

## ORIGINAL ARTICLE

# In silico identified targeted inhibitors of P-glycoprotein overcome multidrug resistance in human cancer cells in culture

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## Keywords

In silico drug screens, MDR inhibitors, multidrug resistance, P-glycoprotein, reversing drug resistance

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## Introduction

Multidrug resistance (MDR), the intrinsic or acquired simultaneous resistance to structurally and chemically

## Abstract

Failure of cancer chemotherapies is often linked to the over expression of ABC efflux transporters like the multidrug resistance P-glycoprotein (P-gp). P-gp expression in cells leads to the elimination of a variety of chemically unrelated, mostly cytotoxic compounds. Administration of chemotherapeutics during therapy frequently selects for cells that over express P-gp and are therefore capable of robustly exporting diverse compounds, including chemotherapeutics, from the cells. P-gp thus confers multidrug resistance to a majority of drugs currently available for the treatment of cancers and diseases like HIV/AIDS. The search for P-gp inhibitors for use as co-therapeutics to combat multidrug resistances has had little success to date. In a previous study (Brewer et al., *Mol Pharmacol* 86: 716–726, 2014), we described how ultrahigh throughput computational searches led to the identification of four drug-like molecules that specifically interfere with the energy harvesting steps of substrate transport and inhibit P-gp catalyzed ATP hydrolysis in vitro. In the present study, we demonstrate that three of these compounds reversed P-gp-mediated multidrug resistance of cultured prostate cancer cells to restore sensitivity comparable to naïve prostate cancer cells to the chemotherapeutic drug, paclitaxel. Potentiation concentrations of the inhibitors were  $<3 \mu\text{mol/L}$ . The inhibitors did not exhibit significant toxicity to noncancerous cells at concentrations where they reversed multidrug resistance in cancerous cells. Our results indicate that these compounds with novel mechanisms of P-gp inhibition are excellent leads for the development of co-therapeutics for the treatment of multidrug resistances.

## Abbreviations

Compound 19, methyl 4-[bis(2-hydroxy-4-oxochromen-3-yl)methyl]benzoate (ZINC 09973259, CID 4694077); Compound 29, 2-[[5-cyclopropyl-1H-1,2,4-triazol-3-yl]sulfanyl]-N-[2-phenyl-5-(2,4,5-trimethylphenyl)pyrazol-3-yl]acetamide (ZINC 08767731, CID 17555821); Compound 34, 2-[1-[4-(4-methoxyphenyl)piperazin-1-yl]-1-oxobutan-2-yl]-4-methyl-[1]benzothio[2,3-d]pyridazin-1-one (ZINC 09252021, CID 22514118); Compound 45, ethyl 1-(1,3-benzodioxole-5-carbonyl)-3-(3-phenylpropyl)piperidine-3-carboxylate (ZINC 15078148, ZINC 15078146, CID 26410703, CID 45252040); DMSO, dimethylsulfoxide; MTT, (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide); NBD, nucleotide-binding domain; P-gp, P-glycoprotein.

unrelated therapeutics, including those to which the cells have never been exposed, is arguably one of the largest barriers to successful chemotherapy treatment of cancer. MDR is not a phenomenon restricted to one particular

type of cancer and approximately 40% of human cancers develop resistances to chemotherapy drugs (Higgins 2007). MDR is most frequently caused by increased or initial high expression levels of membrane efflux proteins belonging to the ATP-binding cassette (ABC) family of transporters that are capable of actively transporting a wide variety of pharmaceutical compounds out of cells (Borst and Elferink 2002; Allen et al. 2003; Eckford and Sharom 2009; Vasiliou et al. 2009).

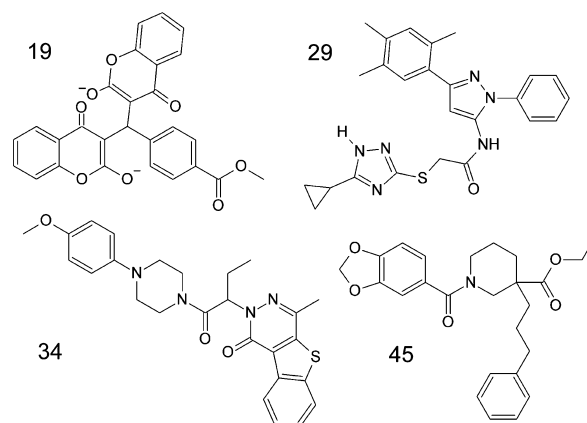
The best-studied mechanism of MDR is that caused by the ABC transporter P-glycoprotein (P-gp). P-gp expression has been shown to increase after chemotherapy treatment and is strongly correlated with decreased response to chemotherapy (Trock et al. 1997; Ambudkar et al. 1999; Leith et al. 1999; Gottesman et al. 2002). P-gp is a plasma membrane protein of 1280 residues that in humans is expressed from the *MDR1* gene. The protein is capable of exporting substrates from the cell by coupling ATP hydrolysis to conformational changes that cause movement of the substrates through the plasma membrane. By reducing intracellular accumulation of drugs, P-gp confers resistance to a wide array of chemotherapeutics, including vinca alkaloids, epipodophyllotoxins, anthracyclines, and taxanes (Gros et al. 1986; Ueda et al. 1986; Croop et al. 1987; Ambudkar et al. 1999; Gottesman et al. 2002; Lage 2008).

Decades of work to overcome P-gp-mediated MDR have identified scores of compounds that are capable of modulating P-gp catalyzed transport of cytotoxic drugs, but have failed clinical trials and were therefore abandoned. Common to many of the previously identified P-gp inhibitors was that they directly competed with chemotherapeutics for interactions at the drug-binding domain (DBD). Mostly due to this characteristic, high doses of these compounds were required for efficacy and resulted in unacceptable toxicities (Fisher et al. 1996). More recently, newer modulators have been developed using structure-activity relationships. The most promising P-gp inhibitor currently under investigation is tariquidar (XR9576) (Martin et al. 1999; Mistry et al. 2001) which appears to be effective at nanomolar concentrations in vitro but so far has shown only limited success in clinical trials (Binkhathlan and Lavasanifar 2013). Early reports suggested that tariquidar inhibits P-gp by binding noncompetitively to sites distinct from the substrate-binding sites (Martin et al. 1999). Others later showed evidence that tariquidar may also compete with transport substrates for access to the drug-binding domains (Pajeva et al. 2013) (Martin et al. 1999). Most recently, it was suggested that tariquidar inhibition was due to “locking” P-gp in an “open to the extracellular side” conformation (Loo and Clarke 2014). This would explain the stimulated ATP hydrolysis activity that seems to result from close

association of the nucleotide-binding domains as seen in (Loo et al. 2010; Verhalen and Wilkens 2011). Some reports show significant accumulation of tariquidar in cells overexpressing P-gp which suggests that tariquidar may be only slowly transported by P-gp, if at all (Martin et al. 1999; Kannan et al. 2011).

We recently reported successful in silico screening methods aimed at identifying P-gp inhibitors with a novel mechanism of inhibition in that they specifically interact with the energy harvesting structures of the transporter, which are the nucleotide-binding domains (NBD) (Brewer et al. 2014). Compounds that were predicted in these screens to significantly interact with the drug transporting structures were eliminated from further evaluation. The goal was to identify small molecules that inhibited P-glycoprotein action without being transport substrates themselves.

By screening for inhibitors that specifically target the nucleotide-binding domains of P-gp, we identified four compounds that inhibited transport substrate (verapamil)-stimulated ATP hydrolysis in vitro (Brewer et al. 2014). Figure 1 shows the chemical structures of the four compounds labeled compounds 19, 29, 34, and 45. None of the compounds stimulated basal ATP hydrolysis activity by P-gp, indicating that they are not transport substrates themselves. With the help of electron spin resonance spectroscopy (ESR) titration experiments using



**Figure 1.** Chemical structures of experimental compounds. Compound 19: methyl 4-[bis(2-hydroxy-4-oxochromen-3-yl)methyl]benzoate (ZINC 09973259, CID 4694077); Compound 29: 2-[(5-cyclopropyl-1H-1,2,4-triazol-3-yl)sulfanyl]-N-[2-phenyl-5-(2,4,5-trimethylphenyl)pyrazol-3-yl]acetamide (ZINC 08767731, CID 17555821); Compound 34: 2-[1-[4-(4-methoxyphenyl)piperazin-1-yl]-1-oxobutan-2-yl]-4-methyl-[1]benzothio[2,3-d]pyridazin-1-one (ZINC 09252021, CID 22514118); Compound 45: ethyl 1-(1,3-benzodioxole-5-carbonyl)-3-(3-phenylpropyl)piperidine-3-carboxylate (ZINC 15078148, ZINC 15078146, CID 26410703, CID 45252040).

a spin-labeled ATP analog (Streckenbach et al. 1980; Delannoy et al. 2005; Hoffman et al. 2010), we were able to show that three of these compounds, 19, 34, and 45 (Fig. 1) directly affected nucleotide binding to P-gp (Brewer et al. 2014). These three compounds had been predicted to interact with P-gp close to the nucleotide-binding sites of P-gp in a kinase-inhibitor-like fashion. The fourth compound, 29 (Fig. 1), had been computationally predicted to interact with the NBD outside the nucleotide-binding sites (Brewer et al. 2014). No effects on nucleotide binding were detected for compound 29 using the ESR spectroscopic methods.

The present study was undertaken to further evaluate these novel P-gp inhibitors and to elucidate their potential as pharmaceutical lead compounds for the development of co-therapeutics for clinically reversing multidrug resistance in cancers. The intrinsic cytotoxicity and ability to resensitize an MDR prostate cancer cell line was assessed for the four identified P-gp inhibitors. The inhibitors presented here show promise as lead compounds for the development of co-therapeutics to overcome multidrug resistance in cancers. More importantly, the success of these inhibitors in cell culture demonstrates that the *in silico* screening methods employed and the subsequent biochemical evaluation have great potential to identify P-gp inhibitors with novel mechanisms of action.

## Materials and Methods

### Materials

Paclitaxel, doxorubicin, verapamil, and MTT 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from MP Biomedicals (Santa Ana, CA). P-gp inhibitors identified through *in silico* screening were purchased in small quantities through SIA MolPort (Riga, Latvia). Stock solutions (10–100 mmol/L) of all drugs and experimental compounds were prepared in DMSO and stored as aliquots at  $-20^{\circ}\text{C}$ . On the day of the experiment, working solutions of compounds prepared in DMSO were further diluted in culture media such that the final DMSO concentration was  $\leq 1\%$  (v/v). MTT solution was prepared as 5 mg/mL in phosphate buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L  $\text{Na}_2\text{HPO}_4$ , 1.8 mmol/L  $\text{KH}_2\text{PO}_4$ ) and sterile filtered through 0.22  $\mu\text{m}$  nylon filter before use. Cell culture materials were purchased from Corning Inc. (Corning, NY) unless otherwise stated. GraphPad Prism™ version 6.05 for Windows was used for plotting data and  $\text{IC}_{50}$  values were calculated from nonlinear, four-parameter logistic curve fitting (GraphPad Software, La Jolla, CA).

### Cell lines and cell culture

Chemotherapeutic naïve DU145 human prostate cancer cells (Stone et al. 1978) and multidrug-resistant DU145TXR (Takeda et al. 2007) were kind gifts from Dr. Evan Keller (University of Michigan, Ann Arbor, MI). Multidrug-resistant DU145TXR cells were derived from DU145 by culturing in the presence of stepwise increasing concentrations of paclitaxel (Takeda et al. 2007). Cells were maintained in complete media consisting of RPMI-1640 with L-glutamine, 10% fetal bovine serum (FBS; BioWest, Logan, UT), 100 U/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin in a humidified incubator at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . The drug-resistant line DU145TXR was maintained under positive selection pressure by supplementing complete media with 10 nmol/L paclitaxel. The noncancerous human fetal lung cell line, HFL1 (Breul et al. 1980) was kindly provided by Dr. Robert Harrod (Southern Methodist University, Dallas, TX) and maintained in complete media consisting of F-12K with L-glutamine, 10% FBS (BioWest, Logan, UT), 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin in a humidified incubator at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . To promote attachment of HFL1 cells, growth surfaces were treated with 0.1 mg/mL rat tail collagen (BD Biosciences, Palo Alto, CA) in 0.02 N acetic acid for 10 min and rinsed with PBS prior to use.

### MTT cell viability assay

The MTT assay is a well-established colorimetric assay which relies on the reduction of the yellow, water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble blue formazan crystals by cellular reductase activities in living cells (Mosmann 1983; Kupcsik 2011). The assay is well suited for medium-throughput evaluations in 96 well plates and was used here for initial *in vitro* toxicity and drug efficacy screening (Alley et al. 1988). Once formazan crystals are solubilized, the absorbance value at 570 nm is directly correlated with the number of metabolically active cells for a wide linear range, and the effects of drugs are reflected in an increase or decrease in the absorbance value (van Meerloo et al. 2011). Experiments were performed for each cell line to determine the optimal seeding density to ensure the cell number was within the linear range for the assay (data not shown).

MTT assays were performed as outlined previously in (Kupcsik 2011; van Meerloo et al. 2011; Riss et al. 2013) with minor modification. Cells were trypsinized from monolayers and diluted in a culture medium to a density of  $2 \times 10^4$  cells/mL before adding 150  $\mu\text{L}$  cell suspension per well in 96-well plates. After 48 h, test

compounds dissolved in DMSO or DMSO controls were diluted 1:50 or 1:100 in complete medium (media, 10% FBS, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin) and 50  $\mu\text{L}$  were added per well. At least three replicate wells per tested compound concentration and twelve replicates for DMSO controls were used. After 48 h, the liquid was aspirated and the wells were rinsed twice with PBS before adding 200  $\mu\text{L}$  of new complete media. After 24 h, 20  $\mu\text{L}/\text{well}$  of a 5 mg/mL MTT solution in PBS was added and the plates returned to the humidified incubators for 4 h to allow formazan development. About 170  $\mu\text{L}$  of the supernatant liquid was removed per well before adding 150  $\mu\text{L}$  DMSO per well. Plates were shaken for 5 min at 900 rpm on a microplate orbital shaker (LabDoctor from MidSci, St Louis, MO) and the absorbance was measured at 570 nm, using a Bio-Tek Eon plate reader (Bio-Tek, Winooski, VT). The absorbance at the 720 nm reference wavelength was subtracted from the 570 nm absorbance of each well as described in (van Meerloo et al. 2011). The mean difference of absorbance (570–720 nm) of control wells with no added cells but containing media and MTT was subtracted from all test wells. Survival was calculated as follows:

$$\% \text{ Survival} = \frac{(\text{Abs}_{570 \text{ nm}} - \text{A}_{720 \text{ nm}} \text{ of test-well average})}{(\text{Abs}_{570 \text{ nm}} - \text{A}_{720 \text{ nm}} \text{ of DMSO-treated control average})}$$

### Drug resistance of paired prostate cancer cell lines

MTT assays were performed as above to evaluate the cytotoxic effects of paclitaxel and doxorubicin on human prostate cancer cells, DU145 and DU145TXR. Stock solutions of paclitaxel and doxorubicin were diluted to 400X final test concentration in DMSO and then further diluted 1:100 into complete media. 50  $\mu\text{L}$  of each dilution was added to give a range of paclitaxel and doxorubicin concentrations. The assay was performed in triplicate wells. Percent survival was calculated using DMSO-treated cells ( $n = 12/\text{plate}$ ) as 100% survival. Results were plotted as the mean with standard deviation (SD) of at least nine replicates per concentration from at least three separate experiments. The reported “fold resistance” are the calculated  $\text{IC}_{50}$  ratios derived from DU145TXR when compared to DU145 cells. The  $\text{IC}_{50}$  values were determined using four parameter variable slope nonlinear regression, using the following equation:  $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{-(\log \text{IC}_{50} - X) * \text{HillSlope}})$  (Version 6.05; GraphPad Prism™, La Jolla CA).

### Potential assays

The MTT assay as described above was used to determine the ability of compounds 19, 29, 34, 45 (see Fig. 1), and verapamil, to increase the cytotoxic effect of 500 nmol/L paclitaxel in DU145TXR cells (Mistry et al. 2001). Paclitaxel in DMSO (0.5  $\mu\text{L}/\text{well}$  of 200  $\mu\text{mol}/\text{L}$  paclitaxel) and 0.5  $\mu\text{L}/\text{well}$  of 400X inhibitor solutions also dissolved in DMSO were added to test wells in triplicate together with 49  $\mu\text{L}/\text{well}$  complete media. The values shown represent the mean survival of cells when using DMSO-only treated cells (no added P-gp inhibitors) as 100% and result from at least six replicates for each concentration of P-gp inhibitor from at least two separate experiments. Mean survival values were used to calculate the  $\text{PC}_{50}$  concentration of inhibitors that resulted in 50% reduction in cell survival in the presence of 500 nmol/L paclitaxel. The same experiment was performed in the absence of paclitaxel to assess potential toxicity of the experimental compounds themselves. The  $\text{PC}_{50}$  values were calculated from four parameter variable slope nonlinear regressions.

### Dose-dependent sensitization

MTT assays were performed as above to evaluate the effects of 5  $\mu\text{mol}/\text{L}$ , 10  $\mu\text{mol}/\text{L}$ , and 25  $\mu\text{mol}/\text{L}$  of the novel P-gp inhibitors on sensitizing the MDR prostate cancer cell line DU145TXR to paclitaxel. The 400X working solutions of both paclitaxel and inhibitors were prepared in DMSO such that test wells received 0.5  $\mu\text{L}$  of paclitaxel (PTX) and 0.5  $\mu\text{L}$  of inhibitor solutions with 49  $\mu\text{L}$  complete media. Control wells received 49  $\mu\text{L}$  complete media and 1  $\mu\text{L}$  of DMSO ( $n = 6$  per plate). The average survival of DU145TXR in the presence of the tested concentration of the inhibitors and paclitaxel was plotted as the mean percent survival with SD representing at least six replicates per concentration from at least two individual experiments. The  $\text{IC}_{50}$  values were determined using four parameter variable slope nonlinear regression. The fold sensitization is the ratio of  $\text{IC}_{50}$  of paclitaxel alone to that of paclitaxel in the presence of inhibitor (Dale et al. 1998; Mistry et al. 2001).

### Isobologram analysis of synergistic drug action

To determine whether the inhibitors showed synergistic effects with the chemotherapeutic, paclitaxel, isobolograms of the  $\text{IC}_{50}$  values of compounds alone on the  $x$ -axis and paclitaxel alone on the  $y$ -axis were plotted. The line connecting these two points is the line of additivity (Eid et al. 2012a,b; Sun and Wink 2014). The  $\text{IC}_{50}$  values of compounds 29, 34, and 45 at 5, 10, and 25  $\mu\text{mol}/\text{L}$  in



the presence of paclitaxel were also plotted. Points located below the line of additivity indicate synergistic action of the drug combinations (Eid *et al.* 2012a,b; Sun and Wink 2014).

### Intrinsic cytotoxicity assays

MTT assays as described above were used to determine the intrinsic cytotoxicity of compounds 19, 29, 34, 45, and verapamil. 200X DMSO stock solutions were prepared for each concentration tested such that each well received 1  $\mu$ L compound solution. Control wells ( $n = 12$  per plate) received 1  $\mu$ L of DMSO. Percent survival was calculated relative to the DMSO only control wells. Mean percent survivals with SD was determined with at least 6 replicates per concentration from at least two individual experiments. Inhibitor concentrations tested were from 0.05  $\mu$ mol/L to 50  $\mu$ mol/L. Due to the limited solubility of some of the compounds, 50  $\mu$ mol/L was the highest concentration of inhibitors tested, and  $IC_{50}$  determination was therefore problematic. In order to estimate the compound  $IC_{50}$  values, four parameter variable slope nonlinear regression as above was employed, except that the bottom constraint was set to equal to 0 and top constraint was set to equal to 100. The  $IC_{50}$  values reported in Table 2 for the experimental compounds alone should therefore be regarded as estimates of the experimental compound toxicities.

## Results

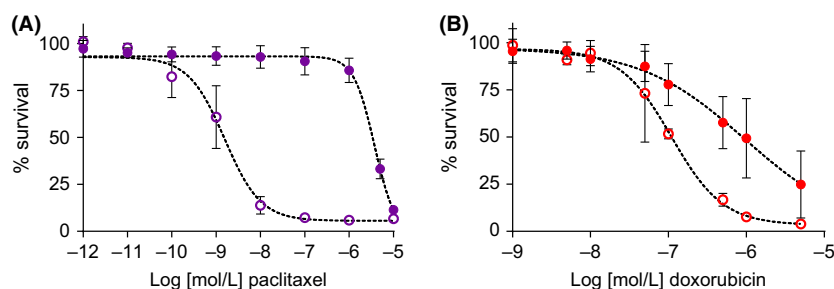
### Drug resistance of paired prostate cancer cell lines

The human prostate cancer cell line DU145 (Stone *et al.* 1978) and a P-gp over-expressing, multidrug-resistant subline, DU145TXR were used to evaluate the potential of identified P-gp inhibitors to reverse multidrug resistance in cancer cells. DU145TXR had been previously

established by exposing the drug-sensitive, DU145 cells to stepwise increased concentrations of paclitaxel (Takeda *et al.* 2007). These authors demonstrated that P-gp expression was likely the main cause of the MDR phenotype of DU145TXR by showing that the knockdown of MDR1 mRNA and P-gp expression, using MDR1 siRNA restored paclitaxel sensitivity of DU145TXR (Takeda *et al.* 2007). To determine whether these paired cell lines would be suitable for testing the potential of the *in silico* identified P-gp inhibitors (Brewer *et al.* 2014) to resensitize multidrug-resistant cells to chemotherapeutics, both DU145 and DU145TXR were exposed to the chemotherapeutics, paclitaxel, and doxorubicin, which are both P-gp substrates, and the effects on cell survival were evaluated by MTT assays. Figure 2 shows the effects of paclitaxel and doxorubicin in panels A and B, respectively, on DU145 (open circles) and the MDR DU145TXR cells (solid circles) in culture. The drug concentration that resulted in 50% reduction in cell viability ( $IC_{50}$ ) was calculated from these graphs and the fold resistance was established as the ratio of the  $IC_{50}$  values of the resistant DU145TXR cells to that of the chemosensitive DU145 cells. The  $IC_{50}$  of DU145 was determined to be about 2 nmol/L for paclitaxel and about 110 nmol/L for doxorubicin. The  $IC_{50}$  of the multidrug-resistant DU145TXR was determined to be about 3760 nmol/L for paclitaxel and 950 nmol/L for doxorubicin. The data indicate that the MDR cell line DU145TXR has >2,400-fold higher resistance to paclitaxel compared to the parental DU145 cell line and over eightfold higher resistance to doxorubicin.

### P-gp inhibitors potentiate cytotoxicity of paclitaxel for the multidrug-resistant DU145TXR cell line

Ultra-high throughput *in silico* screening for drug-like molecules that interact specifically with the nucleotide-binding domains of P-glycoprotein, but are not transport



**Figure 2.** Drug resistance of paired prostate cancer cell lines: Survival of chemotherapy naïve human prostate cancer cell line, DU145 (open circles), and P-gp overexpressing, multidrug-resistant subline, DU145TXR (closed circles), incubated with paclitaxel (A) or doxorubicin (B) for 48 h. Percent survival relative to DMSO-treated controls was determined by MTT assays as described in materials and methods. Values represent the mean  $\pm$  SD of at least nine replicates per concentration from at least three separate experiments. The data were fitted using unconstrained 4-parameter nonlinear slope regressions.

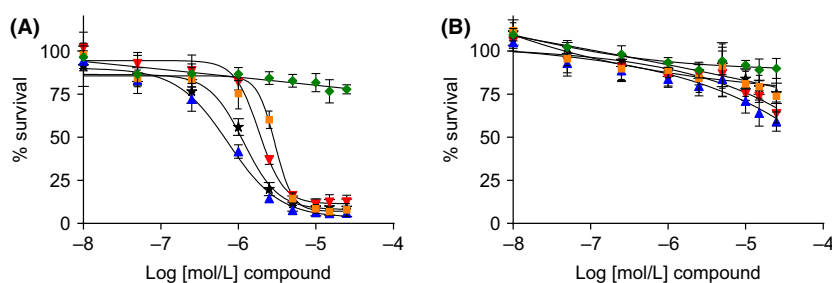
substrates of the pump led to the discovery of four compounds that inhibited P-gp ATPase activity in biochemical assays (Brewer *et al.* 2014). These compounds, compound 19, 29, 34, and 45, see Figure 1, were tested for their ability to increase the cytotoxic effects of paclitaxel in the multidrug-resistant DU145TXR cell line. DU145TXR cells were exposed to 500 nmol/L paclitaxel, a concentration that was shown in experiments as in Figure 2A to result in >85% survival of the multidrug-resistant DU145TXR but <10% survival of the sensitive DU145 cell line. Experimental inhibitors were added at a range of concentrations to the cultures in the presence of 500 nmol/L paclitaxel and cell viability was assessed by MTT assays. The results shown in Figure 3A indicate that three of the four experimental compounds, compounds 29 (squares), 34 (triangles) and 45 (inverted triangles) strongly affected the sensitivity of the resistant DU145TXR cells to 500 nmol/L paclitaxel. The effects of experimental compounds 29, 34 and 45 were similar to those of verapamil (Fig. 3A, stars). Compound 19 (diamonds) had no apparent effect on the sensitivity to paclitaxel of DU145TXR. To test whether the loss in cell viability may have been caused by the experimental compounds themselves, experiments comparable to those shown in Figure 3A were performed in the absence of paclitaxel. The results are shown in Figure 3B and clearly suggest that the resensitization of DU145TXR by compounds 29, 34, and 45 were not a result of the intrinsic toxicity of the P-gp inhibitors at the concentrations tested. Comparing Figure 3A, B shows that while 1–10  $\mu\text{mol/L}$  of the P-gp inhibitors strongly affected the sensitivity of DU145TXR to paclitaxel (Fig. 3A), the same concentrations of compounds alone resulted in more than 80% survival of DU145TXR in the absence of chemotherapeutic (Fig. 3B).

In order to compare the efficacy of the inhibitors to increase toxicity of 500 nmol/L paclitaxel, the potentiation concentrations and  $\text{PC}_{50}$  values of the inhibitors were

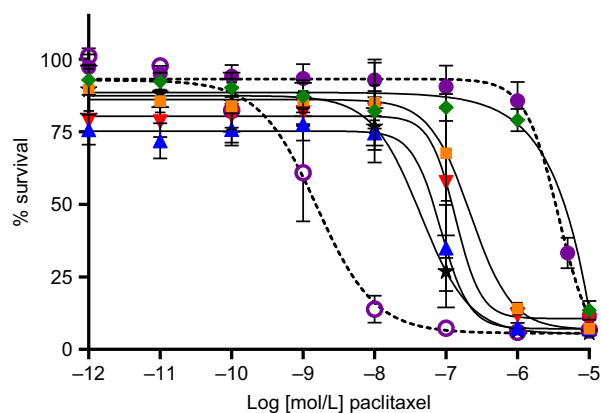
calculated from the data in Figure 3A. The  $\text{PC}_{50}$  values represent the concentrations of inhibitors that result in a 50% reduction in cell viability in the presence of 500 nmol/L paclitaxel and were calculated to be 3  $\mu\text{mol/L}$ , 0.7  $\mu\text{mol/L}$ , and 1.9  $\mu\text{mol/L}$ , for compounds 29, 34, 45, respectively. A similar  $\text{PC}_{50}$  value for the known inhibitor of P-gp, verapamil, was calculated from the data in Figure 3A to be 1.2  $\mu\text{mol/L}$ . A  $\text{PC}_{50}$  value for compound 19 could not be calculated since it had no effect on the toxicity of paclitaxel to DU145TXR at the concentrations used in these experiments.

### Dose-dependent sensitization of MDR prostate cancer cell line

To determine the degree to which the MDR prostate cancer cell line DU145TXR could be sensitized to paclitaxel, we investigated cell survival at increasing concentrations of paclitaxel in the presence of three fixed concentrations of the experimental P-gp inhibitors. Figure 4 shows as an example the survival of DU145TXR at increasing concentrations of paclitaxel in the presence of 5  $\mu\text{mol/L}$  of the experimental compounds (solid lines). For reference, the survival of chemotherapy naïve DU145 (open circles) and resistant DU145TXR (closed circles) in the presence of paclitaxel alone was included (broken lines). Cell survival in the presence of P-gp inhibitors, compound 19 (diamonds), 29 (squares), 34 (triangles), 45 (inverted triangles), and verapamil (stars) is shown. At 5  $\mu\text{mol/L}$  P-gp inhibitor concentrations, an over 10-fold increase in paclitaxel sensitivity of the DU145TXR cells was observed compared to DU145TXR without P-gp inhibitors (Fig. 4, closed circles). Doubling the concentration of experimental compounds to 10  $\mu\text{mol/L}$ , approximately doubled the DU145TXR sensitivity to paclitaxel, see Table 1. At both 5 and 10  $\mu\text{mol/L}$  inhibitor concentrations, the effects of the experimental compounds were comparable to those of the known MDR modulator, verapamil (Fig. 4 and



**Figure 3.** In silico identified P-gp inhibitors potentiate the cytotoxic effects of paclitaxel in the multidrug resistance (MDR) human prostate cancer cell line *DU145TXR*. Cells were incubated with 10 nmol/L–25  $\mu\text{mol/L}$  inhibitor 19 (diamonds), 29 (squares), 34 (triangles), 45 (inverted-triangles), or verapamil (stars) with 500 nmol/L paclitaxel (A) or without paclitaxel (B) for 48 h and survival was determined by MTT assay. Values represent the mean  $\pm$  SD of at least two separate experiments performed in triplicate.



**Figure 4.** Sensitization of multidrug resistance (MDR) prostate cancer cell line DU145TXR. DU145TXR cells (closed symbols) were incubated with various concentrations of paclitaxel and 5  $\mu\text{mol/L}$  of the in silico identified P-gp inhibitors 19 (diamonds), 29 (squares), 34 (triangles), 45 (inverted triangles) and verapamil (stars). For reference, the DU145TXR (closed circle) and DU145 (open circle) incubated with paclitaxel only are also included. Values are mean  $\pm$  SD from at least 2 separate experiments performed in triplicate wells. The data were fitted using nonconstrained four-parameter nonlinear slope regressions.

**Table 1.** Sensitization of MDR prostate cancer cell line DU145TXR to paclitaxel.

	Paclitaxel $\text{IC}_{50}$ (nmol/L)	Fold sensitization
DU145	2	
DU145TXR	3759	
DU145TXR + Compound 29		
5 $\mu\text{mol/L}$	220	17
10 $\mu\text{mol/L}$	76	50
25 $\mu\text{mol/L}$	4	800
DU145TXR + Compound 34		
5 $\mu\text{mol/L}$	85	44
10 $\mu\text{mol/L}$	26	145
25 $\mu\text{mol/L}$	4	895
DU145TXR + Compound 45		
5 $\mu\text{mol/L}$	135	28
10 $\mu\text{mol/L}$	85	44
25 $\mu\text{mol/L}$	7	515
DU145TXR + Verapamil		
5 $\mu\text{mol/L}$	44	85
10 $\mu\text{mol/L}$	30	127
25 $\mu\text{mol/L}$	11	330

$\text{IC}_{50}$  values calculated using nonlinear, four-parameter logistic curve fitting from plotted mean survival determined by MTT assay with at least six replicates per concentration from at least two separate experiments. Fold sensitization is the ratio of  $\text{IC}_{50}$  paclitaxel alone to  $\text{IC}_{50}$  paclitaxel plus compound.

Table 1). At 25  $\mu\text{mol/L}$  P-gp inhibitor concentrations, compounds 29 and 34 sensitized the multidrug-resistant DU145TXR to  $\text{IC}_{50}$  values that were close to those observed for the parental, sensitive DU145 cell line, an

800- and 895-fold sensitization, respectively (Table 1). Sensitization of DU145TXR to paclitaxel by the experimental compounds 29, 34 and 45 exceeded sensitization by verapamil (Table 1). Compound 19 did not show any effect on cell viability in any of the experiments at any of the tested concentrations and was therefore excluded from Table 1. In similar experiments as shown in Figure 4, reversal of resistance of DU145TXR to doxorubicin exposure was evaluated at 25  $\mu\text{mol/L}$  of compounds 29, 34 and 45 (data not shown). At this concentration, all three compounds resensitized the MDR prostate cancer cells to doxorubicin to levels that are comparable with the naïve DU145 cell line. Compound 19 was not evaluated since it did not show any effects in any of the other resensitization experiments.

### Intrinsic toxicity of inhibitors on cancerous and noncancerous cells

The intrinsic in vitro toxicities of the identified compounds were evaluated to determine their potential as therapeutic lead compounds. Toxicity was assessed in the noncancerous human fibroblast cell line HFL1 (Breul et al. 1980) as well as the human prostate cancer cell lines DU145 and its MDR subline DU145TXR (Takeda et al. 2007). In vitro cytotoxic concentrations determined using the HFL1 cell line have been shown to be comparable to whole animal toxicity testing as predictors of human toxicity (Barile and Cardona 1998; Yang et al. 2002). In experiments similar to the one shown in Figure 3B, the three cell lines, HFL1, DU145 and DU145TXR were exposed to verapamil as well as compounds 19, 29, 34, and 45, at concentration ranges from 0.05 to 50  $\mu\text{mol/L}$  for 48 h and survival was determined, using the MTT assay. Unfortunately, some of the compounds displayed very limited solubility at concentrations higher than 50  $\mu\text{mol/L}$  so that no toxicity data were determined for higher concentrations. The  $\text{IC}_{50}$  values of the four experimental compounds and verapamil were therefore estimated using bottom and top constraints in the four parameter variable slope nonlinear regression described in Materials and Methods and are shown in Table 2. No  $\text{IC}_{50}$  could be determined for compound 19.

### Discussion

Over 30 years of research aimed at finding inhibitors of drug export by the multidrug resistance P-glycoprotein have failed to identify compounds that robustly reverse multidrug resistances in clinical cancer chemotherapy (Robert and Jarry 2003; Coley 2010; Crowley et al. 2010; Palmeira et al. 2012). The majority of previously identified P-gp inhibitors were themselves, to varying degrees,

**Table 2.** In vitro cytotoxicity of identified compounds.

IC <sub>50</sub> (μmol/L)	Compound 19	Compound 29	Compound 34	Compound 45	Verapamil
HFL1	ND	42	133	62	ND
DU145	99	34	44	41	75
DU145TXR	ND	39	412	107	207

IC<sub>50</sub> values were calculated using nonlinear, four-parameter logistic curve fitting constrained from 0 to 100 from the plotted mean survival determined by MTT assay with at least six replicates per concentration from at least two separate experiments. ND, not determined.

transport substrates of P-gp and inhibited export of chemotherapeutics by competing for transport cycles or drug binding to P-gp (Saeki et al. 1993a,b). The most promising recent inhibitor candidate, tariquidar, has also been suggested to bind to the drug-binding sites of P-gp (Chufan et al. 2013), but seems more encouraging in that it seems to block conformational changes in P-gp needed for drug transport (Loo and Clarke 2014). Accumulation of tariquidar in cells over-expressing P-gp (Martin et al. 1999; Kannan et al. 2011) indicates that this inhibitor is not transported by P-gp, and may therefore have an improved prospect of being active also in clinical application. A recent computational study (McCormick et al., 2015) from our lab suggested that tariquidar preferably binds to the cytoplasmic extracellular loop region of P-gp. If bound there, the compound was not pumped across the membrane leaflet in our computational studies. A second, less preferred binding site was predicted to be within the drug binding domains of P-gp. Tariquidar bound to these sites was transported by P-gp in these *in silico* studies.

We hypothesized that one potential reason for the failure to identify potent inhibitors of P-gp may have been that many of the early generation inhibitors interact with the drug binding domains of the protein. Targeting the drug binding parts of the protein potentially poses two problems. (1) The drug binding sites are highly flexible, allow binding of chemically very diverse substrates and often bind more than one substrate at a time (Dey et al. 1997; Martin et al. 2000; Aller et al. 2009). These characteristics make structure-activity predictions immensely challenging. (2) Despite the differing toxicities and degrees of efficacies shown by previous inhibitors, the fact that many are removed from cells by P-gp may prevent the necessary therapeutic concentration of inhibitor to accumulate in MDR cancer cells.

In a recent study we presented the use of ultra-high throughput computational methods to search for inhibitors of the multidrug resistance P-glycoprotein that have novel mechanisms of action in that they do not compete with transport substrates but interfere with the energy harvesting steps of the enzyme (Brewer et al. 2014). Drug-like compounds were sought that strongly inter-

acted with the nucleotide-binding domains (NBD) and inhibited P-gp through blocking the steps needed to convert the energy of ATP into conformational changes that cause drug export. In our studies we used unique subtractive screens of several million drug-like compounds that did not discriminate for chemical classes or structures. We identified several hundred that fulfilled our criteria of predicted weak interactions with the drug-binding domains but predicted strong interactions with the NBDs of P-gp. Of these initial hits, about 40 compounds were selected for predicted binding close to and partially overlapping with the nucleotide-binding sites. Some of the 40 that were chosen were predicted to interact at positions outside of the nucleotide-binding sites where allosteric effects may inhibit the protein from adopting crucial catalytic conformations. These 40 compounds were screened biochemically and biophysically and four compounds, 19, 29, 34 and 45 (Fig. 1 and (Brewer et al. 2014)), were found to inhibit ATP hydrolysis of P-gp in the presence of the transport substrate, verapamil (Saitoh and Aungst 1995). None of the compounds stimulated basal ATP hydrolysis activity, suggesting that they were indeed not transport substrates of the pump. Using ESR titration experiments and an ESR active ATP analog, we showed that three of these compounds, 19, 34 and 45, directly interfered with nucleotide binding, while the fourth, compound 29, did not affect nucleotide-binding characteristics. Compound 29 was predicted computationally to bind at an allosteric region of the nucleotide-binding domains outside the nucleotide-binding sites (Brewer et al. 2014). It is interesting to note that compound 19 (Fig. 1) may be considered a flavonoid as it contains both A and C as well as the modified B ring of flavonol. The predicted docking position and docking mode of 19 is very similar to flavonoid binding as studied in (Badhan and Penny 2006). It has previously been shown and predicted by others that flavonoids interact with the nucleotide-binding domains of the enzyme and are strong modulators of P-gp activity (Di Pietro et al. 2002), for recent study see (Saeed et al. 2015).

The computationally identified P-gp inhibitors presented here can be qualified as either kinase-like inhibitors, interacting close to the nucleotide-binding sites and



interfering with nucleotide binding by P-gp, or allosteric inhibitors that bind outside of the nucleotide-binding regions. Compounds such as these make interesting leads for the development of potent P-gp inhibitors which may not encounter the drawbacks of previous P-gp inhibitors resulting from interaction with the drug transporting domains. The kinase-like inhibitors of P-gp, however, potentially impose a different set of problems in that they may interact with other nucleotides utilizing enzymes and result in unacceptable toxicities due to blocking of important cellular functions. We have prescreened some of these potential negative side effects and showed that the kinase and dehydrogenase used in the coupled enzyme assay to determine ATP hydrolysis by P-gp (pyruvate kinase and lactate dehydrogenase) were not affected by the identified P-gp inhibitors (Brewer *et al.* 2014). Preliminary results from our lab furthermore indicated that the close relative of P-gp, the multidrug resistance-associated protein, MRP3, was also not inhibited by the P-gp inhibitors (Brewer, F.K., Wise, J.G., Vogel, P.D., unpublished data).

In this present study, we evaluated the effects of these four inhibitors of P-gp on multidrug-resistant and naïve cancer cells in culture. We also assessed the overall toxicity of the compounds in chemotherapy naïve cancer cells, multidrug-resistant cancer cells, and noncancerous cells.

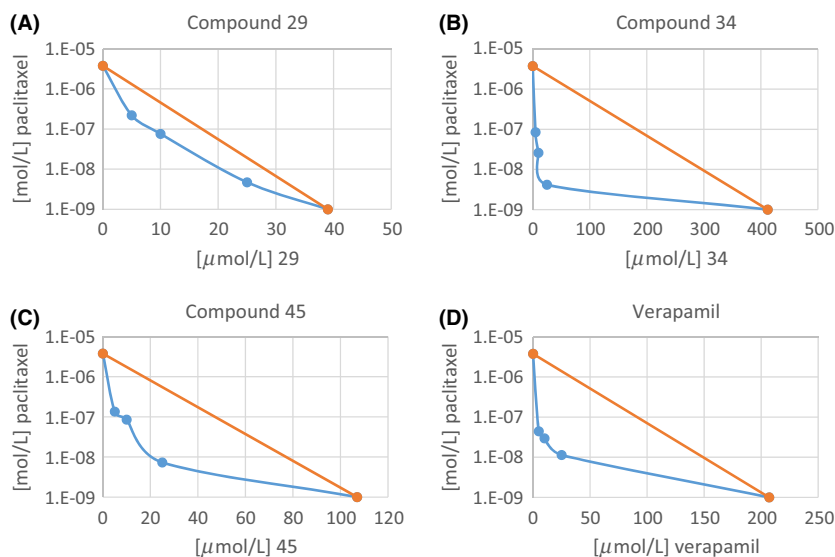
The prostate cancer cell line DU145 and its multidrug-resistant derivative line, DU145TXR, were chosen for this study. DU145TXR was created by exposure of DU145 cells to increasing concentrations of the chemotherapeutic, paclitaxel (Takeda *et al.* 2007), one of the chemotherapeutics of choice for treatment of prostate cancer. These authors showed that multidrug resistance of DU145TXR was attained by overexpression of P-gp compared to the drug-sensitive DU145, which does not express P-gp. Figure 2 shows that in our hands the DU145TXR cells possess approximately 2400-fold increased resistance to paclitaxel and about eightfold increased resistance to the nonstandard (for prostate cancer) chemotherapeutic, doxorubicin, as compared to DU145. This result is consistent with the fact that both chemotherapeutics are transport substrates of P-gp (Ohnishi *et al.* 1995).

To determine the efficacy of resensitizing multidrug-resistant cancer cells, varying concentrations of the experimental compounds were administered together with 500 nmol/L paclitaxel to the multidrug-resistant DU145TXR cells. Each of the P-gp inhibitors 29, 34 and 45, but not compound 19, restored DU145TXR sensitivity to 500 nmol/L paclitaxel (Fig. 3A) with potentiation concentrations ( $PC_{50}$ ) for 29, 34 and 45 in the low micromolar range, very similar to verapamil (Fig. 3A).

When the novel P-gp inhibitors 29, 34 and 45 were added at 25  $\mu$ mol/L to the cancer cells in experiments similar to Figure 2A, sensitivity to both paclitaxel and

doxorubicin of DU145TXR was restored (Table 1, and data not shown). This concentration of inhibitors was previously shown to lead to robust inhibition of P-gp-catalyzed ATP hydrolysis (Brewer *et al.* 2014). The recovered sensitivities to paclitaxel were comparable to that of the parental, non-MDR DU145 cell line (Table 1). The effects of the novel inhibitors exceeded those of the known multidrug resistance modulator, verapamil, which was one of the first MDR inhibitors reported (Tsuruo *et al.* 1981; Ozols *et al.* 1987). Compound 19 showed no effect on DU145TXR cells chemotherapy sensitivity. The lack of activity of 19 may be due to the fact that the two 4-hydroxycoumarin residues of compound 19, see Figure 1, would likely be deprotonated at the neutral pH of the cell culture experiments, making the compound unable to enter cells. When inhibitors 29, 35 or 45 were added at lower concentrations, i.e. 10 or 5  $\mu$ mol/L, correspondingly lower, but still significant re-sensitization to paclitaxel for the MDR cell line was observed (Fig. 4 and Table 1). This result suggests a direct correlation between the concentrations of P-gp inhibitors and the extent of re-sensitization of the MDR DU145TXR to paclitaxel.

Three of the compounds seemed to directly interact with the nucleotide-binding sites of P-gp when assessed using electron resonance spectroscopy (ESR) with an ESR active ATP analog, SL-ATP (Brewer *et al.* 2014). As mentioned above, this finding raised the question whether other ATP-utilizing enzymes would also be affected by the P-gp inhibitors. Even though initial biochemical findings suggested some specificity of protein interaction with the novel P-gp inhibitors as discussed above, there still was the possibility that the compounds would inhibit other essential enzymes. If this was the case, these compounds would likely exhibit high toxicity to both cancerous and noncancerous cells in the absence of the chemotherapeutics. To test for potential cytotoxicity, noncancerous HFL1 cells were incubated with P-gp inhibitors at concentrations between 0.05  $\mu$ mol/L and 50  $\mu$ mol/L similar to Figure 3B. HFL1 cells have been shown to be comparable to whole animal toxicity testing and good predictors of human toxicity (Barile and Cardona 1998; Yang *et al.* 2002). To compare cytotoxicity and estimate a potential therapeutic concentration window for the novel inhibitors, cell viability was tested in these noncancerous cells as well as in the two prostate cancer cell lines, DU145 and DU145TXR. At 25  $\mu$ mol/L, the concentration where compounds 29, 34 and 45 caused robust inhibition of P-gp ATPase activity (Brewer *et al.* 2014) and nearly full reversal of multidrug resistance (see Table 1), compounds 29, 34 and 45 did show some intrinsic cytotoxicity to noncancerous HFL1 cells. Viability of the cells decreased by 35%, 23% and 13%, respectively (not shown). It is interesting that at this same concentration



**Figure 5.** Synergistic effects of experimental compounds in resensitizing multidrug resistant (MDR) DU145-TXR cancer cells to paclitaxel. The  $IC_{50}$  values determined for paclitaxel alone for DU145TXR were plotted on the  $y$ -axis and the estimated  $IC_{50}$  values for experimental compounds 29 (A), 34 (B), 45 (C) and known inhibitor verapamil (D) (from Table 2) were plotted on the  $x$ -axis. Synergistic effects of inhibitor compounds and paclitaxel are obvious when the  $IC_{50}$  values of paclitaxel/inhibitor combinations are below the line that connects the individual  $IC_{50}$  values of inhibitor and chemotherapeutic. Here, the combination  $IC_{50}$  values are plotted for 5, 10, and 25  $\mu\text{mol/L}$  of compound 29 (A), 34 (B), 45 (C) and verapamil (D) (from Table 1) in the presence of paclitaxel. Because 0 is not permitted on a logarithmic scale, data points at  $10^{-9}$  mol/L paclitaxel are meant to represent 0 mol/L paclitaxel, thus indicating the  $IC_{50}$  of compounds in the absence of paclitaxel.

of compounds 34 and 45, significantly higher toxicity was observed for the prostate cancer cell line, DU145, and its P-gp-expressing MDR subline, DU145TXR (not shown). The greatest difference was observed for compound 45 which displayed very low toxicity (13%) to HFL1 at 25  $\mu\text{mol/L}$  but killed 31% and 36% of DU145 and DU145TXR, respectively (not shown).

To evaluate the intrinsic toxicity,  $IC_{50}$  values for compounds 29, 34 and 45 for the noncancerous HFL-1 cell lines as well as the MDR and naïve cancer cell lines were calculated. Unfortunately, concentrations of inhibitors above 50  $\mu\text{mol/L}$  could not be tested due to solubility limitations. At 50  $\mu\text{mol/L}$  the compounds did exhibit cytotoxicity, but even though the curves were clearly on a downward trend (not shown), there was still significant cell viability.  $IC_{50}$  values therefore were estimated by non-linear fit constraining the top and bottom to 100 and 0, respectively. The estimated  $IC_{50}$  values shown in Table 2 indicate that compound toxicity was observed at concentrations significantly above the low micromolar concentrations needed to sensitize the multidrug-resistant cancer cells to paclitaxel (Fig. 3A). These results suggest that no major interaction of the experimental P-gp inhibitors with other, nontarget proteins have occurred.

To determine whether the inhibitors showed synergistic effects with the chemotherapeutic, paclitaxel, isobolograms of the  $IC_{50}$  values of compounds alone on the

$x$ -axis and paclitaxel alone on the  $y$ -axis were plotted, Figure 5. The  $IC_{50}$  values of compounds 29, 34, and 45 at 5, 10, and 25  $\mu\text{mol/L}$  in the presence of paclitaxel, similar to (Eid *et al.* 2012a,b; Sun and Wink 2014) were plotted. Analysis of the data clearly suggests that compounds 29, 34, and 45 have a strong synergistic effect with paclitaxel toxicity. Compound 19 was not evaluated since it did not show any significant effects.

## Conclusion

P-glycoprotein inhibitors are presented here that were computationally identified on the premise that they bind preferentially to the nucleotide-binding domains of the protein and inhibit drug export by interfering with the energy harvesting mechanism of the pump. Three of four of these molecules reversed chemoresistance in a multidrug-resistant prostate cancer cell line while not showing significant cytotoxicity to noncancerous HFL1 cells at the concentrations where they reversed multidrug resistance.  $PC_{50}$  values were determined to be in the low micromolar range. These compounds may be considered excellent leads for optimization as P-glycoprotein inhibitor co-therapeutics that are likely not transport substrates themselves. These inhibitor candidates may therefore have improved characteristics compared to earlier generation inhibitors. Further studies regarding drug-drug interac-

tions as well as optimization of drug characteristics like solubility and toxicity are needed before these compounds may become interesting for clinical use.

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## Author Contributions

Follit, Brewer, Wise, and Vogel participated in research design, while Follit conducted the experiments; Follit, Wise, and Vogel performed data analysis; and Follit, Brewer, Wise, and Vogel wrote or contributed to the writing of the manuscript.

## Disclosure

None declared.

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