Identification of Compounds Selectively Killing Multidrug-Resistant Cancer Cells

Dóra Türk,1 Matthew D. Hall,2 Benjamin F. Chu,2 Joseph A. Ludwig,4 Henry M. Fales,3 Michael M. Gottesman,2 and Gergely Szakács1

Abstract

There is a great need for the development of novel chemotherapeutic agents that overcome the emergence of multidrug resistance (MDR) in cancer. We catalogued the National Cancer Institute's DTP drug repository in search of compounds showing increased toxicity in MDR cells. By comparing the sensitivity of parental cell lines with MDR derivatives, we identified 22 compounds possessing MDR-selective activity. Analysis of structural congeners led to the identification of 15 additional drugs showing increased toxicity in Pgp-expressing cells. Analysis of MDR-selective compounds led to the formulation of structure activity relationships and pharmacophore models. This data mining coupled with experimental data points to a possible mechanism of action linked to metal chelation. Taken together, the discovery of the MDR-selective compound set shows the robustness of the developing field of MDR-targeting therapy as a new strategy for resolving Pgp-mediated MDR. [Cancer Res 2009;69(21):8293–301]

Introduction

Members of the ATP binding cassette (ABC) transporter superfamily are widely recognized as major contributors to controlling drug distribution and pharmacokinetics, and the acquisition of anticancer drug resistance. Expressed largely in the plasma membranes of cancer cells, ABC transporters mediate cellular resistance to anticancer agents by the ATP-dependent efflux of toxic chemotherapeutics from cells. Although several ABC transporters have been shown to transport anticancer drugs, P-glycoprotein (Pgp/MDR1/ABCB1) stands out by conferring the highest level of resistance to a vast array of drugs and because of its shown association with clinical multidrug resistance (MDR) and poor clinical outcome (1). Pgp transports large, hydrophobic, positively charged molecules that can have strikingly dissimilar structures, including clinically relevant compounds such as anticancer drugs, HIV-protease inhibitors, immunosuppressive agents, and antiepileptics (1–3). Despite promising in vitro results obtained with several generations of Pgp inhibitors, successful modulation of clinical MDR through the chemical blockade of drug efflux from cancer cells has not been successful (1), and new strategies are required.

Intriguingly, Pgp can contribute not only to acquired resistance but also to paradoxical drug sensitivity (4–14). “Collateral sensitivity” of otherwise MDR cells represents a promising but yet unrealized strategy for targeting Pgp-mediated MDR. Given the importance of drug-transporter interactions and the desire to uncover compound classes with new modalities, we recently analyzed the Developmental Therapeutics Program (DTP) drug activity data set, and reported the prediction and validation of small molecules recognized by various ABC transporters (7). The drug database compiled by the DTP of the National Cancer Institute (NCI) contains the cytotoxicity profiles of >100,000 compounds across 60 human cancer cell lines (NCI-60 panel). This data set, combined with other descriptors of the cell panel, provides clues to mechanisms of chemosensitivity and resistance (7, 15, 16). Statistical correlations between the sensitivity of these cell lines to a panel of anticancer drugs and the expression of Pgp across the NCI-60 cell panel identified new Pgp substrates, but also suggested that cells expressing higher levels of Pgp are more sensitive to the thiosemicarbazone NSC73306. This agent was selected for in vitro evaluation (17), and we have subsequently shown that NSC73306 is truly selective for functional Pgp; cells are hypersensitive to NSC73306 in proportion to their Pgp function, and this selectivity is abrogated by functional inhibition of Pgp or downregulation of Pgp expression by siRNA (18). Furthermore, NSC73306 itself is not an inhibitor or substrate of Pgp, suggesting that this compound does not confer its toxicity in the same fashion as the so-called “collateral sensitivity” agents.

We are currently undertaking preclinical assessment of NSC73306. In parallel, we sought to identify new “MDR-selective” compounds to expand the scope of bioactive agents and to gain insight into the Pgp-specific mechanism of action. To this end, here, we report the screening of 42,000 compounds from the DTP data set in silico for MDR-selective activity, predicted by the correlation of cytotoxicity with Pgp expression across the NCI-60 cell line panel. This prediction set was then validated in vitro, focusing on clusters of structurally related compounds. Twenty-two compounds possessing MDR-selective activity were identified and analysis of structural congeners in the DTP repository led to the identification of 15 additional compounds showing increased toxicity in Pgp-expressing cells. The discovery of these MDR-selective compounds shows the robustness of the developing field of MDR-targeting therapy as a new strategy for resolving Pgp-mediated MDR.

Materials and Methods

Chemicals. Unless otherwise stated, compounds were obtained from the NCI DTP drug repository. NSC716765, NSC716766, and NSC716772 were synthesized by us (17); the octapeptide NSC633657 (D-Phe-Cys-Tyr-D-Trp-Lys-Ser-Cys-Thr-NH2) was prepared by John Stonik, NCI. NSC617961 and NSC617963 were sourced from the Auckland Cancer Society Research Institute’s DTP drug repository in search of compoundsshowingincreasedtoxicityinPgp-expressingcells. Analsis of these cell lines to a panel of anticancer drugs and the expression of Pgp across the NCI-60 cell panel identified new Pgp substrates, but also suggested that cells expressing higher levels of Pgp are more sensitive to the thiosemicarbazone NSC73306. This agent was selected for in vitro evaluation (17), and we have subsequently shown that NSC73306 is truly selective for functional Pgp; cells are hypersensitive to NSC73306 in proportion to their Pgp function, and this selectivity is abrogated by functional inhibition of Pgp or downregulation of Pgp expression by siRNA (18). Furthermore, NSC73306 itself is not an inhibitor or substrate of Pgp, suggesting that this compound does not confer its toxicity in the same fashion as the so-called “collateral sensitivity” agents.

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Centre, New Zealand (19). NSC697124 and NSC697125 were sourced from Cancer Research Drug database.

**Drug database.** The DTP Human Tumor Cell Line Screen has screened tens of thousands of compounds for growth inhibition of human cancer cell lines. Publicly available screening results of ∼43,000 compounds were downloaded from the DTP Web site (July 2007 Release). Scrutiny of the data set indicated that it was not immediately amenable to stringent statistical analysis due to missing dose-response values or lack of activity in the dose window tested (12.1% of the cell line screen data points are missing and 44.9% of growth inhibition values indicate inactivity; ref. 20). Because uninformative drug profiles are not expected to yield useful leads, we cause uninformative drug profiles are not expected to yield useful leads, we

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**Case in point.** To validate predicted MDR-selective activity of a compound, IC50 values were determined using the MTT cytotoxicity assay on the parental KB-3-1 cell line, and its P-glycoprotein-expressing derivative, KB-V1. Those unavailable for testing are noted as such (NA). MDR1 selectivity is calculated as the ratio of a compound’s IC50 against KB-3-1 cells divided by its IC50 against KB-V1 cells. A value of >1 indicates that the compound kills Pgp-expressing cells more effectively than parental cells—so-called MDR-selective activity (bolded), as assessed by two-sided paired Student’s t test of multiple MTT assays. Compounds with a ratio of ≤1 inhibit growth in both cell lines with similar potency (0.5–1x1), indicating that they are either Pgp substrates or their toxicity is not modulated by Pgp. Compounds with an IC50 of >50 μmol/L were considered to be inactive (not toxic, NT), and as such their MDR1 selectivity could not be determined.

### Table 1. Putative MDR-selective compounds

<table>
<thead>
<tr>
<th>Compound (NSC)</th>
<th>Pearson Selectivity ratio</th>
<th>Compound (NSC)</th>
<th>Pearson Selectivity ratio</th>
<th>Compound (NSC)</th>
<th>Pearson Selectivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>10580</td>
<td>0.41</td>
<td>1.71 ± 0.26a</td>
<td>693743</td>
<td>0.42</td>
<td>NA</td>
</tr>
<tr>
<td>43320</td>
<td>0.56</td>
<td>2.08 ± 0.37‡</td>
<td>641208</td>
<td>0.43</td>
<td>2.13 ± 0.09†</td>
</tr>
<tr>
<td>73306</td>
<td>0.51</td>
<td>3.64 ± 0.93a</td>
<td>647574</td>
<td>0.42</td>
<td>NT</td>
</tr>
<tr>
<td>86715</td>
<td>0.53</td>
<td>≤1</td>
<td>649816</td>
<td>0.47</td>
<td>1.17 ± 0.12‡</td>
</tr>
<tr>
<td>168468</td>
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<td>3.82 ± 0.85‡</td>
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<td>NA</td>
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<tr>
<td>292408</td>
<td>0.45</td>
<td>2.80 ± 0.76‡</td>
<td>651859</td>
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<td>NA</td>
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<tr>
<td>356777</td>
<td>0.4</td>
<td>1.64 ± 0.42‡</td>
<td>653864</td>
<td>0.41</td>
<td>NA</td>
</tr>
<tr>
<td>403148</td>
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<td>NT</td>
<td>654724</td>
<td>0.41</td>
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<tr>
<td>617961</td>
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<td>≤1</td>
<td>669341</td>
<td>0.41</td>
<td>≤1</td>
</tr>
<tr>
<td>617963</td>
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<td>672027</td>
<td>0.55</td>
<td>≤1</td>
</tr>
<tr>
<td>621481</td>
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<td>≤1</td>
<td>672036</td>
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<td>1.27 ± 0.16‡</td>
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<tr>
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<td>673999</td>
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<td>626670</td>
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<td>681125</td>
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<tr>
<td>632591</td>
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<td>NA</td>
<td>688942</td>
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<td>632731</td>
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<td>692419</td>
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<td>693630</td>
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<td>693871</td>
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<tr>
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<td>3.90 ± 1.37‡</td>
</tr>
<tr>
<td>635977</td>
<td>0.42</td>
<td>NA</td>
<td>695331</td>
<td>0.44</td>
<td>2.60 ± 0.86*</td>
</tr>
<tr>
<td>636097</td>
<td>0.42</td>
<td>≤1</td>
<td>695333</td>
<td>0.58</td>
<td>3.11 ± 1.99*</td>
</tr>
</tbody>
</table>

**NOTE:** Compounds identified as having MDR-selective activity, listed with their Pearson’s correlation coefficients—the correlation between Pgp expression and DTP determined efficacy of each compound across the NCI-60 cell line panel. To validate predicted MDR-selective activity in vitro, compounds were sourced by a variety of methods, predominantly through the NCI DTP drug library, but also via other investigators and synthesis in our laboratories. For compounds that were available, IC50 values were determined using the MTT cytotoxicity assay on the parental KB-3-1 cell line, and its P-glycoprotein-expressing derivative, KB-V1. Those unavailable for testing are noted as such (NA). MDR1 selectivity is calculated as the ratio of a compound’s IC50 against KB-3-1 cells divided by its IC50 against KB-V1 cells. A value of >1 indicates that the compound kills Pgp-expressing cells more effectively than parental cells—so-called MDR-selective activity (bolded), as assessed by two-sided paired Student’s t test of multiple MTT assays. Compounds with a ratio of ≤1 inhibit growth in both cell lines with similar potency (0.5–1x1), indicating that they are either Pgp substrates or their toxicity is not modulated by Pgp. Compounds with an IC50 of >50 μmol/L were considered to be inactive (not toxic, NT), and as such their MDR1 selectivity could not be determined.

*Significance level, P < 0.05.

†Significance level, P = 0.01.

‡Significance level, P < 0.001.

http://discover.nci.nih.gov

Pgp (MDR1) as a result of selection in vinblastine (21). NIH-MDR-G185 is an isogenic subclone of the parental line NIH3T3 transfected with wild-type pHMDR1/A (22). MES-SA Dx5, a drug-resistant cell line expressing high levels of Pgp, was derived from a human uterus sarcoma line (MES-SA) by doxorubicin selection (23). KB-3-1, KB-V1, NIH3T3, and NIH-MDR-G185 cells were grown in DMEM cell culture medium at 37°C in 5% CO2. MES-SA and MES-SA Dx5 cells were grown in McCoy’s medium. NIH-MDR-G185, KB-V-1, and Dx5 cells were maintained in 60 ng/mL colchicine, 1 μg/mL vinblastine, and 500 nmol/L doxorubicin (Adriamycin), respectively, to maintain Pgp expression. All cell culture media (Life Technologies) was supplemented with 10% fetal bovine serum (Life Technologies), 5 mmol/L glutamine (Life Technologies), and 50 unit/mL penicillin and streptomycin (Life Technologies). Retroviral expression of ABCB1 in A431 cells was achieved by a method described previously (24). The human skin-derived, epidermoid carcinoma cells, A431, were maintained in α-MEM (Life Technologies) supplemented as above.

**Cell viability assay.** Viability was assessed as described previously (7). Cytotoxicity assays were performed in triplicate, and curves were fitted by Prism software (GraphPad Software, Inc.) using nonlinear least-squares regression in a normalized sigmoidal dose–response model with variable slope. Curve fit statistics were used to determine the concentration of test compound that resulted in 50% toxicity (IC50). Differences between the GI50 values were analyzed by two-sided paired Student’s
test and results were considered statistically significant at a \( P \) value of <0.05.

**Results**

Correlative information from the NCI-60 screen identifies putative MDR-selective compounds that are more active in cells that overexpress Pgp. In an effort to identify candidate MDR-selective compounds, we used quantitative real-time PCR expression data (7) to correlate Pgp mRNA levels and the publicly available DTP drug screening toxicity profiles across the NCI-60 panel. Our analysis included DTP's complete public data set, consisting of 42,657 candidate anticancer agents that had been submitted for screening. Dose-response data of the compounds screened against the NCI-60 cell panel were downloaded from DTP's Web site (July 2007 release) and curated to remove uninformative dose-response profiles.

![Figure 1: Dendrogram showing the average linkage hierarchical clustering of 64 putative MDR-selective compounds. The distance matrix is derived from Tanimoto similarity indices (http://pubchem.ncbi.nlm.nih.gov). Numbers, NSC codes. Black, compound not available for testing; in bold, confirmed MDR-selective activity; italicized, lack of MDR-selective activity; *, metal complex. Clusters of structurally related compounds where one or more analogues are active are highlighted by their core common structure (structures for all compounds are shown in Supplementary Table S1).](image-url)
97.4% of drug-Pgp correlations fall within the range $-0.4 < r < 0.4$, considered to accommodate compounds with poor or no correlation with Pgp expression, indicating that for most drugs Pgp is not a key determinant of toxicity. Although the analysis identified several putative substrates at $r < -0.4$, we focused on the positively correlated Pgp-drug correlations ($r \geq 0.4$). Data conditioning reduced the 42K set to 64 compounds ("Discovery set") showing a strong positive correlation ($r \geq 0.4$) between Pgp expression and efficacy patterns across the NCI-60 cell line panel (Supplementary Table S1; Table 1). Based on our earlier validation of the bioinformatic correlation used here, we hypothesized that these compounds would likely possess MDR-selective activity. Indeed, NSC73306, reported previously by us as an MDR-selective agent, was again identified in this screen (Table 1; ref. 7).

To characterize the structural coherence of the Discovery set, the 64 compounds were clustered based on commonality in their structural features (chemical descriptors), revealing distinct Tanimoto clustering (Fig. 1). Strikingly, 9 of the 64 compounds contain a thiosemicarbazone functional group and 5 of these are isatin-$\beta$-thiosemicarbazones: NSC73306, NSC716765, NSC716766, NSC716768, NSC716772, NSC669341, NSC693336, NSC695331, and NSC695333 (isatin-$\beta$-thiosemicarbazones in italics). This suggests that the isatin-$\beta$-thiosemicarbazone core of NSC73306, the only MDR-selective agent reported in the DTP drug database thus far, is strongly associated with MDR-selective activity. Notably, the biological activity of thiosemicarbazones often involves endogenous metal ion coordination (25, 26), and the metal chelates of thiosemicarbazones are regularly reported to be more active than the compound alone (27). The 64 compounds also include eight metal complexes (Fig. 1). Seven of these contain 1,10-phenanthroline (phen) or 2,2′-bipyridine (bipy) bidentate ligands, and six contain lanthanoid metal ions. Phen and bipy are well-characterized chelating agents known to show antiproliferative activity (28). Two highly similar 8-hydroxyquinoline analogues, NSC693871 and NSC693872, are also potent chelators with known antiproliferative activity (29–31).

A distinct cluster is formed by seven natural product-derived sesquiterpene benzoquinones based on the natural product perezone [NSC697125, 2-(1,5-dimethyl-4-hexenyl)-3-hydroxymethyl-p-benzoquinone; ref. 32]. Perezone and six closely related analogues NSC697125 (aminoperezone), NSC697124 (isoaminoperezone), NSC697129 (anilineperezone), NSC697120, NSC697128, NSC697137, and NSC697135 are known to display a range of pharmacologic properties including redox activity and mitochondrial decoupling (33). The benzoquinone moiety of perezones responsible for redox activity can also be found in other NSC compounds such as the catechol of NSC10580 (34).

Beyond these three major classes (encompassing 24 of 64 compounds), a variety of structural motifs and known drug derivatives exist, presenting a number of promising opportunities for lead drug validation. For example, there are three podophyllotoxin (or etoposide) analogues (NSC403148, NSC651859, and NSC653864), and an olivine analogue (NSC86715) that was previously identified in a screen for MDR1-related agents (35), and a recently patented Bcl-2 inhibitor (NSC168468).

**Verification of MDR-selective activity in cytotoxicity assays.** The 64 compounds listed in Table 1 correlated with elevated toxicity in proportion to Pgp expression across the NCi60 cell lines. To validate the predicted Pgp-potentiated activity, 35 compounds that were made available by DTP were tested using a cell line pair not included in the NCI-60 panel. MTT cytotoxicity assays were performed against KB-3-1, a human adenocarcinoma cell line, and KB-V1, a MDR derivative of KB-3-1 that overexpresses MDR1/Pgp (ref. 21; Table 1). By definition, MDR-selective compounds show selective toxicity toward cells expressing Pgp, defined here as the ratio of a compound's IC$_{50}$ against KB-3-1 and KB-V1 cells. A compound that is more toxic to KB-V1 cells yields a ratio >1 and indicates MDR-selective activity. Of the 35 compounds tested, 3 compounds had an IC$_{50}$ of >50 μmol/L and thus were considered non toxic. Ten of the remaining 32 compounds that were tested inhibited growth in both cell lines with similar potency, suggesting that Pgp does not modulate the toxicity of these compounds.

Conversely, 22 compounds showed preferential growth inhibition in the Pgp-overexpressing KB-V1 cell line. To assess whether the potentiation of cytotoxicity against Pgp-expressing cells is robust and not cell line specific, four compounds that were available in sufficient quantities were tested on additional MDR cell lines. Irrespective of the selecting drug used to maintain Pgp expression, the tissue of origin, and in otherwise isogenic, Pgp-transfected cell lines, all four compounds showed elevated toxicity.

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**Table 2. MDR-selective agents show elevated toxicity in relation to Pgp expression and function in Pgp-expressing cell lines**

<table>
<thead>
<tr>
<th>NSC</th>
<th>KB-3-1/KB-V1 (PSC)</th>
<th>KB-V1 (PSC)</th>
<th>NIH-3T3/G185</th>
<th>MES-SA/A431</th>
<th>MES-SA/A431</th>
<th>A431/Dx5</th>
<th>A431/B1</th>
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</thead>
<tbody>
<tr>
<td>10569</td>
<td>1.71</td>
<td>2.20</td>
<td>4.27</td>
<td>4.78</td>
<td>4.32</td>
<td></td>
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</tr>
<tr>
<td>168468</td>
<td>3.82</td>
<td>2.87</td>
<td>3.10</td>
<td>3.82</td>
<td>1.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>292408</td>
<td>2.80</td>
<td>3.20</td>
<td>1.77</td>
<td>3.50</td>
<td>4.13</td>
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<tr>
<td>713048</td>
<td>1.71</td>
<td>4.90</td>
<td>3.98</td>
<td>5.06</td>
<td>2.18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: MDR1 selectivity of NSC10580, NSC168468, NSC292408, and NSC713048 comparing parental (KB-3-1, NIH-3T3, MES-SA, and A431) and their respective Pgp-expressing derivatives (KB-V1, G185, MES-SA Dx5, and A431 B1) were determined by MTT cytotoxicity assays. For KB-V1, cytotoxicity was also assayed in the presence and absence of the Pgp inhibitor PSC833 (2 μmol/L), to determine whether functional Pgp is required for MDR-selective activity.
in Pgp-expressing cells relative to their parental line, demonstrating that the MDR-selective activity is not restricted to the KB-3/KB-V1 cell pair (Table 2). Furthermore, PSC833, a high affinity Pgp-inhibitor, was used to establish the requirement for functional Pgp to mediate sensitization in KB-V1 cells (Table 2). For each drug, inhibition of Pgp rendered the MDR cells less sensitive to the compounds, confirming that functional Pgp is required for the increased toxicity of the identified MDR-selective agents. This activity profile is shared by NSC73306 (18), and shows that the MDR1-selective activity of NSC73306 is not unique, but represents a robust modality for targeting MDR.

**Effect of structural diversification on MDR-selective activity.** We searched the DTP database for structural analogues of the confirmed MDR-selective compounds NSC10580, NSC168468, NSC292408, NSC713048 (Table 2), and NSC73306 (18). It was hypothesized that some of the close structural analogues of the five MDR-selective compounds used as seeds would show increased toxicity in KB-V1 cells. For each lead, inhibition of Pgp rendered the MDR cells less sensitive to the compounds, confirming that functional Pgp is required for the increased toxicity of the identified MDR-selective agents. This activity profile is shared by NSC73306 (18), and shows that the MDR1-selective activity of NSC73306 is not unique, but represents a robust modality for targeting MDR.

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In total, the approach of analyzing the correlation between gene expression and drug activity combined with structural similarity analysis yielded 37 MDR-selective agents. This set contains several structurally coherent subgroups, which were further analyzed for structure activity relationships. To identify common features that may be responsible for MDR-selective activity, we generated pharmacophore models for two prominent clusters, containing the thiosemicarbazones and the 10580 analogues (Supplementary Fig. S1), using the most active compounds as templates (NSC693871 and NSC337743 for the TSC and 10580 clusters, respectively). Both models contain at least two hydrogen bond acceptors, a hydrogen bond donor as well as an aromatic ring (Supplementary Fig. S1). QSAR analysis of the thiosemicarbazones and analogues of NSC168468 identified descriptors that predict MDR1-selective activity with very high accuracy \( r^2 = 0.98 \) and \( r^2 = 0.85 \), respectively (see Supplementary Fig. S2 and Supplementary Table S3 for details). Given the low number of compounds tested, these predictions are preliminary and further refinement and testing of the model is currently under way with synthetic libraries.

**The role of metal binding in MDR-selective activity.** Most compounds identified here are poorly characterized—28 of the 64 putative MDR-selective compounds do not appear in any peer-reviewed publication (SciFinder 2008 structure-search). The
NCI-60 screening data represent a unique, publicly available, information-rich source that has the potential to interface screening profiles with gene expression data and structural analysis, and to assign a putative mechanism of action to compounds (36). Self-organizing maps provide a visually compelling clustering algorithm to analyze the relation of drug activity patterns to functional categories representing distinct modes of action (37). To find out if the MDR-selective compounds are associated with a common mechanism of action, the Discovery set was projected on a self-organizing map representing cytotoxicity measurements of the DTP tumor cell screen. Most of the confirmed MDR-selective compounds project to a distinct region populated by metal chelation complexes and chelators whose activity is linked to nucleic acid metabolism (31, 37). A particularly strong cluster, suggestive of a shared mechanism of action, is formed by structurally diverse compounds including NSC73306 and NSC693871 (Supplementary Fig. S3).

Along with metal-ion chelators, such as thiosemicarbazones or the hydroxyquinolines (NSC693871 and NSC693872), the Discovery set contains several metal complexes. The relationship between chelating molecules and coordination complexes is inextricable; solution equilibria results in dissociation of a complex into ligands and metal ions, and it may be either the complex or a component such as the liberated ligand, metal ion, or counter ion that is responsible for biological activity (38). To explore the role of the ligands and metal ions in the activity of MDR-selective compounds, experiments were performed with free ligands and chelated complexes. First, we examined the actual metal binding capacity of NSC73306. The two likely coordination modes of NSC73306 are the N,S bistidentate and N,O tridentate coordination additionally through the ind-2-one oxygen (Fig. 2A). Addition of Fe3+, Cu2+, and Zn2+ resulted in immediate color changes associated with rapid complexion (Fig. 2B). Electrospray ionization mass spectrometry of each mixture confirmed the molecular weight of NSC73306 ([M-L-H]2+ + H+) and revealed a 2:1 ligand/metal complex, with each ligand singly deprotonated [M(L-H)2 + H+] indicating avid metal binding (Table 3).

If a stable metal-bound species was responsible for the enhanced activity of NSC73306, its preformed complexes should show improved activity. To address this hypothesis, metal ions were premixed with NSC73306 and cytotoxicity of the complexes was determined by MITT assays (metal ions alone were not toxic). The Cu-TSC complex, which is not stable in the reducing environment of the cell, proved significantly more toxic to both cell lines (Table 4). However, intracellular reduction of Cu2+ to Cu+ results in the dissociation of the complex and the liberation of NSC73306 (data not shown). Therefore, the increased toxicity of the Cu-TSC complex in both KB-3-1 and KB-V1 cells is probably due to the chaperoning of highly toxic Cu2+ into the cell as a complex with NSC73306. Complexation with iron lowered cytotoxicity and slightly diminished selectivity, probably by chelation that is relatively stable even within the cell. Zinc(II) generally forms more stable complexes than iron(II) (in accordance with the Irving-Williams series), yet zinc coordination did not influence MDR-selective activity. Although it is possible that the Zn-NSC73306 complex retains its activity, it seems that a stable complexed species of NSC73306 is not the MDR-selective active species; if it were, significant improvement in activity and selectivity would be observed when using preformed complexes.

Given that phen complexes with a range of metal ions (Nb3+, Ce3+, Sn4+, and La3+) were uncovered, we tested the MDR-selective activity of the free ligand. Following our initial article on the expression of ABC genes and drug-gene pairs (7), a lanthanoid tris-phenanthroline complex (KP772 ([tris(1,10-phenanthroline)lanthanum(III)] trithiocyanate)) was reported to possess selective toxicity in MDR cells (39). This compound was also tested by the DTP (NSC632737) and, notably, our screen identifies it as a putative MDR-selective compound (structural analogue of NSC292408, see Supplementary Table S2). Phen and NSC292408 showed a similar MDR-selective activity (3.5- and 2.8-fold, respectively), suggesting that it is the free ligand that is active. The La3+ and the SCN− counter-ion components that complete KP772, as well as the other metal ions, were not toxic in the dose windows studied. The ruthenium(II) complex ([Ru(phen)3]Cl2) showed significant improvement in activity and selectivity would be observed when using preformed complexes.

### Table 3. Formation of metal complexes with NSC73306

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Peak mass</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC73306</td>
<td>327.1</td>
<td>L-H</td>
</tr>
<tr>
<td>NSC73306 + Fe(II)</td>
<td>707.1</td>
<td>[Fe(L-H)2]2+ + H+</td>
</tr>
<tr>
<td>NSC73306 + Cu(II)</td>
<td>714.1</td>
<td>[Cu(L-H)2]2+ + H+</td>
</tr>
<tr>
<td>NSC73306 + Zn(II)</td>
<td>715.1</td>
<td>[Zn(L-H)2]2+ + H+</td>
</tr>
</tbody>
</table>

NOTE: Electrospray ionization mass spectrometry of NSC73306 (L, mw = 326.1 g/mol), and mixtures with metal ions (+II oxidation state) in 1:1 methanol/H2O. The highest mass peak observed is shown, in each case corresponding to a 2:1 ligand/metal complex.

### Table 4. Cytotoxicity of MDR-selective ligands and their preincubated metal complexes against the KB-3-1 parental adenocarcinoma cell line, and the MDR subline KB-V1 that expresses high levels of Pgp

<table>
<thead>
<tr>
<th>Compound</th>
<th>KB-V1, IC50 (μmol/L)</th>
<th>KB-3-1, IC50 (μmol/L)</th>
<th>MDR1 selectivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chelators</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSC73306</td>
<td>3.3 ± 1.3</td>
<td>14.2 ± 1.2</td>
<td>4.30</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>1.16 ± 0.16</td>
<td>4.04 ± 0.19</td>
<td>3.50</td>
</tr>
<tr>
<td>Desferrioxamine</td>
<td>43.1 ± 8.6</td>
<td>0.75 ± 0.12</td>
<td>0.020</td>
</tr>
<tr>
<td>Triapine</td>
<td>5.9 ± 2.6</td>
<td>1.4 ± 2.5</td>
<td>0.20</td>
</tr>
<tr>
<td>Phen</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KP772</td>
<td>—</td>
<td>—</td>
<td>2.0*</td>
</tr>
<tr>
<td>[Ru(phen)3]Cl2</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>—</td>
</tr>
<tr>
<td>KSCN</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>—</td>
</tr>
<tr>
<td>[NdCl3(C2H6)(phen)]Cl2</td>
<td>1.05 ± 0.66</td>
<td>7.73 ± 2.87</td>
<td>2.80</td>
</tr>
<tr>
<td>NSC73306</td>
<td>10.2 ± 0.9</td>
<td>19.6 ± 2.6</td>
<td>1.90</td>
</tr>
<tr>
<td>Cu(II)-NSC73306</td>
<td>0.56 ± 0.02</td>
<td>0.57 ± 0.1</td>
<td>1.00</td>
</tr>
<tr>
<td>Zn(II)-NSC73306</td>
<td>4.8 ± 4.7</td>
<td>20.8 ± 5.4</td>
<td>4.30</td>
</tr>
<tr>
<td>Fe(II)-NSC73306</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>Cu2+</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>Zn2+</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>—</td>
</tr>
</tbody>
</table>

*From Heffeter et al. Biochem. Pharmacol. 2007, 73, 1873-1886, against the KB-3-1 and P-gp expressing KBC-1 cell line pair.
by forming metal chelates. As the rate of ligand exchange is approximately eight orders of magnitude slower in Ru(II) complexes than La(III) complexes (40), insignificant levels of phen would be exchanged from the Ru(II) complex in a 72-hour cytotoxicity experiment. The inert ruthenium complex was completely inactive. Of the NSC292408 (a tin-phenanthroline complex) analogues available for testing, only one, the platinum-phenanthroline NSC615541, showed MDR-selective activity (Supplementary Table S2). The inactive NSC292408 analogues either lack the MDR-selective phen ligands or are such stable complexes that they may not be released to exert their activity. The fact that these chelating ligands alone possess cytotoxic response profiles similar to their metal chelates indicates that the active metal complexes serve as carriers for the chelators, which themselves are the active drug molecules, perhaps by forming metal chelates in situ (31).

Discussion
An ultimate goal in cancer therapy is to devise individually tailored treatment protocols targeting specific pathways of cellular proliferation or drug resistance. Pgp represents one of the best-studied mechanisms of resistance to hydrophobic anticancer drugs. Pgp-mediated drug transport of cytotoxic drugs is modulated by a wide range of agents, many of which have been tested in clinical trials. However, despite the clear rationale for the use of efflux inhibitors in combination with cytotoxic agents, the development of these compounds has been slow, and the clinical benefit of Pgp inhibition is still in question. Our aim was to explore the DTP drug database for compounds that may offer a radically different solution by exploiting, rather than suppressing, Pgp function to induce cytotoxicity. Such drugs (termed "MDR-selective compounds") target otherwise MDR cycling cells, without inhibiting the transporter and avoiding side effects associated with the damage of resting cells that constitutively express Pgp. We based our approach on the presumption that a positive correlation between a compound's cytotoxicity profile and the expression of Pgp in the NCI-60 cell panel may be the result of a causal interaction, where the activity of Pgp sensitizes the cell to the cytotoxicity of the compound. It may be argued that this approach has several limitations: the cytotoxicity profiles across the NCI60 panel are derived from single MTT assays, leading to variability in the quality of the primary data; the possibility that compounds are not pure as submitted; and the activity of drug metabolism and resistance pathways may confound the statistical approach, producing false negative and false positive results. Given these and other confounding factors, it is remarkable that 37 MDR-selective compounds were validated in this study. Future studies will determine if any of the compounds not available for testing (Table 1) are also MDR selective. Of particular interest is the series of perezone derivatives that appear in a structurally coherent group among the top-scoring untested compounds (Fig. 1).

The new MDR-selective compounds presented here have several features reminiscent of NSC73306, the first Pgp-targeting drug identified in our initial report (7).

Because the MDR-selective toxicity is reversed in the presence of Pgp inhibitors, we can conclude that the enhanced toxicity of the compounds shown in Table 2 is indeed dependent on Pgp activity, and is not due to an off-target effect linked to the genetic drift of KB-V1 cells. Furthermore, the results obtained with an isogenic cell line pair support that functional expression of Pgp is necessary and sufficient to convey increased sensitivity to these compounds. The degree of Pgp-dependent toxicity varied; whereas some compounds showed a minor (albeit statistically significant) MDR-selective activity, others, such as NSC693871, were considerably more toxic in KB-V1 cells. As observed with NSC73306, expression of Pgp in MDR cell lines decreased upon exposure to the MDR-selective agents, supporting a causal link between the toxicity and Pgp function.7

Analysis of the MDR-selective molecules in the context of the DTP drug database has the potential to relate the information in the tumor cell line data set to structural feature analysis. Granted that the general toxicity of the MDR-selective compounds identified here is probably mediated by a number of mechanisms, the structural coherence of the Discovery set may imply shared modes of MDR-selective toxicity that pertain to structurally related compound subsets. There is a significant enrichment in certain chemical features within the Discovery set: the nine TSCs comprise 14% of the Discovery set, while representing only 1% of the original set of the screened compounds, and there are only 25 phen and 56 bipy compounds in the original data set (0.059% and 0.12%, respectively). Thus, the enrichment of TSC (~12-fold), phen (over 1,500-fold), and bipy (over 750-fold) reinforces that these features are associated with MDR-selective activity. In all three structural cohorts, there are two nitrogen atoms in a cis(1,4) arrangement, separated by two carbons. The initial QSAR (Supplementary Table S3) is a first step toward a systematic substructure analysis coupled with statistical correlation of compound activity, to highlight structural features necessary for MDR selectivity (41). Recently, the TSC Dp44mT was reported to be more toxic to KB-V1 cells (42). Dp44mT was not submitted to the DTP; therefore, it does not appear in our list of putative MDR-selective agents. Interestingly, it also contains at least two hydrogen bond acceptors, a hydrogen bond donor as well as an aromatic ring, underscoring the pharmacophore model derived from the TSCs analyzed in this study (Supplementary Fig. S2).

The abundance of metal chelators in the Discovery set is striking. The eight metal-containing compounds represent 12.5% of the Discovery set, whereas only 2.7% of the compounds found in the DTP library form complexes (including sodium and calcium cation formulations). Several MDR-selective compounds project to the S region of the self-organizing map representing the DTP drug response data (Supplementary Fig. S3), along with chelating agents targeting metallo enzymes, especially iron- and copper-dependent proteins (31). Typically, metal complexes projecting to the S region show some degree of similarity in both structure and cytotoxic response profiles. Interestingly, the type of the chelated metal is varied, suggesting that the cytotoxicity of S-region complexes is determined primarily by the organic component of the complex (the ligand) and the type of metal is only playing a minor role (31). Our results indicate that the MDR-selective chelators can form complexes with a range of biologically important metals ions, including iron (Fig. 2). Extensive research efforts have been dedicated to the design of iron or copper chelators as anticancer agents, and several drugs in the clinic such as bleomycin, the anthraquinones, triapine, and hydroxyurea show the validity and diversity of metal interaction as a viable chemotherapeutic target (38). Based on the results obtained by this correlative observational study, we speculate that metal ion interaction is key to the cytotoxicity of at least a subset of the MDR-selective compounds. Iron-chelating drugs may kill cancer cells by a number of mechanisms, including (1) deprivation of...
nutritionally essential iron, (2) iron-drugs associations that lead to redox cycling (6), or (3) highly specific metal enzyme targets, such as ribonucleotide reductase (43). We speculate that the activity of Pgp may increase the susceptibility of the cells to these mechanisms. At the same time, our results also suggest that metal chelation alone is not sufficient for Pgp-potentiated activity. Because metal complexes were as active as the ligands alone (Table 4), chelates may serve as chaperones facilitating free diffusion of the ligands in to the cells (44). In line with this hypothesis, NSC73306 does not interact with Pgp (18), whereas triapine (a TSC ribonucleotide reductase inhibitor devoid of MDR-selective activity) is actively effluxed by Pgp (45). Thus, it seems that evasion of Pgp-mediated efflux as well as the ability to chelate metals are necessary, if not sufficient requirements for the MDR-selective activity of the chelator compounds. Dissociation of the complexes within the cells leading to the release of the free ligand and MDR-selective activity may be related to reactive oxygen species generated by redox cycling, as has been shown for the activity of other TSCs (46).

Taken together, it seems that the target of the MDR-selective compounds is not Pgp per se. The paradoxical vulnerability uncovered by the MDR-selective compounds may be linked to the efflux of an endogenous substrate providing, e.g., redox resistance to the influence of Pgp on membrane lipid composition, or to other, Pgp-related metabolic changes that characterize MDR cells. The results here indicate that MDR-selective compounds may be used against MDR cells, providing a range of active pharmacophores. According to a recent report, the TSC Dp44mT has broad spectrum activity against a wide range of cancer cell types in vivo, including those possessing the MDR phenotype mediated by Pgp (42). The fact that a related TSC shows strong in vivo anticancer activity in human xenografts is promising, and suggests that the MDR-selective chelators identified in this study may have potent and broad antitumor activity. Further evaluation of the compounds identified here will help to elucidate this intriguing modality, and provide the basis of a fresh therapeutic approach to resolving MDR cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Identification of Compounds Selectively Killing Multidrug-Resistant Cancer Cells

Dóra Türk, Matthew D. Hall, Benjamin F. Chu, et al.


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