Abstract

Purpose: The aim of these studies was to characterize the action of STX140 in a P-glycoprotein–overexpressing tumor cell line both in vitro and in vivo. In addition, its efficacy was determined against xenografts derived from patients who failed docetaxel therapy.

Experimental Design: The effects of STX140, Taxol, and 2-methoxyestradiol (2-MeOE2) on cell proliferation, cell cycle, and apoptosis were assessed in vitro in drug-resistant cells (MCF-7_DOX) and the parental cell line (MCF-7_WT). Mice bearing an MCF-7_DOX tumor on one flank and an MCF-7_WT tumor on the other flank were used to assess the in vivo efficacy. Furthermore, the responses to STX140 of three xenografts, derived from drug-resistant patients, were assessed.

Results: In this study, STX140 caused cell cycle arrest, cyclin B1 induction, and subsequent apoptosis of both MCF-7_DOX and MCF-7_WT cells. Taxol and 2-MeOE2 were only active in the MCF-7_WT parental cell line. Although both STX140 and Taxol inhibited the growth of xenografts derived from MCF-7_WT cells, only STX140 inhibited the growth of tumors derived from MCF-7_DOX cells. 2-MeOE2 was ineffective at the dose tested against both tumor types. Two out of the three newly derived docetaxel-resistant xenografts, including a metastatic triple-negative tumor, responded to STX140 but not to docetaxel treatment.

Conclusions: STX140 shows excellent efficacy in both MCF-7_WT and MCF-7_DOX breast cancer xenograft models, in contrast to Taxol and 2-MeOE2. The clinical potential of STX140 was further highlighted by the efficacy seen in xenografts recently derived from patients who had failed on taxane therapy.

The taxanes, paclitaxel (Taxol) and docetaxel (Taxotere), are routinely used in metastatic breast cancer and have also been successfully tested in the adjuvant and neoadjuvant setting for early breast cancer (1). Although initially responsive, many tumors quickly become resistant to taxane therapy, leading to disease progression. Over recent years, the understanding of clinical drug resistance has progressed significantly, with the role of drug efflux pumps and changes in tubulin subtype expression becoming well characterized. Conversely, the frequently reported role of tubulin point mutations in mediating drug resistance in vitro has not been supported to date by any clinical observations (2). Monzo et al. (3) reported a correlation between point mutations in βIII tubulin and patient response to Taxol in non–small cell lung cancer; however, subsequent studies have shown that the mutations reported are in nontranslated tubulin pseudo genes (4). Another postulated mechanism of drug resistance is the change in expression of microtubule-associated proteins such as MAP4 (5) and stathmin (6). Although a subset of breast cancers overexpress stathmin, no association was found with overall disease-free survival and associations with drug resistance have not been reported (7).

The causes of all clinically observed drug resistance have yet to be fully resolved. Two mechanisms of resistance, which have been identified in the clinic, are the overexpression of drug efflux pumps, such as P-glycoprotein (8), and changes in β-tubulin isoform expression (9). The P-glycoprotein efflux pump is a member of the ATP-binding cassette (ABC) membrane transporter protein family, and is encoded by the ABCB1 (MDR1) gene. These membrane-based proteins decrease drug accumulation within cells by rapid energy-dependent drug efflux. Juliano and Ling (10) first identified the P-glycoprotein pump in 1976. Since then, numerous studies have correlated P-glycoprotein expression with many multidrug-resistant tumor phenotypes, including breast cancer in the clinic (11–14). The P-glycoprotein pump has a wide range of substrates and is able to confer resistance to many anticancer drugs, including doxorubicin, epirubicin, paclitaxel, docetaxel, vincristine, vinblastine, and mitoxantrone (14).
The two primary strategies for overcoming P-glycoprotein-mediated drug resistance have been the development of compounds that inhibit the P-glycoprotein pump and the discovery of new chemotherapeutic agents that are not substrates for the drug efflux pump. To date, the success of P-glycoprotein pump inhibitors has been disappointing due to a combination of factors. Many initial trials were badly designed, with poorly defined patient populations, inaccurate measurements of the P-glycoprotein status of patients, and the inclusion of non-P-glycoprotein substrates in the combined chemotherapeutic regimens tested (14). A further complicating factor is the inhibition of P-glycoprotein in normal tissues, such as the liver, which affects the pharmacokinetics of the chemotherapy drugs, necessitating dose reductions as drug clearance is impaired. A new third generation of highly specific P-glycoprotein inhibitors is under development, and well-designed clinical trials of these are ongoing (15).

An alternative strategy has been the design of new chemotherapeutic agents that are not substrates for the P-glycoprotein pump. The epothilones, currently in phase II trials, are a new class of microtubule-stabilizing agents whose chemotherapeutic activity is under development, and well-designed clinical trials of these are ongoing (15).

Materials and Methods

Drug synthesis. 2-Methoxyestradiol (2-MeOE2) was synthesized by literature routes (35). STX140 (Fig. 1A) was synthesized by reaction of 2-MeOE2 with sulfamoyl chloride in dimethylacetamide (28).

Cell culture. MCF-7 (estrogen receptor-positive) breast cancer cells were obtained from the American Type Culture Collection (LGC Promochem) and MCF-7/DOX cells were kindly donated by Dr. G.L. Scheffer (Department of Pathology, Free University Hospital, Amsterdam, the Netherlands). Cells were maintained in DMEM containing phenol red, supplemented with 10% fetal bovine serum, 2 mmol/L l-glutamine, 1% nonessential amino acids, and 0.075% sodium bicarbonate (Sigma). All cells were cultured at 37°C under 5% CO2 in a humidified incubator. The resistance factor (RF) values were calculated from the IC_{50} values (RF = IC_{50} drug-resistant cell line/IC_{50} parental WT cell line) for cell proliferation after 96 h compound treatment (0.1 nmol/L to 10 μmol/L), using the CellTiter96 Aqueous One assay reagent microtiter plate assay (Promega). All compounds were dissolved at 10^{-2} mol/L in tetrahydrofuran for in vitro experiments (10^{-6}% to 10^{-4}% final tetrahydrofuran concentration).

Substrate accumulation flow cytometric analysis. Cells were plated at 1 × 10^5 to 1.5 × 10^5 per well in 12-well plates (Triple Red). After 24 h, they were pretreated with 40 μmol/L verapamil (Sigma) for 1 h before treatment with 10 μmol/L doxorubicin (Sigma) in either the presence or absence of verapamil. Control cells were untreated or vehicle treated. One hour later, the cells were harvested by trypsinization (0.25%
trypsin 0.05% EDTA) and placed immediately on ice. Collected cells were pelleted by centrifugation at 1,000 × g, resuspended in 0.5 mL ice-cold PBS with 2.5% fetal bovine serum, and the accumulation of doxorubicin was analyzed using a flow cytometer (FACScan, Becton Dickinson) using the FL-2 channel as previously reported (36). The relative amount of doxorubicin in each sample was calculated as a percentage of the median fluorescence in the MCF-7WT + doxorubicin samples using the median fluorescence in the control MCF-7WT + 40 μmol/L verapamil samples as a blank.

Cell cycle analysis. Cells were plated at 60% to 70% confluency in T-25 flasks (Triple Red). After 24 h, they were treated with 500 nmol/L of each compound (STX140, Taxol, and 2-MeOE2) for 48 h. Control cells were untreated or treated with tetrahydrofuran vehicle only. To harvest cells for flow cytometric DNA analysis, cells were washed with PBS before being trypsinized (0.25% trypsin 0.05% EDTA). Medium containing nonadherent cells was also collected and pooled with the trypsinized cells. The cells and PBS washings were pelleted by centrifugation at 1,500 rpm, washed twice with PBS, fixed in cold 70% ethanol, treated with 100 μg/mL RNase for 5 min, stained with 50 μg/mL propidium iodide, and analyzed using a flow cytometer (FACScan, Becton Dickinson).

Apoptosis analysis. Cells were plated at 60% to 70% confluency in T-25 flasks (Triple Red). After 24 h, they were treated with 500 nmol/L of each compound (STX140, Taxol, and 2-MeOE2) for 72 h. Cells were harvested as described above for cell cycle analysis. The cells and washings were pelleted by centrifugation at 1,500 rpm, washed twice with PBS, and resuspended in binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl2] at 1 × 106 cells/mL. Cells were then stained with fluorescein-conjugated Annexin V (BD Biosciences) antibody and propidium iodide (5 μg/mL) before flow cytometric analysis. Apoptotic cells are defined as cells positive for Annexin V and negative for propidium iodide.

Immunoblotting. Cells were seeded at ~2.5 × 105 per T-25 flask (Triple Red) in 5 mL medium and incubated at 37°C, 5% CO2 in a humidified incubator. After 24 h, the compounds were added and the cells were incubated for a further 24, 48, and 72 h. Protein was prepared from treated cells using radioimmunoprecipitation assay lysis buffer (Sigma) and a Bradford assay was undertaken to determine protein concentration. Equal amounts of protein were loaded in all wells (15 μg). Proteins were separated by electrophoresis through a 4% to 12% NuPAGE Bis-Tris gel (Invitrogen) and subsequently transferred to Hybond-P membrane (GE Healthcare). To ensure equal protein loading and successful transfer, the membranes were stained and visualized with Ponceau S (Sigma) before proceeding with detection.

Detection was carried out using anti-human cyclin B1 (Santa Cruz Biotechnology, Inc.), anti-human p53 (Abcam), or anti-human phosphospecific Bcl-2 (Cell Signaling) primary antibodies. An alkaline phosphatase–conjugated anti-rabbit IgG secondary antibody (Cell Signaling) was used for detection. Band intensities were quantified using Kodak 1D software, version 3.5 (Eastman Kodak Company).

Reverse transcription-PCR. Total mRNA was purified from T-25 flasks (Triple Red) at ~80% confluence using QIAshredder and RNeasy kits (Qiagen) and stored at −80°C. For in vivo samples, 20 to 40 mg of tissue was excised from the tumor xenograft and transferred to 2 mL of RNALater solution (Ambion, Inc.). The tissue sample was transferred to 600 μL of RNeasy Lysis Buffer (Qiagen) containing 1% β-mercaptoethanol (Promega) and homogenized. The homogenate was centrifuged (3 min at 10,000 × g), and the RNA was isolated from the supernatant using the RNeasy kit (Qiagen) and stored at −80°C.

A 5 μg aliquot of each mRNA sample was reverse transcribed in a final volume of 33 μL to generate cDNA using the First-Strand cDNA Synthesis Kit (GE Healthcare) and stored at −20°C. Reverse transcription-PCR reactions were done in a "Rotor Gene 2000 Real-time Cycler" (Corbett Research) with 0.5 μL cDNA in a final volume of 10 μL, using Taqman universal PCR master mix (Applied Biosystems) and the MDR1 (Hs00184491) Taqman primer and probe (Applied Biosystems). The large ribosomal protein (RPL0, 4310879E) was used as an internal housekeeping gene to normalize each sample (Applied Biosystems).

The following conditions were used for amplification: 10 min at 95°C followed by 30 cycles of 10 s denaturation at 95°C and 30 s of annealing/amplification at 72°C, in accordance with the recommended conditions for these primers and probes (Applied Biosystems). Relative mRNA expression for P-glycoprotein was calculated using the comparative quantification algorithm in the Rotor Gene 6 software (Corbett Research).

Xenograft models. For the MCF-7 xenograft models, nonovariecto- mized female MF-1 nu/nu mice (Harlan) were injected s.c. in one flank with 5 × 105 MCF-7WT cells and in the other with 5 × 106 MCF-7DOX cells in ice-cold Matrigel (0.1 mL), resulting in a single tumor per flank. All experiments were carried out under conditions that complied with institutional guidelines. Daily oral administration of STX140 (20 mg/kg) in 0.1 mL 10% tetrahydrofuran/90% propylene glycol, 2-MeOE2 (75 mg/kg) in 0.1 mL 10% tetrahydrofuran/90% propylene glycol, or once weekly i.v. administration of 15 mg/kg Taxol (Bristol-Myers Squib) in 0.2 mL saline was initiated when the tumors reached 50 to 150 mm3 in volume (n = 5 per group). Animal weights and tumor measurements were taken every 7 days using electronic calipers. Tumor volume (V), in mm3, was determined using the following equation: length × width2/2. Results are expressed as a percentage of the tumor volume at day of measurement (Vn) over the volume at day 0 (Vo). At the end of study (day 28), before being sacrificed, mice were anesthetized and digital photographs were taken of their tumors. After the animals were sacrificed, tumor tissue (20-40 mg) was excised and transferred to RNALater solution (Ambion) for subsequent RNA purification.

For the patient-derived xenograft studies, tumor xenografts were maintained by serial transplantation into immunodeficient mice (34). Fragments of 30 to 60 mm3, dissected in culture medium, were grafted s.c. into the interscapular fat pad of 8- to 12-week-old female Swiss nude mice, under Avertin anesthesia. Tumors were allowed to attain a size of 60 to 150 mm3 before initiating dosing. STX140 was dosed 5 of 7 days (Monday to Friday) for 42 days (25 mg/kg orally) as a micronized suspension in 0.5% methylcellulose. The micronized suspension in 0.5% methylcellulose is equally efficacious as the 10% tetrahydrofuran/90% propylene glycol solution of STX140 when administered orally at the same concentrations. Docetaxel (Taxotere, Sanofi-Aventis) was used at a previously described efficacious dose of 20 mg/kg i.p. at 3-week intervals diluted in its specific excipient (34). Animal weights and tumor measurements were taken twice weekly.

Statistics. In vitro experiments were carried out in triplicate and data presented are representative of one of three such experiments. All errors shown are the mean ± SE. Student’s t test was used to assess significance.

Results

Cell proliferation assays. Previous studies have shown STX140 to be a potent inhibitor of cell proliferation in a wide range of tumor types, with an IC50 value of 250 nmol/L in MCF-7WT cells (30). In this study, the efficacy of STX140, and two known P-glycoprotein substrates, Taxol and doxorubicin, were assessed in both the MCF-7DOX cell line and the MCF-7WT cell line. Although the MCF-7WT cells are sensitive to all three compounds, the micrographs presented in Fig. 1B show that MCF-7DOX cells exposed to Taxol (500 nmol/L) or doxorubicin (500 nmol/L) are still able to proliferate and reach confluency. In contrast, STX140 (500 nmol/L) inhibits proliferation of the MCF-7DOX and the MCF-7WT cells to a similar extent. These observations are supported by the calculated RF values. The RF value reflects the fold increase in the drug-resistant cell line

5 Unpublished data.
compared with the parental wild-type cell line. MCF-7_{DOX} cells have very little resistance (RF = 1.5) to STX140 in vitro. In contrast, these cells have significant resistance to both doxorubicin (RF = 150) and Taxol (RF = 1000) in vitro.

**Cell cycle and apoptosis.** STX140, Taxol, and 2-MeOE2 were used at the same dose for a direct comparison of efficacy in vitro. The dose selected was 500 nmol/L, which is ~2 × IC_{50} value of STX140 in MCF-7_{WT} cells (30) and is an achievable plasma concentration in vivo following oral dosing with STX140 (24). This dose is more than 30-fold the IC_{50} value of Taxol in MCF-7_{WT} cells, but is not a dose that significantly affects the proliferation of MCF-7_{DOX} cells in response to Taxol. Although 500 nmol/L is ~10-fold less than the reported IC_{50} value of 2-MeOE2 in MCF-7_{WT} cells (30), it is unlikely that 2-MeOE2 would achieve even this plasma concentration after oral dosing due to its well-reported lack of oral bioavailability (23, 24, 37, 38); thus, using a higher dose would be clinically irrelevant.

STX140 and Taxol both caused significant G_{2-M} cell cycle arrest after 48 h, with 45% and 72% of cells in G_{2-M} cell cycle arrest in response to STX140 and Taxol, respectively, in the MCF-7_{WT} cell line (Fig. 2A). No significant cell cycle arrest was observed in response to 2-MeOE2 in this cell line. Corresponding results were seen for apoptosis in the MCF-7_{WT} cell line after 72 h (Fig. 2B), with an increase in the number of cells undergoing apoptosis in response to STX140 (2-fold versus control) and Taxol (3-fold versus control). A small increase was also seen in response to 2-MeOE2 (1.7-fold versus control) in the MCF-7_{WT} cell line. In the MCF-7_{DOX} cell line, the highest number of cells in G_{2-M} cell cycle arrest was seen after STX140 treatment (68%; Fig. 3A). In contrast, only 23% of cells were in G_{2-M} cell cycle arrest after Taxol treatment. In both control cells and 2-MeOE2–treated cells, only 8% of cells were in G_{2-M} arrest. Despite Taxol inducing some cell cycle arrest in the MCF-7_{DOX} cell line, there was no corresponding increase in the number of cells undergoing apoptosis after 72 h (Fig. 3B). Only STX140 caused increased apoptosis in the MCF-7_{DOX} cell line after 72 h, with twice as many cells (36%) undergoing apoptosis relative to the untreated cells.

**Fig. 2.** Cell cycle and apoptosis analysis of MCF-7_{WT} cells. A, cell cycle: MCF-7_{WT} cells were treated with 500 nmol/L of each compound for 48 h, harvested, stained with propidium iodide, and analyzed using a flow cytometer. STX140 and Taxol both caused G_{2-M} cell cycle arrest after 48 h, with 45% and 72% of cells in G_{2-M} cell cycle arrest, respectively. No cell cycle arrest was observed with 2-MeOE2. B, apoptosis: MCF-7_{WT} cells were treated with 500 nmol/L of compound for 72 h before being stained with fluorescenc-conjugated Annexin V antibody and propidium iodide for fluorescence-activated cell sorting analysis. STX140 (2-fold vs control), Taxol (3-fold vs control), and 2-MeOE2 (1.7-fold vs control) all increased the number of apoptotic cells. M2, apoptotic cells on histogram. C, cyclin B1, p53, and phospho-Bcl-2: MCF-7_{WT} cells were treated for 24, 48, and 72 h with 500 nmol/L of each compound and total protein was immunoblotted using the appropriate antibodies. STX140, Taxol, and, to a lesser extent, 2-MeOE2, all induced cyclin B1 after 24 h; by 48 h, the cyclin B1 had degraded. Only STX140 and Taxol induced p53 protein and phosphorylation of Bcl-2.
The cell cycle regulatory protein, cyclin B1, is induced during cell cycle arrest. Cyclin B1 blocks both progression through the cell cycle and apoptosis (39); however, once cyclin B1 is degraded, the cells can either progress through the cell cycle or undergo apoptosis. Figure 2C shows that both STX140 and Taxol cause a rapid accumulation of cyclin B1 protein after 24 h in the MCF-7WT cells, and that after 48 h the levels of cyclin B1 protein decrease, allowing the cells to proceed through the apoptotic pathway (Fig. 2B). STX140 causes a strong induction of cyclin B1 protein (7-fold versus control); in contrast, Taxol only weakly induces cyclin B1 (1.3-fold versus control) in the MCF-7DOX cell line (Fig. 3C). These data are in agreement with the apoptosis observed in response to Taxol and STX140 in both cell lines. 2-MeOE2 only causes a weak induction of cyclin B1 in the MCF-7WT cell line (Fig. 2C).

A key mediator of apoptosis, p53, was induced by both Taxol and STX140 in the MCF-7WT cell line after 24 h, and protein levels remained elevated for 72 h (Fig. 2C). 2-MeOE2 did not cause an induction of p53 at the concentration tested. These data correlate well with the observed changes in apoptosis seen in response to all three compounds (Fig. 2B). The immunoblots show that the MCF-7DOX cell line constitutively overexpresses p53 and no changes in p53 protein expression were seen with any compound (Fig. 3C).

The antiapoptotic protein Bcl-2 was inactivated by phosphorylation in response to STX140 in both cell lines after a 24 h exposure (Figs. 2C and 3C). The results with Taxol in the MCF-7WT cell line were similar (Fig. 2C), but the changes in phosphorylation were much weaker in the MCF-7DOX cell line (2-fold induction versus 7-fold induction with STX140; Fig. 3C).

P-glycoprotein expression and activity. The MCF-7DOX cell line has been reported to be resistant to Taxol and doxorubicin due to expression of the P-glycoprotein drug efflux pump (33). Figure 4A and B confirms that whereas MCF-7WT cells express little ABCB1 mRNA and no P-glycoprotein, MCF-7DOX cells...
express high levels of both *ABCB1* mRNA (2,150-fold that of MCF-7<sub>WT</sub> cells) and P-glycoprotein. Immunoblotting with an anti-BCRP antibody, and reverse transcription-PCR with *ABCG2* (BCRP)– or *ABCC1* (MRP1)–specific primers, indicated that MCF-7<sub>DOX</sub> cells do not express the BCRP or MRP1 eflux pumps (data not shown), which can expel doxorubicin from cells.

To confirm that the expressed P-glycoprotein is active, eflux studies were undertaken with doxorubicin as a fluorescent substrate in the presence or absence of the P-glycoprotein inhibitor verapamil. Figure 4C shows that in the presence of doxorubicin, the fluorescence of the MCF-7<sub>WT</sub> cells dramatically increases due to the intracellular accumulation of doxorubicin, as seen by a shift to the right on the graph. The shift is much less pronounced in the MCF-7<sub>DOX</sub> cell line, with 75% less doxorubicin accumulating in MCF-7<sub>DOX</sub> cells relative to the MCF-7<sub>WT</sub> cells (Fig. 4C and D). However, in the presence of verapamil, the MCF-7<sub>DOX</sub> cells no longer expel doxorubicin, and doxorubicin accumulates to the same extent as in the parental MCF-7<sub>WT</sub> cells.

**Xenograft studies.** The MCF-7 breast xenograft model is widely used for the preclinical assessment of potential anticancer therapies. In this study, the efficacies of the preclinical compound, STX140, a drug in development, 2-MeOE2 (Panzem), and the routinely used anticancer drug, Taxol, were...
compared. To further evaluate these compounds, the previously described multidrug-resistant MCF-7_{DOX} cell line was also used to generate xenografts. To allow a direct comparison between the two cell types, mice were inoculated on one flank with the MCF-7_{WT} cells and on the opposite flank with the MCF-7_{DOX} cells.

The MCF-7_{WT} tumors grew significantly quicker (20-fold; Fig. 5A and C) than the MCF-7_{DOX} tumors (3.5-fold; Fig. 5B and C) over the 28 days of the study ($P < 0.001$). Both Taxol (15 mg/kg i.v., weekly) and STX140 (20 mg/kg orally, daily) significantly inhibited MCF-7_{WT} tumor growth ($P < 0.001$ versus control; Fig. 5A and C). In contrast, Taxol did not significantly inhibit the growth of the MCF-7_{DOX} tumors, whereas STX140 caused significant regression ($P < 0.01$ versus control) of the multidrug-resistant tumors (Fig. 5B and C). 2-MeOE2, despite being used at a previously reported efficacious dose (40), failed to significantly inhibit the growth of either tumor type. No significant weight loss was seen in any treatment group (data not shown).

Tumor tissue samples were taken at the end of the study. The RNA was isolated and subsequent reverse transcription-PCR was done for the ABCB1 gene. Figure 5D shows that after the 28-day study, the MCF-7_{DOX} tumors still overexpressed ABCB1 mRNA relative to MCF-7_{WT} tumors at a similar level to the in vitro cell lines (1,663-fold vs 2,150-fold).

![Fig. 5. MCF-7 xenograft studies. Female MF-1 nu/nu mice were injected s.c. in one flank with $5 \times 10^6$ MCF-7_{WT} cells and in the other with $5 \times 10^6$ MCF-7_{DOX} cells in ice-cold Matrigel ($n = 5$ per group). Daily oral administration of vehicle (0.1 mL 10% tetrahydrofuran/90% propylene glycol), STX140 (20 mg/kg), 2-MeOE2 (75 mg/kg), or once weekly i.v. administration of Taxol (15 mg/kg) was initiated when the tumors reached 50 to 150 mm$^3$ in volume (day 0). A, growth of MCF-7_{WT} tumors: Dosing with either Taxol or STX140 caused significant inhibition of tumor growth (**, $P < 0.001$). B, growth of MCF-7_{DOX} tumors: Only dosing with STX140 caused significant inhibition of tumor growth ($^*$, $P < 0.001$) and tumor regression (**, $P < 0.05$ vs tumor volume at day 0). C, tumor images: At the end of study, mice were anesthetized and digital photographs were taken of their tumors. The pictures clearly show the efficacy of STX140 in the MCF-7_{DOX} model, in contrast to Taxol. The decreased growth rate of the MCF-7_{DOX} tumors relative to the MCF-7_{WT} tumors can also be seen (dotted line, tumor edge). D, ABCB1 mRNA expression: After 28 d dosing, the MCF-7_{DOX} tumors still overexpressed ABCB1 mRNA relative to MCF-7_{WT} tumors at a similar level to the in vitro cell lines (1,663-fold vs 2,150-fold).]

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experiments had shown to be equally efficacious as 20 mg/kg oral STX140 in 0.1 mL 10% tetrahydrofluran/90% propylene glycol (data not shown).

In two out of the three patient-derived breast cancer xenografts, STX140 exhibited significant antitumor activity as a single agent, resulting in regression of HBCx-3 (Fig. 6A), an estrogen receptor-positive, hormone-dependent tumor, and growth arrest of HBCx-14 (Fig. 6B), a triple-negative tumor. Complete tumor regression was seen in 4 of 10 mice in the HBCx-3 xenograft and 3 of 10 mice in the HBCx-14 xenograft. In the third xenograft, HBCx-12 (Fig. 6C), also a triple-negative tumor, STX140 resulted in a modest growth delay that did not reach statistical significance. In contrast, docetaxel did not have significant antitumor activity in any of the three xenografts. When STX140 was administered orally at 25 mg/kg, 5 of 7 days for 42 days, the average body weight loss was <10% and no treatment-related deaths were observed in mice bearing any of the three xenografts (data not shown).

Discussion

The development of multidrug resistance in cancer patients limits the long-term efficacy of many drugs used to treat cancer. The discovery of new anticancer agents that are not substrates for the multidrug efflux pumps is an ongoing priority for cancer research. This study shows that the recently developed 2-substituted estrogen sulfamate, STX140, is highly efficacious in in vivo models of clinical drug resistance.

To investigate the activity of STX140 in multidrug-resistant cancer, the MCF-7DOX cell line was used in conjunction with three xenografts derived from patients who had failed on taxane therapy. Immunoblotting and reverse transcription-PCR experiments confirmed the overexpression of P-glycoprotein and the lack of expression of the other common drug efflux pumps, BCRP and MRP1, in the MCF-7DOX cell line. Furthermore, fluorescence-activated cell sorting analysis showed that P-glycoprotein was active and could be successfully blocked by the inhibitor verapamil. Thus, any observed drug resistance is most likely mediated by the overexpression of the P-glycoprotein drug efflux pump in the MCF-7DOX cells. In contrast, no MDR1 up-regulation has been detected in the xenografts derived from taxane-treated patients. The exact mechanism of resistance remains to be fully elucidated for these tumors, despite extensive profiling (34).

STX140 induced cell cycle arrest and apoptosis in both MCF-7WT and MCF-7DOX cells. Taxol also induced cell cycle arrest and apoptosis in MCF-7WT cells, but in MCF-7DOX cells it was significantly less effective at inducing cell cycle arrest and failed to induce apoptosis. These data are supported by the RF calculations. STX140 was similarly potent in both cell lines, but Taxol was nearly 1,000-fold less effective in the MCF-7DOX cell line relative to the MCF-7WT cells. To further investigate the nature of the antiproliferative activities of these compounds, cell cycle and apoptosis regulatory proteins were examined by immunoblotting. Both STX140 and Taxol blocked the activity of Bcl-2, an inhibitor of apoptosis, by phosphorylation and induced the proapoptotic protein p53 in the MCF-7WT cells. However, in the MCF-7DOX cells, there was no phosphorylation of Bcl-2 in response to Taxol, but Bcl-2 was phosphorylated in response to STX140.
In this study, MCF-7\textsubscript{DOX} cells constitutively overexpressed p53 and no changes were seen in p53 levels in response to any compound. Chen et al. (41) showed increased levels of p53 in an MCF-7 cell line overexpressing P-glycoprotein. However, in contrast to data presented here, Bcl-2 protein could not be detected in these cells. They concluded that despite being a multidrug-resistant cell line, these cells were more susceptible to some inducers of apoptosis as their apoptosis pathway was already primed, in contrast to the wild-type cells. As p53 overexpression in this study does not induce an increase in the apoptotic rate of untreated MCF-7\textsubscript{DOX} cells relative to MCF-7\textsubscript{WT} cells, this suggests that either the expressed p53 is inactive or apoptosis is blocked downstream. Chorna et al. (42) showed increased expression of a mutant p53 in a MCF-7 cell line overexpressing P-glycoprotein, which did not significantly affect the induction of cell death by ionizing radiation. Despite the differences in p53 protein expression, STX140 induces apoptosis in both cell lines. This concurs with previous studies that have shown STX140 to be active in cell lines lacking active p53 (29). The failure of Taxol to induce apoptosis, cyclin B1, and Bcl-2 phosphorylation in the MCF-7\textsubscript{DOX} cells is most likely caused by increased drug efflux preventing efficacious intracellular concentrations of the drug being achieved.

Ueda et al. (43) proposed some basic principles to try to identify P-glycoprotein substrates, although many substrates still do not meet these criteria. Most substrates are between 300 and 2,000 in molecular weight, are hydrophobic, possess two planar aromatic rings, have a basic nitrogen atom, and for many steroids the presence of either C-11, C-16, or C-17 hydroxyl groups indicates they may be substrates. Although STX140 is a weakly hydrophobic steroidoidal compound, data in this article show that it is not a substrate for P-glycoprotein. This is probably due to substitution of the C-17 hydroxy group by a sulfamate group, the lack of C-11 or C-16 hydroxyl groups, the presence of only one planar aromatic ring, and/or the lack of a basic nitrogen atom in STX140. Due to the lack of efficacy both \textit{in vitro} and \textit{in vivo} of 2-MEOE2 in this study, no conclusions can be inferred on whether 2-MEOE2 interacts with P-glycoprotein. Given that many steroids are substrates for P-glycoprotein, and the presence of a C-17 hydroxyl group, it is surprising that 2-MEOE2 is reported not to be a substrate for P-glycoprotein (44).

To assess \textit{in vivo} efficacy, we established a novel dual tumor xenograft model, with an MCF-7\textsubscript{WT} tumor on one flank and an MCF-7\textsubscript{DOX} tumor on the alternate flank. This model allows direct comparison between the two cell types in one animal and from an ethical standpoint reduces the number of animals required for each study. Previous studies have shown STX140 to be a potent oral inhibitor of \textit{in vivo} tumor growth in MDA-MB-435 xenografts (24) and in the Lewis lung model (28). A significant potential advantage of STX140 over existing chemotherapeutic agents is the ability to dose daily by the oral route, thus ensuring ease of administration and a constant exposure of the tumor to the drug. In contrast, it is necessary to dose Taxol i.v., and due to its severe toxicity it can only be administered intermittently (25, 26). Previous \textit{in vitro} studies with MCF-7 cells overexpressing P-glycoprotein have reported a decreased growth rate relative to the parental cell line (42). In this study, using the novel dual xenograft model with MCF-7\textsubscript{DOX} and MCF-7\textsubscript{WT} xenografts, the MCF-7\textsubscript{DOX} tumors grew significantly slower than their wild-type counterparts. One possible explanation is that the MCF-7 cell lines are dependent on estradiol to stimulate their proliferation, and estradiol is a possible substrate for P-glycoprotein (43).

The clinical potential of STX140 was further highlighted by data generated from a new preclinical model for breast cancer, which has been developed by Maranoni et al. (34) to reflect the diverse range of tumor types. Two of the three xenografts used in this study were metastatic, triple negative, and Ki67 positive (HBCx12 and HBCx14). Both of these xenografts could be inhibited by a combination of doxorubicin and cyclophosphamide (34) but not docetaxel. The HBCx3 xenograft was estrogen receptor–positive, nonmetastatic, Ki67 positive, and displayed markers of aggressiveness, such as mutated p53 and no progesterone receptor expression. The HBCx3 xenograft did not respond to the combination of doxorubicin and cyclophosphamide but responded to hormone deprivation induced by Degarelix, a GHRH antagonist (34). In this study, STX140 significantly inhibited the growth of both the HBCx14 and HBCx3 xenografts, demonstrating the potential clinical utility of STX140 in both hormone-dependent and hormone-independent drug-resistant tumors. STX140 failed to significantly inhibit the growth of the other triple-negative xenograft, HBCx12. Maranoni et al. (34) showed that whereas HBCx12 and HBCx14 were both triple negative, HBCx12, in contrast to HBCx14, was HER3-positive and overexpressed both RB and P-AKT. How this contributes to the poor efficacy of STX140 in this xenograft remains to be elucidated. The triple-negative xenografts respond to the doxorubicin and cyclophosphamide combination (34). However, doxorubicin, in contrast to STX140, is a substrate for P-glycoprotein and thus is not efficacious in tumors in which drug resistance is mediated by P-glycoprotein.

This study shows for the first time that STX140 induces cell cycle arrest and apoptosis via the inactivation of Bcl-2 in both wild-type and multidrug-resistant hormone-dependent breast cancer cells. In contrast to 2-MEOE2 and Taxol, STX140 was efficacious \textit{in vivo} in both wild-type and multidrug-resistant tumors which overexpress P-glycoprotein. The activity of STX140 in two out of three drug-resistant human breast cancer xenografts is very encouraging. These xenografts do not overexpress P-glycoprotein and their mechanism of resistance remains to be fully characterized, thus reflecting many clinical situations. The activity against multidrug-resistant tumors combined with the previously reported excellent bioavailability and lack of toxicity suggest that STX140 is a highly promising anticancer drug. It is envisaged that STX140 will enter the clinic in 2008.

References
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