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The Selectivity of Austocystin D Arises from Cell-Line-Specific Drug Activation by Cytochrome P450 Enzymes

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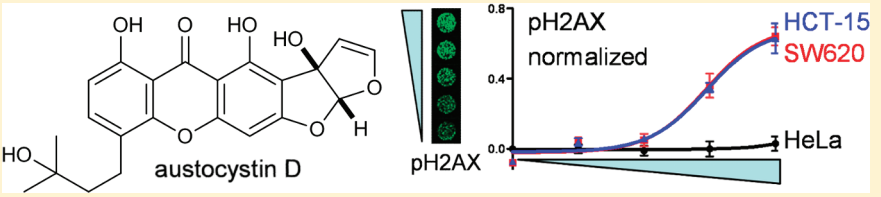
Supporting Information

ABSTRACT: The natural product austocystin D was identified as a potent cytotoxic agent with in vivo antitumor activity and selectivity for cells expressing the multidrug resistance transporter MDR1. We sought to elucidate the mechanism of austocystin D's selective cytotoxic activity. Here we show that the selective cytotoxic action of austocystin D arises from its selective activation by cytochrome P450 (CYP) enzymes in specific cancer cell lines, leading to induction of DNA damage in cells and in vitro. The potency and selectivity of austocystin D is lost upon inhibition of CYP activation and does not require MDR1 expression or activity. Furthermore, the pattern of cytotoxicity of austocystin D was distinct from doxorubicin and etoposide and unlike aflatoxin B_1, a compound that resembles austocystin D and is also activated by CYP enzymes to induce DNA damage. Theses results suggest that austocystin D may be of clinical benefit for targeting or overcoming chemoresistance.



Chemoresistance is a serious challenge to successful cancer treatment. A common mechanism of acquired resistance to chemotherapeutics is the overexpression of ATP-binding cassette transport proteins (ABC transporters) including MDR1, the multidrug resistance-associated protein 1 (MRP1), and the breast cancer resistance protein (BCRP).[1] These transporters mediate the ATP-dependent efflux of a variety of chemicals. MDR1 serves the physiological role of protecting the body from toxic chemicals.[2] Cancer cells, however, use it to pump out chemotherapeutic agents, thereby reducing treatment efficacy. To circumvent this resistance to chemotherapeutic agents, effort has focused on the discovery and development of small molecules that are selectively toxic to cancer cells that overexpress ABC transporters.

The natural product austocystin D (**1**, Figure 1) was isolated from *Aspergillus* isolate UGM218 as a potent and selectively cytotoxic agent in a 26-cell-line panel.[3] On the basis of its selective cytotoxicity, austocystin D generated interest as a promising lead anticancer compound. Austocystin D is unusual in that it is cytotoxic to cell lines that overexpress MDR1, also known as P-glycoprotein. In fact, austocystin D was observed to be 20-fold more potent in cell lines in which MDR1 was overexpressed by drug selection or transfection.[3] The compound was subsequently found to be active in vivo; it inhibited the growth of human colon carcinoma cell line LS174T subcutaneously implanted in nude mice.[3] While several austocystins have been identified as bacterial mutagens,[4] it was unclear whether DNA damage accounted for the antitumor and unique cell-line selective activity of austocystin D.

There have been a number of attempts to identify the target of austocystin D, including the use of affinity chromatography and gene expression profiling in yeast. These attempts have not led to the discovery of the cellular target for and the mechanism of action of austocystin D's selective antiproliferative activity.[3] Here we describe the elucidation of the mechanism of action of austocystin D. The selective cytotoxicity of austocystin D did not fully correlate with ABC transporter expression, although austocystin D did act as an MDR1 substrate. Instead, austocystin D cytotoxicity correlated with activation of a DNA damage response pathway and cytochrome P450 (CYP) activity. Both ketoconazole, an inhibitor of CYP 3A4 at low doses and other CYP enzymes at higher doses,[5] and chemical modification to block the likely site of CYP activation diminished austocystin D cytotoxicity. Finally, we observed that austocystin D directly damaged DNA in vitro, but only in the presence of mammalian liver microsomes. Therefore, austocystin D is activated by CYP enzymes to directly damage DNA, and the selective antiproliferative action is due to selective CYP enzyme activity. These studies provide an explanation for the selective antiproliferative action of austocystin D and point to the possible utility of

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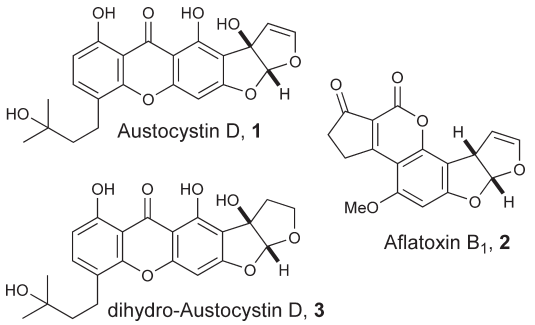

Figure 1. Structures of austocystin D (**1**), aflatoxin B_1 (**2**), and dihydro-austocystin D (**3**).

Table 1. Growth Inhibition by Austocystin D

tissue type	cell line	GI_{50} nM (n)
Normal		
umbilical vein endothelial	HUVEC	4249 (2)
breast	MCF-10A	3513 (3)
Cancer		
non-small-cell lung	A549	>5000 (5)
cervix	HeLa	>5000 (5)
leukemia	SR	16 (2)
brain glioblastoma	U87	4946 (3)
breast	MCF-7	<1 (3)
breast	MDA-MB-231	549 (3)
prostate	PC-3	3 (2)
colon	SW620	27 (2)
colon	HCT-15	42 (2)
uterine	MES-SA	>10 000 (2)
uterine	MX2	3358 (2)

targeting differences in CYP enzyme expression to overcome chemoresistance.

RESULTS AND DISCUSSION

Austocystin D Is a Potent and Highly Selectively Cytotoxic Natural Product with Activity Unlike That of Doxorubicin, Etoposide, and Aflatoxin B_1. Austocystin D was tested for cytotoxicity after 72 h treatment in a panel of cell lines. As indicated in Table 1, austocystin D displayed potent cytotoxicity, with GI_{50} values below 10 nM for three cell lines, and marked selectivity, with greater than 10 000-fold selectivity between the most sensitive cell line (MCF7) and the least sensitive cell line (MES-SA). The selectivity of austocystin D was not easily explained by tissue origin, as demonstrated by a comparison of MCF7 and MCF10A breast cell lines, where a selectivity of greater than 1000-fold was observed for MCF7 cells (Figure 2a). Furthermore, the selectivity and activity was unlike that of chemotherapeutic agents such as doxorubicin and etoposide, both ~50-fold more toxic to MCF10A cells, or the structurally related aflatoxin B_1, which did not display selectivity or potency, with GI_{50} values in each cell line above 5 μM (Figure 2). The differential selectivity to that of doxorubicin and etoposide indicates that austocystin D may have the potential to overcome resistance to these agents, and the differential potency to aflatoxin B_1 indicates that these structurally related natural products (Figure 1) may not share a related mechanism of action or cellular activity.

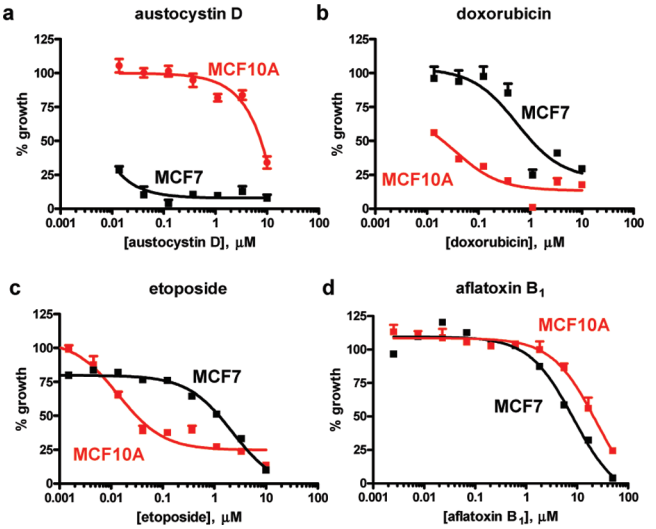

Figure 2. Austocystin D potency and selective cytotoxicity is distinct from doxorubicin, etoposide, and aflatoxin B_1. Dose-dependent growth inhibition of tissue-matched transformed, MCF7, and nontransformed, MCF10A, cells after 72 h treatment with (a) austocystin D, (b) doxorubicin, (c) etoposide, and (d) aflatoxin B_1. Vertical bars represent SEM, representative experiments shown.

Austocystin D Directly Interacts with MDR1, But This Interaction Is Not Required for Its Cytotoxic Activity. Since austocystin D was previously shown to display selective cytotoxicity to cells overexpressing the ABC transporter MDR1, we investigated the possibility that austocystin D interacts directly with this transporter. MDR1 drug efflux activity is accompanied by ATP hydrolysis and therefore can be monitored in vitro by measuring the production of inorganic phosphate from membranes containing MDR1.[6] Similar to known MDR1 substrates, verapamil and vinblastine,[1] austocystin D stimulated ATPase activity in this assay, suggesting that it is also likely to be an MDR1 substrate (Figure 3a). Furthermore, cyclosporin A, a known inhibitor of the substrate-stimulated MDR1 ATPase activity,[7] fully inhibited austocystin D-stimulated ATPase activity.

To determine whether austocystin D also modulated drug efflux activity in cells, we measured the abundance of the fluorescent dye calcein in live cells.[8] In this assay, the nonfluorescent calcein acetoxymethyl ester (calcein-AM) is added to cells and is hydrolyzed to calcein by cellular esterases. Calcein-AM is an MDR1 and MRP1 family substrate and thus is actively effluxed from cells expressing those transport proteins, resulting in low levels of retained fluorescence.[9] MDR1 and MRP1 family substrates or inhibitors prevent calcein-AM efflux, leading to high levels of retained fluorescence. Similar to MDR1 substrate verapamil and inhibitor cyclosporin A, austocystin D inhibited efflux of calcein-AM, indicating that it can also modulate drug efflux activity in cells (Figure 3b).

Although austocystin D modulated ABC transporter activity in vitro and in cells, this activity does not account for the cytotoxicity of austocystin D. Verapamil and austocystin D both act as MDR1 substrates with similar cellular activity, yet verapamil is more than 1000-fold less cytotoxic than austocystin D (Figure 3c, d). Furthermore, we evaluated a panel of cell lines for ABC transporter activity in relation to austocystin D sensitivity. We measured calcein-AM efflux as an indication of the presence of functional MDR1 or MRP1 family members and Hoechst 33342

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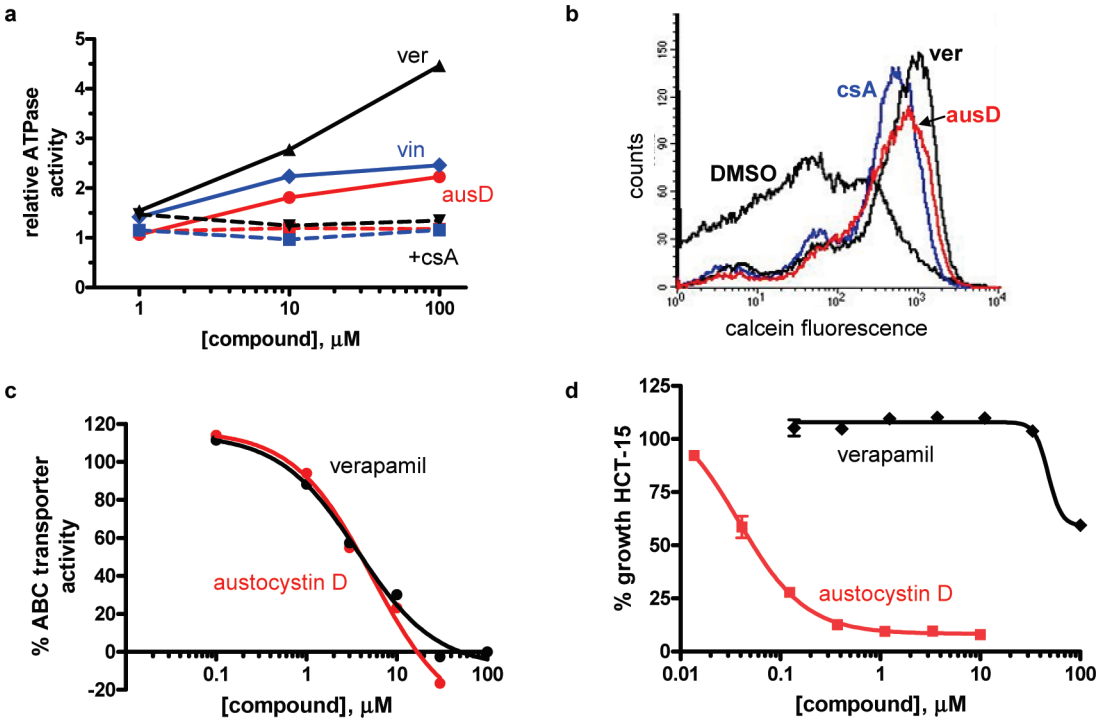

Figure 3. Austocystin D modulates MDR1 activity in vitro and in cells. (a) Effect of austocystin D (ausD) and MDR1 substrates verapamil (ver) and vinblastine (vin) on the ATP hydrolysis by membranes containing MDR1 in the presence (dashed lines) or absence (solid lines) of 1 μM MDR1 inhibitor cyclosporin A (csA). Austocystin D, verapamil, and vinblastine each activated MDR1 ATP hydrolysis that was inhibitable by cyclosporin A. (b) Effect of 10 μM austocystin D, 40 μM verapamil, and 10 μM cyclosporin A on the retention of calcein in HCT-15 cells. Austocystin D behaved similarly to verapamil and cyclosporin A to inhibit calcein-AM efflux and thereby increase intracellular calcein fluorescence relative to vehicle (DMSO)-treated cells. (c, d) Effect of austocystin D and verapamil on (c) calcein-AM efflux and (d) growth in HCT-15 cells. Vertical bars represent SEM, representative experiment shown.

Table 2. Austocystin D Cytotoxicity Does Not Correlate with Drug Efflux Activity[a]

cell line	GI_{50} (nM)	MDR1/MRP activity	BCRP activity
MCF7	<1	−	+ (subset of cells[b])
MCF10A	3513	−	−
HCT-15	42	+	not determined
SW620	27	−	−
MES.SA	>10 000	−	not determined
MES.SA/MX2	3358	+	not determined

[a] At least two independent experiments were performed for each data point. [b] <50% of MCF7 cells displayed BCRP activity.

efflux as an indication of the presence of functional BCRP family members.[9,10] As indicated in Table 2, SW620 cells lacked activity for known ABC transporters yet were sensitive to austocystin D. Taken together, the lack of correlation between verapamil and austocystin D cytotoxicity and ABC transporter activity provides strong evidence that the cytotoxicity of austocystin D is not due to its interaction with ABC transporters.

Austocystin D Is a Cytochrome P450-Activated DNA Damage Agent. Because of its structural similarity to aflatoxin B_1, a CYP-activated mutagen,[11] we investigated whether the selective cytotoxic activity of austocystin D was due to CYP-activated DNA damage. Austocystin D and aflatoxin B_1 share a vinyl ether moiety, critical in aflatoxin B_1 for its biological activity (Figure 1). This moiety in aflatoxin B_1 undergoes epoxidation catalyzed by CYP enzymes, and the resulting epoxide forms DNA adducts.[11] To detect cellular DNA damage, we established an in-cell Western assay measuring the phosphorylation levels of histone H2AX. Histone H2AX is phosphorylated by the kinase ATM at serine 139 in response to DNA double strand breaks. This signal occurs in response to DNA-damaging agents such as bleomycin and camptothecin[12,13] and is a sensitive readout of DNA damage response pathways.[14] In this assay, we observed that known DNA damage agents camptothecin, doxorubicin, and bleomycin induced dose-dependent phosphorylation of histone H2AX after 4 h of drug treatment (Figure 4a, Figure S1a,b). We subsequently determined that austocystin D induced the phosphorylation of histone H2AX in cell lines for which it was cytotoxic (Figure 4a,b). Thus, austocystin D caused a cell-line selective induction of the DNA damage response that matched its cell-line selective cytotoxicity.

To investigate the likelihood that austocystin D required CYP activation to induce the DNA damage response and be cytotoxic, we treated cells with both ketoconazole, a cell-permeable inhibitor of CYP 3A4 activity,[5] and austocystin D. Ketoconazole fully prevented austocystin D cytotoxicity and induction of the DNA damage response, demonstrating that CYP activity is required for austocystin D's antiproliferative activity (Figure 5a,b). This effect was specific to austocystin D and did not result from general inhibition of the DNA damage response (Figure 5c).

CYP activation of aflatoxin B_1 occurs via epoxide formation at the vinyl ether. The epoxide forms a direct covalent interaction with guanine nucleotides, causing DNA damage.[11] We sought to

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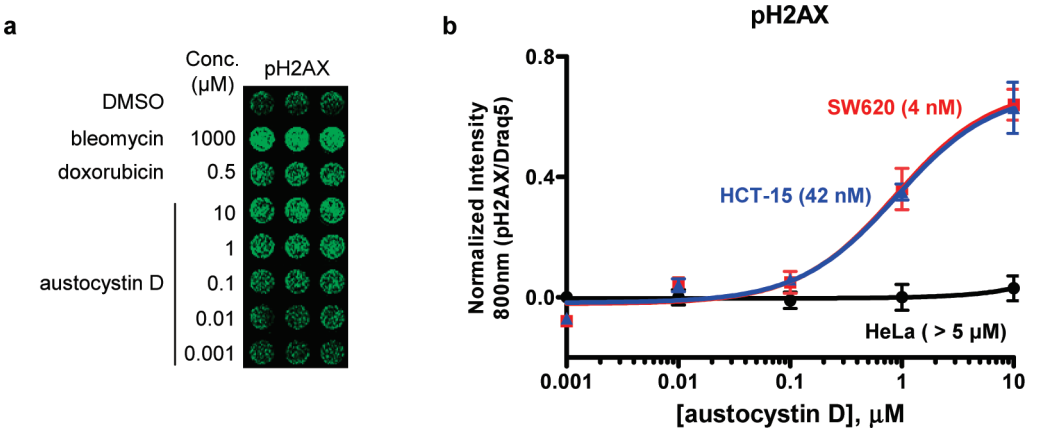

Figure 4. Austocystin D displays cell-line-selective DNA damage that correlates with cytotoxicity. (a) In-cell Western showing the effect of austocystin D, doxorubicin, bleomycin, and DMSO (vehicle) on levels of phosphorylated histone H2AX (pH2AX) after 4 h treatment of HCT-15 cells. (b) In-cell Western data showing the effect of austocystin D on pH2AX in SW620, HCT-15, and HeLa cells following 1 h of treatment. 72 h GI_{50} values are shown for comparison in parentheses. Vertical bars represent SEM.

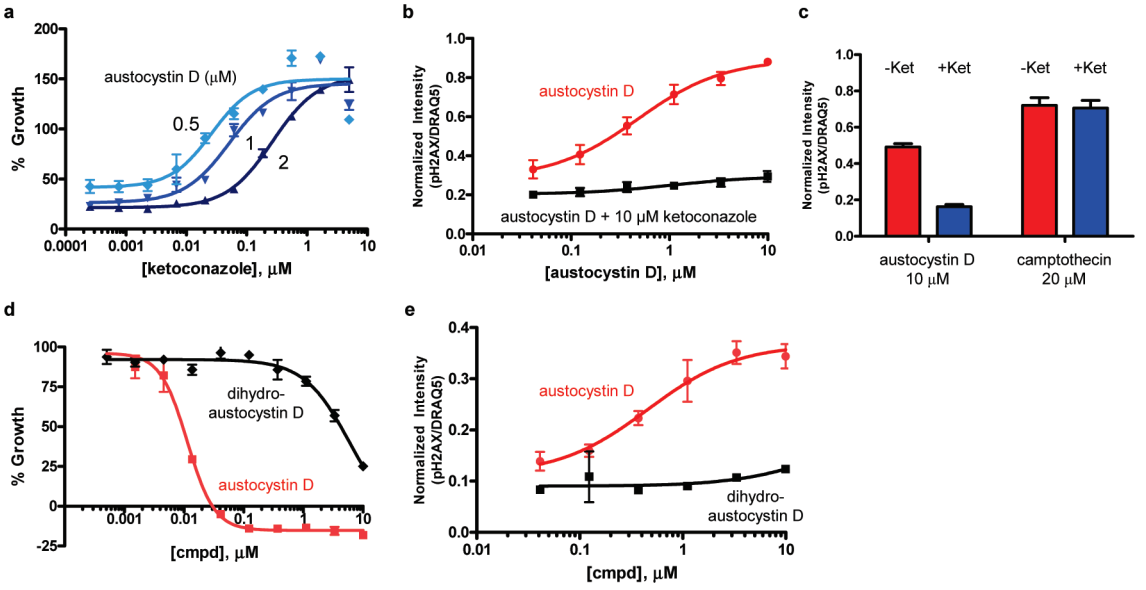

Figure 5. Inhibition of CYP or hydrogenation of austocystin D abrogates austocystin D DNA damage and cytotoxicity. (a) Inhibitory effect of the CYP inhibitor ketoconazole on HCT-15 cell growth inhibition of 0.5 μM (light blue), 1 μM (blue), and 2 μM (dark blue) austocystin D. (b) Inhibitory effect of 10 μM ketoconazole on austocystin D-induced phosphorylation of histone H2AX in HCT-15 cells following 1 h ketoconazole pretreatment and 4 h austocystin D treatment. (c) Specific inhibition of 10 μM ketoconazole (Ket) on 10 μM austocystin D-induced phosphorylation of histone H2AX in SW620 cells in comparison to 20 μM camptothecin. (d, e) Comparison of austocystin D and dihydro-austocystin D for (d) growth inhibition after 72 h treatment of MCF7 cells and (e) induction of phosphorylation of histone H2AX after 4 h in MCF7 cells. Vertical bars represent SEM.

probe the involvement of an austocystin D epoxide intermediate by hydrogenation of the vinyl ether, since the aflatoxin B_1 epoxide intermediate is known to be short-lived under physiological conditions,[15] and we therefore expected the austocystin D epoxide to also be short-lived. We reduced the vinyl ether of austocystin D, yielding dihydro-austocystin D[16] (**3**, Figure 1) and observed that dihydro-austocystin D displayed only very weak cytotoxicity and induction of the DNA damage response, further supporting the importance of metabolic activation for the mechanism of action of austocystin D (Figure 5d,e).

To support the cellular data indicating that austocystin D induced DNA damage following CYP activation, we applied an in vitro reconstituted DNA damage assay.[17,18] In this assay, supercoiled plasmid DNA is incubated with compound in the presence or absence of human liver microsomes, a rich source of CYP enzymes, and an NADPH regenerating system to generate in situ the NADPH that is required for the CYP activity. Alkylation of DNA leads to single strand breaks, which relax the supercoiled DNA. The resulting nicked DNA can be detected by agarose gel electrophoresis. In this assay, austocystin D caused direct DNA damage at 100 nM, consistent with the compound's GI_{50} in cells (Figure 6 and Table 1). This in vitro DNA damage activity required CYP activity, as increased nicked DNA was observed only in the presence of both liver microsomes and the NADPH regenerating system required for activity of CYP enzymes in vitro.

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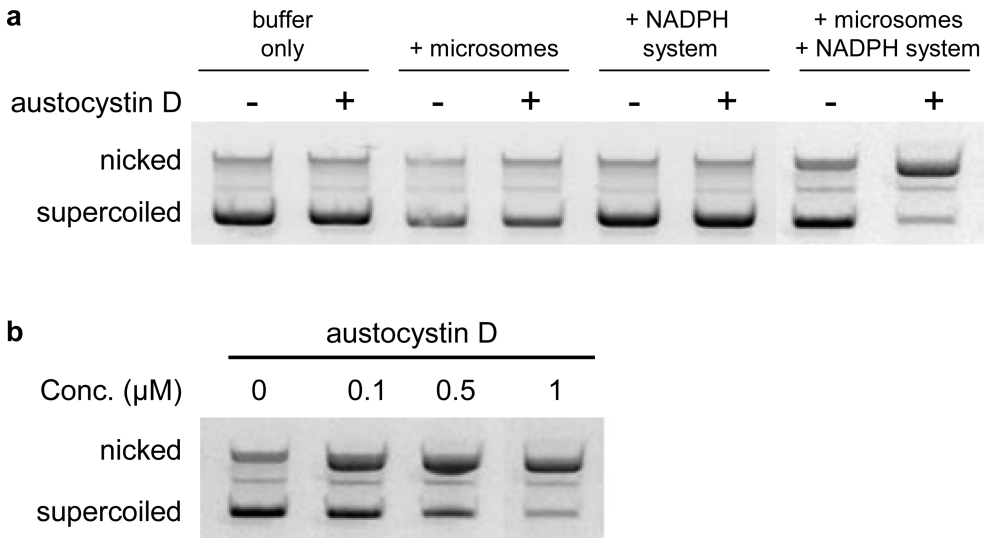

Figure 6. Austocystin D mediates direct DNA damage in vitro in the presence of liver microsomes. (a) DNA agarose gel showing the effect of 1 μM austocystin D on converting supercoiled plasmid DNA to nicked DNA after treatment with different additives. (b) DNA agarose gel showing the potency of austocystin D-induced DNA nicking.

DISCUSSION

Austocystin D was originally discovered more than 30 years ago in an effort to identify and characterize secondary metabolites in maize meal cultures of *Aspergillus ustus* (Bainier) Thom. and Church.[16] More recently, it was reported to act as a selectively cytotoxic agent to certain cancer cell lines with an apparent selectivity toward cells expressing MDR1.[3] We sought to elucidate the mechanism of action of austocystin D to uncover a potentially useful approach to selectively target chemoresistant cancer cells that act by expression of ABC transporters. Our approach involved the investigation of two possible leads: that ABC transporter activity might be required for austocystin D antiproliferative activity and that austocystin D might act by a mechanism similar to aflatoxin B_1, a well-characterized structurally related compound that is activated by CYP enzymes to induce DNA damage.

We began by confirming the cell-line selectivity of austocystin D and found it to be remarkably potent and selective, displaying over 1000-fold selectivity between sensitive and resistant cell lines. In comparison to the chemotherapeutic agents doxorubicin and etoposide, austocystin D displayed a reverse sensitivity pattern between the breast cancer cell line MCF7 and the proliferative nontransformed breast cell line MCF10A with the advantage of being more selective for the cancer cell line. Austocystin D was also only weakly active against the normal human umbilical vein endothelial cell line (HUVEC), suggesting the possibility of selectively targeting cancer cells, particularly in the setting of resistance to standard chemotherapy. Further characterization of a subset of cell lines revealed that ABC transporter activity did not correlate with austocystin D antiproliferative activity. For example, SW620 cells were highly sensitive to austocystin D with a GI_{50} of 27 nM but did not display ABC transporter activity.

To investigate the possibility that austocystin D induced DNA damage in cells, we measured the induction of phosphorylation of histone H2AX, which occurs in response to DNA damage. Austocystin D induced histone H2AX phosphorylation, and this activity fully correlated with its cellular cytotoxicity. The histone H2AX phosphorylation and antiproliferative activity could be fully inhibited by treatment with ketoconazole, a CYP inhibitor with highest potency toward CYP 3A4, or reduction of austocystin D to dihydro-austocystin D, a form that was not expected to undergo CYP-catalyzed epoxidation like aflatoxin B_1.[11] Therefore, a mechanism emerged in which austocystin D is activated by CYP enzymes to induce DNA damage leading to cell death. Further support for this mechanism of action came from in vitro experiments in which we directly observed DNA damage in the presence of liver microsomes expected to contain CYP enzymes.

While austocystin D and aflatoxin B_1 share a similar mode of action in vitro and in the Ames test,[4] namely, CYP-activated DNA damage, the compounds differ dramatically in both potency and cell-line selectivity. In the comparison between MCF7 and MCF10A, aflatoxin B_1 displayed little selectivity and poor potency, while austocystin D was highly selective and potent, being 1000-fold more active against MCF7 cells. Although both compounds contain a vinyl ether moiety, the remainder of the structures may encode CYP enzyme specificity or sensitivity, or differences in DNA recognition. Alternatively, the reactivity of the vinyl ether or epoxide may vary between the compounds, leading to differences in rate of epoxide formation or covalent interaction with cellular DNA. One possibility for the difference in activity between austocystin D and aflatoxin B_1 is the absence of a tertiary alcohol in aflatoxin B_1 adjacent to the site of epoxidation. This hydroxyl is present in aflatoxin M_1, a metabolite of aflatoxin B_1.[19] Neal et al. directly compared the cytotoxicity of aflatoxins B_1 and M_1 and reported differences; the human B lymphoblastoid cell line MCL-5 that stably expresses CYPs 1A2, 2A6, 3A4, and 2E1 and microsomal epoxide hydrolase was sensitive only to aflatoxin B_1, while the untransfected line, cHol, was sensitive only to aflatoxin M_1.[20] A comparison of the biological activity of austocystin D to aflatoxin M_1 will help reveal the importance of the tertiary alcohol to differences between austocystin D and aflatoxins. In addition, an investigation into CYP selectivity and hepatoxicity will help reveal distinctions between austocystin D and aflatoxins and the potential clinical utility of austocystin D.

Despite its initial discovery as an MDR+ cell selective agent, we have determined here that the unusual selectivity of austocystin D is mediated by CYP activation, rather than by a direct

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interaction with ABC transporters. This unanticipated finding is likely explained by coordinate expression of CYPs and ABC transporters by the pregnane xenobiotic receptor (PXR) or the constitutive androstane receptor (CAR) nuclear hormone receptors. These receptors induce the expression of a coordinated cellular detoxification program in response to xenobiotic small molecules, and each is known to upregulate both ABC transporters and CYP enzymes.[21,22] Thus, our findings support a model whereby transcriptional upregulation of ABC transporters by PXR/CAR can lead to a multiple drug resistance phenotype and that this transcriptional program also induces tumoral expression of CYP enzymes. This model is also supported by the observations that PXR agonists lead to multiple drug resistance and that chemotherapeutics prone to multiple drug resistance induce PXR-mediated transcription.[22−24]

These findings suggest a context-dependent cytotoxicity strategy for the design of new therapeutics for multiple drug resistant cancers, where the co-regulation of MDR and CYP enzymes creates a vulnerability to CYP-activated prodrugs that are directly activated in the tumor tissue. Previous CYP-activated anticancer agents, such as cyclophosphamide, have relied on metabolic activation of the drug in the liver.[25,26] Such drugs have limited cancer cell autonomous effects and require liver activation for activity. This is typically associated with systemic toxicity as the activated drug enters circulation.[25] In the present work, we show that austocystin D has significant cancer cell autonomous activity, suggesting that the molecule or derivatives could be suitably activated directly within tumors. Due to the coordinate expression of CYPs and ABC transporters such as MDR1, the natural product austocystin D or compounds with a similar mechanism of action have the potential to target multiple drug resistant tumors, and thus may address a significant clinical need.

EXPERIMENTAL SECTION

General Experimental Procedures. Austocystin D was isolated as previously described,[27] and dihydro-austocystin D was synthesized as described in the Supporting Information and has previously been synthesized.[16] All other compounds were obtained from commercial suppliers.

Cytotoxicity Testing. HUVEC cells were obtained from Lonza, and other cell lines were obtained from ATCC. All cell lines were maintained according to manufacturer specifications. Cells were plated in 96-well plates at densities ranging from 2500 to 10 000 cells/well 24 h prior to the addition of compounds. Compound stocks in 100% DMSO were 3-fold serially diluted in 100% DMSO in 96-well U-bottom plates to generate 1000× dose curves. These were subsequently diluted 1:100 in RPMI-1640 medium and then diluted 1:10 into the wells plated with cells for a final DMSO concentration of 0.1%. The Cell-Titer Blue viability assay (Promega) was performed at 0 and 72 h of compound treatment. GI_{50} values for compounds were determined relative to DMSO control samples measured at 0 h (0% growth) and 72 h (100% growth). All curve fitting was performed using nonlinear regression with GraphPad Prism5 software.

In Vitro MDR1 ATPase Assay. Drug-stimulated MDR1 ATPase activity was determined by measuring inorganic phosphate release.[6] Human MDR1 and control membranes (BD Biosciences) were prepared in assay buffer (50 mM Tris-Mes pH 6.8, 1 mM EGTA, 1 mM dithiothreitol, 25 mM KCl, 2.5 mM sodium azide) with and without cyclosporin A (50 μM final concentration). Compounds were added to membranes and incubated for 10 min at 37 °C, and the ATPase reaction was initiated by the addition of ATP. The final concentrations of membrane, ATP, and DMSO in the reactions were 200 μg/mL, 4 mM, and 1%, respectively. The reactions were stopped by the addition of SDS solution containing antifoam A (final concentration of 3%) after 0 and 60 min incubation periods. Two equal volumes of 35 mM ammonium molybdate in 15 mM zinc acetate:/10% ascorbic acid (1:4) were subsequently added followed by a 20 min incubation at 37 °C and measurement of absorbance at 800 nm. The released inorganic phosphate was determined by comparison to a phosphate standard curve at 800 nm. The MDR1-specific ATPase activity was calculated by comparing ATPase activity from MDR1 membranes to that of control membranes.

MDR1/MRP Activity. MDR1/MRP cellular activity was determined by measuring calcein-AM efflux.[28] Cells were plated in triplicate wells at a density of 5×10^5 cells per six-well 2 days prior to the assay. For the assay, cells were treated with compounds for 60 min at 37 °C followed by the addition of calcein-AM (0.25 μM). After 30 min at 37 °C, the cells were washed twice with PBS, trypsinized, suspended in cold PBS, and analyzed with a FACS Calibur equipped with a 488 nm argon laser (Becton−Dickinson). In Figure 3c, the mean fluorescence of each histogram was calculated for austocystin D and verapamil at the indicated doses in duplicate, and the average of two samples was plotted with GraphPad Prism5, as above. DMSO was set to 100% ABC transporter activity, and 100 μM verapamil was set as 0% ABC transporter activity for normalization.

BCRP Activity. BCRP cellular activity was determined by measuring Hoechst 33342 efflux.[10] Cells were trypsinized from semiconfluent 150 cm^2 plates, resuspended at 10^6 cells/mL in media (RPMI-1640, 10% FBS), and incubated with 5 μg/mL Hoechst 33342 at 37 °C. After 45 min, cells were washed once with cold Hanks balanced saline solution supplemented with 1 mM HEPES and 2% FCS (HBSS+) and resuspended in media (RPMI-1640, 10% FBS) with 10 μM fumitremorgin C at 37 °C. After 20 min, cells were washed with cold HBSS+, resuspended in cold HBSS+ containing 2 μg/mL propidium iodide (PI) to exclude nonviable cells, and analyzed with a FACS LSRII (BD Biosciences) equipped with 360 nm UV and 488 nm argon lasers.

In-Cell Western Assay for Phosphorylation of Histone H2AX. Cells were plated in 96-well black-walled plates at 10 000 cells/well 24 h prior to the addition of compounds. Compound stocks in 100% DMSO were 3-fold serially diluted in 100% DMSO in 96-well U-bottom plates to generate 1000× dose curves. These were subsequently diluted 1:100 in RPMI-1640 medium and then diluted 1:10 into the wells plated with cells for a final DMSO concentration of 0.1%. After 4 h at 37 °C, cells were fixed in 4% paraformaldehyde for 20 min, washed briefly in 0.1% Tween-20, and permeabilized in 0.1% Triton-X 100 in PBS. After 20 min, cells were incubated in blocking buffer (Licor Biosciences, Odyssey blocking buffer) for 1 h and incubated overnight with antiphospho-H2AX antibody (Cell Signaling Technology, phospho-H2A.X Ser139) at 1:400 in blocking buffer. The cells were subsequently washed and incubated with goat-anti-rabbit IR800CW (Licor Biosciences) at 1:800 in blocking buffer and DRAQ5 (Biostatus Limited) at 1:10 000. After 1 h, cells were washed with PBS with 0.1% Tween-20. Signals were detected with a Licor Odyssey infrared scanner (Licor Biosciences) at 700 and 800 nm. Each well was normalized within the well by taking the ratio of 800 nm/700 nm. Ratios of duplicate or triplicate wells were averaged and normalized to the in-plate controls 0.1% DMSO (0% induction) and 10 μM etoposide (100% induction). Curve fitting was performed as for cytotoxicity testing. For ketoconazole experiments, cells were pretreated with 10 μM ketoconazole for 1 h prior to the 4 h treatment with austocystin D or camptothecin.

In Vitro DNA Damage Assay. Supercoiled pLenti6 plasmid DNA (120 μg/mL) was incubated at 37 °C with compound (100 nM, 500 nM, or 1 μM; 1% DMSO final concentration) in the absence or presence of human liver microsomes (0.25 mg/mL; CellzDirect) and an NADPH regenerating system (1.5 mM NADP, 12.5 mM glucose-6-phosphate, 0.25 U/mL glucose-6-phosphate dehydrogenase, 100 mM phosphate pH 7.5, 5 mM $MgCl_2$). After 4 h, the reaction was stopped by

addition of an equal volume of phenol/chloroform (1:1) solution. After vigorous vortexing, the emulsion was separated by centrifugation, and the DNA was recovered from the aqueous phase by using a gel extraction kit (Qiagen). The purified DNA was then subjected to electrophoresis in TAE buffer using a 0.8% agarose gel, and the bands were photographed using Biorad Gel Doc.

■ ASSOCIATED CONTENT

Supporting Information. Phosphorylation of histone H2AX in response to DNA damage agents camptothecin, doxorubicin, and bleomycin. Synthesis and ^{1}H NMR of dihydro-austocystin D. This material is available free of charge via the Internet at http://pubs.acs.org.

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