Identifying a Resistance Determinant for the Antimitotic Natural Products Disorazole C₁ and A₁^S

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ABSTRACT

Disorazoles are macrocyclic polyketides first isolated from the fermentation broth of the myxobacterium *Sorangium cellulosum*. Both the major fermentation product disorazole A₁ and its much rarer companion disorazole C₁ exhibit potent cytotoxic activity against many human tumor cells. Furthermore, the disorazoles appear to bind tubulin uniquely among known antimitotic agents, promoting apoptosis or premature senescence. It is uncertain what conveys tumor cell sensitivity to these complex natural products. Therefore, we generated and characterized human tumor cells resistant to disorazole C₁. Resistant cells proved exceedingly difficult to generate and required single step mutagenesis with chronic stepwise exposure to increasing concentrations of disorazole C₁. Compared with wild-type HeLa cells, disorazole C₁-resistant HeLa/DZR cells were 34- and 8-fold resistant to disorazole C₁ and disorazole A₁ growth inhibition, respectively. HeLa/DZR cells were also re-

markably cross-resistant to vinblastine (280-fold), paclitaxel (2400-fold), and doxorubicin (47-fold) but not cisplatin, suggesting a multidrug-resistant phenotype. Supporting this hypothesis, MCF7/MDR cells were 10-fold cross-resistant to disorazole C₁. HeLa/DZR disorazole resistance was not durable in the absence of chronic compound exposure. Verapamil reversed HeLa/DZR resistance to disorazole C₁ and disorazole A₁. Moreover, HeLa/DZR cells expressed elevated levels of the drug resistance ATP-binding cassette ABCB1 transporter. Loss of ABCB1 by incubation with short interfering RNA restored sensitivity to the disorazoles. Thus, the multidrug resistance transporter ABCB1 can affect the cytotoxicity of both disorazole C₁ and A₁. Disorazole C₁, however, retained activity against cells resistant against the clinically used microtubule-stabilizing agent epothilone B.

The myxobacterium *Sorangium cellulosum* has been a rich source of pharmacologically interesting secondary metabolites, including the clinically used semisynthetic analog of epothilone B ixabepilone (Chin et al., 2006). Disorazole polyene macrodiolides were isolated first in 1994 and observed to have significant antifungal activity with no antibacterial activity (Jansen et al., 1994). Initial biochemical and pharmacological studies focused on the major fermentation product disorazole A₁, which blocks cell proliferation, causes G₂/M phase arrest and loss of microtubules, and induces apoptosis (Elnakady et al., 2004; Kopp et al., 2005). More recently, we synthesized and explored the actions of the minor fermenta-

tion component, disorazole C₁, in part because it lacked the reactive epoxide found on disorazole A₁ (Supplemental Fig. 1). Remarkably, disorazole C₁ has potent antiproliferative activity against a wide variety of human tumor cells, it disrupts cellular microtubule integrity, it blocks microtubule polymerization in vitro, it binds tubulin in a unique manner, and it causes apoptosis and premature cellular senescence, all attributes associated with a promising anticancer agent (Wipf and Graham, 2004; Wipf et al., 2006; Tierno et al., 2009).

Disorazole resistance has not been extensively studied; the limited existing literature suggests the natural product disorazole A₁ is not a substrate for the ABCB1 multiple drug resistance transporter (Elnakady et al., 2004). To help clarify further the actions of the disorazoles and the biochemical factors that might impart resistance to their pharmacological actions, we attempted to generate tumor cells that were resistant to their growth inhibitory properties.

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ABBREVIATIONS: ABC, ATP-binding cassette; DMEM, Dulbecco's modified Eagle's medium; Rev, revertant; siRNA, short interfering RNA; EMEM, Eagle's minimal essential medium.

Our initial efforts to establish resistant cell populations by exposing cells to stepwise increased concentrations of disorazole C_1 were unsuccessful (Tierno et al., 2009). In the current study, we have successfully made the first disorazole-resistant cell population using a combination of single step chemical mutagenesis and multistep exposure to increasing concentrations of disorazole C_1 . These cells displayed a multidrug-resistant phenotype and overexpressed the ABCB1 transporter. Use of chemical inhibitors or small interfering RNA against the ABCB1 transporter restored growth inhibition by disorazole C_1 . Significantly, disorazole C_1 retained growth inhibitory activity against epothilone B-resistant cells.

Materials and Methods

Cell Culture Reagents and Proliferation Assays. HeLa, MCF7, MCF7/MDR, A549 (ATCC, Manassas, VA), and epothilone B-resistant A549/EpoB40 cells (a gift from Susan Band Horwitz, Albert Einstein College of Medicine) were cultured in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Cellgro, Mediatech, Herndon, VA). The A549/EpoB40 cells were grown in the presence of 40 nM epothilone B, and 24 h before experimental use the medium was removed, cells were detached with 0.05% trypsin/EDTA, and the washed cells were replated in epothilone B-free medium. Disorazole C_1 was synthesized as described previously (Wipf and Graham, 2004). Unless otherwise indicated, all media, sera, and supplements were obtained from Invitrogen (Carlsbad, CA), and other reagents were from Sigma-Aldrich (St. Louis, MO).

Inhibition of growth was determined using our previously described method (Vogt et al., 2004, 2008). In brief, we fixed cells in 3.7% formaldehyde and stained nuclei with 2 μ g/ml Hoechst 33342

dye to quantify cells using an ArrayScan VTi imaging cytometer (Thermo Fisher Scientific, Waltham, MA) (Vogt et al., 2004, 2008). The 50% growth inhibitory concentration (IC_{50}) of a test agent was calculated after a 72-h incubation. For all studies cells were continuously exposed to compounds in complete medium.

Development of the HeLa/DZR Subline. HeLa cells at 90% confluence were mutagenized by a 24-h exposure to 4 mM ethyl methane sulfonate in DMEM without fetal bovine serum. Cells were rinsed twice with sterile phosphate-buffered saline, once with complete DMEM, and then allowed to recover for 5 days in complete DMEM. Resistant cells were selected by a stepwise increase in the concentration of disorazole C_1 over a period of 9 months. Cells (90% confluent) were first exposed for 24 h to 100 pM disorazole C_1 in complete DMEM. After rinsing twice with sterile phosphate-buffered saline and once with complete DMEM, the cells were allowed to recover in complete DMEM until they reached 90% confluence. The cells were then treated with increasing concentrations of disorazole C_1 (~300 pM/treatment cycle), washed, and allowed to recover again. Cells were periodically tested for increased resistance to disorazole C_1 compared with wild-type HeLa cells. Resistance was quantified by comparing IC_{50} values of the mutagenized, disorazole-treated cells with those of wild-type HeLa cells. Stepwise increase of disorazole C_1 treatment was stopped at 10.8 nM with a level of ~30-fold resistance. To maintain resistance, HeLa/DZR cells were exposed to 10.8 nM disorazole C_1 every 2 weeks and then rinsed and allowed to recover as described above. A revertant cell line (HeLa/DZR/Rev) was developed by culturing HeLa/DZR cells for 32 passages without exposure to disorazole C_1 .

Verapamil Reversal. Wild-type HeLa and HeLa/DZR cells were seeded in 96-well plates at a density of 1500 cells per well and allowed to attach overnight. After attachment, cells were incubated for 72 h in the presence or absence of 5 μ M verapamil with various concentrations of disorazole C_1 , disorazole A_1 , and vinblastine. Cells

Fig. 1. Disorazole C_1 -resistant cells are cross-resistant to other chemotherapeutic agents. Cells were incubated with various concentrations of compounds for 72 h, and the total cell number determined by Hoechst staining nuclei using an ArrayScan II reader as described under *Materials and Methods*. HeLa wild-type cells (circles) and HeLa/DZR cells (squares). Each value is the mean of three determinations. Bars equal S.E.M. unless the value is less than the size of the symbol. A, disorazole C_1 . B, disorazole A_1 . C, vinblastine. D, doxorubicin. E, paclitaxel. F, cisplatin.

were then fixed with 3.7% formaldehyde, and nuclei were stained with 2 μ g/ml Hoechst 33342 dye to quantify cells using an ArrayScan VTi imaging cytometer as described previously (Vogt et al., 2004, 2008).

siRNA Knockdown of ABCB1. Three unique siRNA duplexes targeting ABCB1 were obtained from Invitrogen: ABCB1HSS107917 sequences (5'–3') GAGUGGGCACAAACCAGAUUAUU and AAUAUUAUCUGGUUUGUGCCCACUC; ABCB1HSS107918 sequences CCAUAAAUGUAAGGUUUCUACGGGA and UCCCGUAGAAACCUUACAUAUUAUGG and AACUUGACCAGCAUCGG; and ABCB1HSS107919 sequences GCUCGCCAUGAUGCUGCUCAAGUU and AACUUGAGCAGCAUCAUUGGCGAGC. HeLa/DZR cells were transfected with 20 nM ABCB1 siRNA or scrambled (Stealth siRNA Negative Control Hi GC; Invitrogen) siRNA as follows: HeLa/DZR cells were plated at a density of 7.5×10^4 cells/well into a six-well tissue culture plate in antibiotic-free EMEM. The next day, three wells each were transfected with 20 nM ABCB1 siRNA or scrambled siRNA using 5 μ l/well Dharmafect 1 reagent (Dharmacon RNA Technologies, Lafayette, CO) and 480 μ l/well Opti-MEM transfection medium (Invitrogen) in a total volume of 2 ml/well. Dharmafect and siRNAs were preincubated separately in half the final volume of Opti-MEM for 5 min before being combined in an RNase-free tube and incubated for 20 min. Antibiotic-free EMEM was then added to a final volume of 2 ml/well. Culture medium was removed from the cells and replaced with the medium containing transfection reagents. Five hours later, this medium was removed and replaced with complete EMEM. After 24 h, cells were detached with 0.05% trypsin, seeded into 96-well plates at a density of 1000 cells/well, and allowed to attach overnight. For some studies, cells were treated 48 h after transfection with compounds and growth inhibition determined 72 h later.

Western Blotting. To determine relative ABCB1 expression, we allowed HeLa, HeLa/DZR, HeLa/DZR/Rev, MCF7, and MCF7/MDR cells to grow almost to confluence in six-well plates and then harvested them in ice-cold lysis buffer (50 mM Tris, pH 7.6, containing 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100) supplemented with protease and phosphatase inhibitors as described previously (Achiwa and Lazo, 2007). Protein concentrations in cell lysates were determined with the bicinchoninic acid protein assay kit (Promega, Madison, WI). Equivalent protein amounts from cell lysates were resolved on 6 or 8% Tris glycine gels (Invitrogen) and transferred to nitrocellulose membranes via iBlot. ABCB1 and β -tubulin expression were detected by Western blotting with the ABCB1 antibody (C-19), and the β -tubulin antibody (CLT9003; Cedarlane Laboratories, Burlington, ON, Canada). As a control for ABCB1 protein, we used MES-SA/Dx5 cell lysate from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). To determine the protein depletion by siRNA, we transfected HeLa/DZR cells with siRNA duplexes targeting ABCB1 (Stealth Select RNAi; Invitrogen) or scrambled siRNA negative control at concentrations of 20 and 40 nM. After 24 to 72 h, cells were lysed and analyzed by Western blot as described above using β -tubulin as the loading control.

Results

Development of Disorazole C_1 -Resistant Cells. We initially treated exponentially growing wild-type human HeLa or A549 cells with various concentrations of disorazole C_1 , attempting to harvest compound-resistant surviving cells, but this proved nonproductive. Thus, we adopted a traditional single step mutagenesis compound selection strategy. HeLa cells were treated for 24 h with 4 mM ethyl methane sulfonate, and after washing with DMEM cells were allowed to recover for 5 days in complete DMEM. Resistant cells were selected by a stepwise increase in the concentration of disorazole C_1 over a period of 9 months. The resulting HeLa/DZR cells had a disorazole C_1 IC_{50} value of 5.1 nM

compared with an IC_{50} value of 0.15 nM for the wild-type HeLa cells (34-fold resistance; Fig. 1). The disorazole A_1 IC_{50} values for HeLa and HeLa/DZR cells were 0.05 and 0.40 nM (8.4-fold resistance), respectively. Surprisingly, the HeLa/DZR cells displayed a higher cross-resistance to vinblastine, doxorubicin, and paclitaxel relative to HeLa cells: 279-, 47- and 2408-fold, respectively (Fig. 1). We found no cross-resistance to cisplatin, indicating that the resistance did not extend to all cytotoxic chemotherapeutic agents.

Disorazole C_1 -Resistant Cells Overexpress ABCB1 and Loss of ABCB1 Restores Sensitivity to Disorazoles.

Because of the resistance profile in HeLa cells, we next examined the disorazole sensitivity of naive cells that previously had not been treated with disorazole but expressed elevated levels of the drug transporter ABCB1. MCF7/MDR cells had significantly more ABCB1 than MCF7 cells and were 10-fold more resistant to disorazole C_1 than MCF7 cells (Fig. 2A). These ABCB1-overexpressing cells were 79-fold resistant to vinblastine, as expected (Fig. 2B).

We noted during the development of the HeLa/DZR cells that disorazole C_1 resistance required repeated biweekly exposures to low (10.8 nM) concentrations of disorazole C_1 to enforce the resistant phenotype. We next determined the stability of the resistance phenotype by culturing HeLa/DZR cells for 32 passages without exposure to disorazole. This led to a revertant cell line called HeLa/DZR/Rev, which was

Fig. 2. Multidrug-resistant cells are cross-resistant to disorazole. Cells were incubated with various concentrations of compounds for 72 h, and the total cell number was determined by Hoechst staining nuclei using an ArrayScan reader as described under *Materials and Methods*. MCF-7 cells (closed triangles) and MCF-7/MDR cells (open triangles). Each value was the mean of four determinations. Bars equal S.E.M. unless the value was less than the size of the symbol. A, disorazole C_1 . B, vinblastine.

almost as sensitive to disorazole C_1 as the parental HeLa cells (Fig. 3A). The HeLa/DZR/Rev also lacked any significant resistance to vinblastine (Fig. 3B). These results indicate that the disorazole C_1 -resistant phenotype developed in HeLa/DZR cells was reversible.

Disorazole C_1 -Resistant Cells Have a Phenotype That Is Similar to ABCB1-Overexpressing Cells. Because the HeLa/DZR cells exhibited a drug resistance profile reminiscent of classical multidrug resistant cells, we next

examined the effect of a classical reversing agent, verapamil, on the growth inhibition caused by disorazole C_1 , disorazole A_1 , and vinblastine (Fig. 4). Cotreatment of HeLa/DZR cells with verapamil caused a complete reversal of resistance to disorazole C_1 , whereas the growth inhibitory effects of disorazole C_1 with HeLa cells were unaffected (Fig. 4A). Similar results were found with disorazole A_1 (Fig. 4B). Verapamil also sensitized HeLa/DZR and HeLa cells to vinblastine (Fig. 4C). These results suggest that the disorazole-resistant cells might overexpress one of the major contributors of a multidrug resistant phenotype: ABCB1.

Disorazole C_1 -Resistant Cells Overexpress ABCB1 and Loss of ABCB1 Restores Sensitivity to Disorazole C_1 .

We next examined the disorazole-resistant cells for expression of ABCB1. As illustrated in Fig. 5A, lane 2, the ABCB1 protein levels in HeLa/DZR cells were markedly elevated, exceeding those in the well characterized MES-SA/Dx5 cells (Fig. 5A, lane 4). Significantly, ABCB1 levels in the HeLa/DZR/Rev cells were comparable with the levels found in the parental HeLa cells (Fig. 5A, lanes 1 and 3). To further evaluate the role of ABCB1 in disorazole resistance, we transiently suppressed ABCB1 levels in HeLa/DZR cells with siRNA (Fig. 5B). We found 20 and 40 nM siRNA caused a >75% reduction in ABCB1 protein levels after 24- to 72-h exposure (Fig. 5B, lanes 2 and 3). HeLa/DZR cells regained complete sensitivity to disorazole C_1 after treatment with ABCB1 siRNA (Fig. 6A), although the transfection process appeared to cause partial sensitization in that the cells treated with scrambled siRNA were slightly more responsive to disorazole C_1 than the untreated cells. HeLa/DZR cells were also sensitized to disorazole A_1 by ABCB1 siRNA. In addition, it is interesting to note that these cells were not affected by scrambled siRNA (Fig. 6B). Although HeLa/DZR cells were sensitized to vinblastine by ABCB1 siRNA, the sensitization was not complete (Fig. 6C). In contrast, ABCB1 siRNA had no effect on the cisplatin sensitivity of HeLa/DZR (Fig. 6D).

Epothilones are the most recently FDA-approved and clinically used microtubule-targeting anticancer agents. Despite their size and functional group complexity, these natural product-based agents are generally believed to be unaffected by ABCB1 (Bollag et al., 1995). Epothilone-resistant cells, however, are cross-resistant to another common clinically used natural product, which is affected by ABCB1: paclitaxel

Fig. 3. Disorazole C_1 resistance is reversible. Cells were incubated with various concentrations of compounds for 72 h, and the total number of HeLa (closed circles), HeLa/DZR (closed squares), and HeLa/DZR/Rev (open diamonds) cells was determined by Hoechst staining nuclei using an ArrayScan II reader as described under *Materials and Methods*. Each value was the mean of four determinations. Bars equal S.E.M. unless the value was less than the size of the symbol. A, disorazole C_1 . B, vinblastine.

Fig. 4. Disorazole C_1 resistance is reversible with verapamil treatment. HeLa (circles) or HeLa/DZR (squares) cells were incubated in the presence (open symbols) or in the absence (closed symbols) of 5 μ M verapamil and with various concentrations of compounds for 72 h, and the total number of cells was determined by Hoechst staining nuclei using an ArrayScan reader as described under *Materials and Methods*. Each value was the mean of four determinations. Bars equal S.E.M. unless the value was less than the size of the symbol. A, disorazole C_1 . B, disorazole A_1 . C, vinblastine.

Fig. 5. Disorazole C_1 -resistant cells overexpress ABCB1 and siRNA suppression of ABCB1. A, expression of ABCB1. Western blots of lysates were obtained from HeLa (1), HeLa/DZR (2), HeLa/DZR/Rev (3), and MES-SA/Dx5 (4) cells. The loading control of β -tubulin is shown below the ABCB1 panel. B, siRNA-mediated loss of ABCB1. Protein lysates were obtained from HeLa cells that were not transfected (1) or were transfected with 20 nM (2) or 40 nM (3) ABCB1 siRNA or 20 nM (4) or 40 nM (5) scrambled siRNA. Protein lysates were obtained 24 h later. β -Tubulin was used as the loading control and is shown below the ABCB1 panel.

(Fig. 7B). Therefore, we examined the sensitivity of epothilone-resistant cells to disorazole C_1 . The Epo40 cells were $\sim$ 100-fold resistant to epothilone B (Fig. 7A) and $\sim$ 20-fold resistant to paclitaxel (Fig. 7B). In contrast Epo40 cells were not cross-resistant to disorazole C_1 (Fig. 7C).

Discussion

The highly electrophilic divinyl oxirane moiety of disorazole A_1 has been observed to be a potent inhibitor of tubulin polymerization, and it induces apoptosis at low concentrations, i.e., <100 pM (Elnakady et al., 2004). Our previous studies indicated that the bioactive disorazole pharmacophore does not require the divinyl oxirane moiety. Disorazole C_1 represents one family member that retains considerable potency to inhibit tubulin polymerization and antiproliferative

activity against a large number of human tumor cells, but it also is obtainable by synthetic chemistry (Wipf and Graham, 2004; Tierno et al., 2009). Moreover, disorazole C_1 appears to exploit a unique binding mechanism to disrupt microtubule integrity and causes senescence (Tierno et al., 2009). Our attempts to generate disorazole-resistant cells met with considerable difficulties. We ultimately used both mutagenesis and stepwise exposure to increasing concentrations of disorazole C_1 . Furthermore, the resulting resistant population was not stable.

The only previous study examining potential disorazole resistance, albeit minimally, suggested cells overexpressing ABCB1 might not be resistant to disorazole A_1 (Elnakady et al., 2004). Elnakady et al. (2004) used a multidrug-resistant KB cell line with elevated ABCB1 produced by increased stepwise exposure to vinblastine. They observed the KB-V1 cells were only 2-fold resistant to disorazole A_1 , whereas were 13-fold resistant to vinblastine (Elnakady et al., 2004). Treatment of KB-V1 cells with 11 μ M verapamil increased sensitivity 2-fold against disorazole A_1 , while multiplying vinblastine sensitivity 100-fold. Thus, it was surprising to find that the disorazole C_1 -resistant cells we generated were cross-resistant to anticancer agents often associated with multiple drug resistance derived from high ABCB1 expression. Furthermore, we found that both the disorazole C_1 -resistant cells and MCF7 with elevated ABCB1 were resistant to disorazole A_1 . Our siRNA and pharmacological results support the hypothesis that at least a portion of the disorazole C_1 and A_1 resistance in HeLa/DZR was due to ABCB1. We recognize the methodology used to generate the disorazole C_1 -resistant cells could cause other alterations that could produce resis-

Fig. 6. siRNA knockdown of ABCB1 restores sensitivity to Disorazole C_1 . HeLa/DZR cells were transfected with 20 nM ABCB1 (open squares) or scrambled siRNA (open diamonds) and 5 h later the medium was removed and replaced with complete EMEM. HeLa wild-type (closed circles) and HeLa/DZR (closed squares) cells that were not transfected were also included. After 24 h, cells were detached with 0.05% trypsin, replated, and allowed to attach overnight. Cells were treated 48 h after transfection with compounds, and growth inhibition was determined 72 h later as described above. A, disorazole C_1 . B, disorazole A_1 . C, vinblastine. D, cisplatin.

Fig. 7. Disorazole C₁ retains activity against epothilone B-resistant cells. A549 lung cancer cells (closed inverted triangles) and their epothilone B-resistant counterparts (epob40) (open inverted triangles) were plated at 1000 cells/well in 384-well collagen-coated plates and allowed to adhere overnight. The cells were treated the next day and incubated for 72 h. The number of cells was determined by Hoechst 33342 stained nuclei using an ArrayScan reader as described under *Materials and Methods*. Data are the mean results of four determinations. A, epothilone B. B, paclitaxel. C, disorazole C₁.

tance and these should be studied in the future. Nonetheless, it would appear that at least one protein, ABCB1, can yield cellular resistance to the disorazole pharmacophore. We have no adequate explanation for why resistance was not observed previously with the KB model. It is possible the differences reside in the selected cell type and further studies are required to address this question.

The disorazole pharmacophore remains a potent and promising platform for the development of novel anticancer agents. It is notable that disorazole C₁ retained activity against epothilone-resistant cells, which could be one attribute for stimulating additional studies on this unique natural product pharmacophore. Efforts should be extended to remove sensitivity to the ABCB1 transporter.

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