation of mRNA and protein levels of antiapoptotic proteins XIAP, cIAP-2, Mcl-1, survivin (27) and BclX\(_L\) (27, 28), mitochondrial...
Localization of Bax, release of cytochrome c to the cytoplasm, activation of caspase 9 (29) and caspase 3 (27, 29, 30), and cleavage of poly(ADP-ribose) polymerase (27, 28) in a Bcl-2- (29, 31) and p53-independent manner (32, 33). A recent report determined that one potential mechanism of a proapoptotic effect of flavopiridol may be through inhibition of Mdm2 and p21WAF1/Cip1 and sensitization to tumor necrosis factor in a p53-independent manner (34).

Some of the effects of flavopiridol are cell type specific. The activation of caspase 9 may be mediated through activation of ERK and p38 (29), whereas the down-modulation of Mcl-1 is thought to be stabilized by E2F1 (35). In this study, flavopiridol did not significantly affect the activation state of either ERK 1/2 or p38, although it did decrease their total protein levels, thus effectively diminishing phosphoproteins. Chemical inhibition of these signal pathways individually also decreased the survival of dormant clones, demonstrating their contribution to survival. Thus, it seems that multiple parallel signal pathways contribute to keeping dormant clones alive and flavopiridol affects more than one of these targets. Although some dormant clones survived even flavopiridol treatment, it will be useful to determine whether in combination with chemotherapy (36, 37) or radiation (38, 39), this drug will be able to eradicate all surviving dormant clones. Investigations of signal pathways responsible for survival of dormant breast cancer cells are ongoing.
REFERENCES


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