

Targeting the Achilles Heel of Multidrug-Resistant Cancer by Exploiting the Fitness Cost of Resistance

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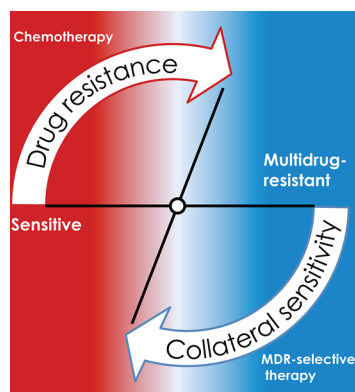
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CONTENTS

1. Introduction and Scope of the Review	5753
2. Multidrug Resistance (MDR)	5754
2.1. ABC Transporters That Confer MDR	5754
2.2. Efforts to Overcome MDR	5755
2.3. Collateral Sensitivity: Strength into Weakness	5756
3. MDR-Selective Compounds Targeting Cells Overexpressing P-GP	5756
3.1. Serendipitous Findings	5756
3.2. NCI-60 Cell Panel: Data Mining in the Database of the Developmental Therapeutics Program (DTP) of the National Cancer Institute	5757
4. Structure–Activity Studies with MDR-Selective Compounds	5759
4.1. Isatin- β -thiosemicarbazones and Analogues	5759
4.2. Desmosdumotin B Analogues	5761
5. Compounds Targeting Multidrug-Resistant Cells Overexpressing MRP1	5762
5.1. Small Molecules Preferentially Targeting MRP1-Overexpressing Multidrug-Resistant Cells by Promoting GSH Efflux	5762
5.1.1. Verapamil and Derivatives	5762
5.1.2. Xanthones	5763

5.1.3. Flavonoids	5763
5.1.4. Additional Compounds Targeting MRP1-Expressing Cells through Induced GSH Depletion	5764
5.2. GSH-Independent Collateral Sensitivity of MRP1-Expressing Multidrug-Resistant Cells	5764
6. Compounds Targeting ABCG2-Expressing Multidrug-Resistant Cells	5764
6.1. Serendipitous Findings	5765
6.2. Mining the DTP Database for ABCG2-Related Compounds	5765
6.3. Photodestruction of ABCG2-Rich Extracellular Vesicles	5765
7. Common Mechanistic Features: Targeting the Fitness Cost of Resistance	5765
7.1. Collateral Sensitivity of Multidrug-Resistant Cells Might Not Be Linked to Transporter Expression	5766
7.2. Role of Transporters in the Collateral Sensitivity of Multidrug-Resistant Cells	5766
7.2.1. Transporters as Targets	5767
7.2.2. Unshielding	5767
7.2.3. Activation	5767
7.2.4. Depletion	5767
8. Perspectives	5769
Author Information	5769
Corresponding Authors	5769
Notes	5769
Biographies	5769
Acknowledgments	5771
References	5771
Note Added after ASAP Publication	5774

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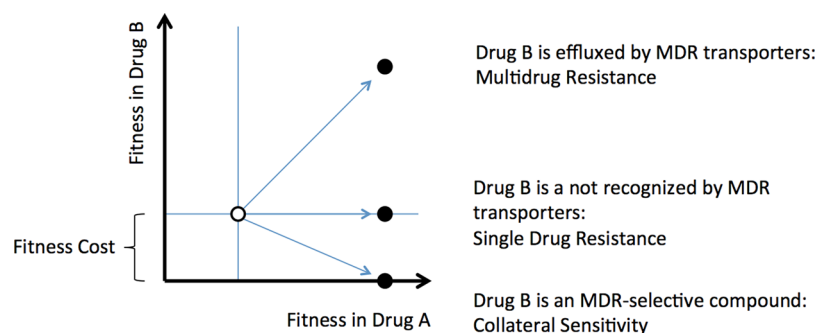


Figure 1. Collateral sensitivity. Changes accompanying acquired resistance to drug A can be beneficial, neutral, or detrimental in the presence of drug B. Cancer cells tend to increase their fitness through the overexpression of efflux transporters that keep the concentration of drug A below a cell-killing threshold. If drug B is not a transported substrate, resistant cells can be eradicated. However, given the wide substrate specificity of the transporters, cancer cells selected in drug A often survive despite treatment with drug B (multidrug-resistant cells show increased fitness in both environments). Conversely, resistance against drug A can be accompanied by decreased fitness in drug B (collateral sensitivity).

1. INTRODUCTION AND SCOPE OF THE REVIEW

The development of multidrug resistance (MDR) in patients suffering cancer remains a significant clinical challenge, with drug efflux by ABC (ATP-binding cassette) transporters contributing significantly. Strategies to circumvent the reduced drug accumulation conferred by these polyspecific efflux transporters have relied on attempts to develop drugs that bypass extrusion (often with a sacrifice in activity) or the exploration of clinical inhibitors that, although showing promise *in vitro*, have not translated to the clinic.

Alterations that confer selective advantage during the evolution of cancer cells might also create vulnerabilities that can be exploited therapeutically.¹ As defined by Szybalski and Bryson, collateral sensitivity is a “phenomenon in drug-resistant cells (prokaryotic or eukaryotic) identified during most *in vitro* studies... [whereby] the development of resistance in cells to one agent can confer higher sensitivity to an alternate agent than seen in the original (parental) line”.² In other words, the resistant cell line is *more sensitive* to a cytotoxin than the parental line from which it is derived (Figure 1). From this perspective, resistance can be interpreted as a trait that could be targeted by new drugs. In this review, we discuss general mechanisms underlying collateral sensitivity and focus on small molecules reported to elicit increased toxicity in cells overexpressing one of the three major multidrug transporters. Such molecules (termed MDR-selective compounds) target multidrug-resistant cycling cells, suggesting that MDR ABC transporters could be considered as the ultimate “Achilles’ heel”—the exquisite spot to fatally wound a multidrug-resistant cancer cell. Herein, we discuss the potential of this emerging technology, cataloging MDR-selective compounds reported in the literature and highlighting chemical features that are associated with MDR-selective toxicity.

2. MULTIDRUG RESISTANCE (MDR)

Despite major advances in therapy, diagnosis, and prevention, cancer remains a deadly disease, claiming 1500 lives every day in the United States. Most who succumb to cancer die because their disseminated cancer does not respond to available chemotherapies. Although cures might be achieved with better drugs, cancer cells usually respond by deploying a variety of mechanisms that result in the loss of their initial hypersensitivity to anticancer drugs.³ Much has been learned about drug action, and efforts to elucidate the molecular basis for resistance have revealed a variety of mechanisms that either

prevent a drug from reaching its target, deploy compensatory mechanisms promoting survival, or lull cancer cells into a dormant state. Theoretically, one could restore the efficacy of first-line drugs by circumventing these resistance mechanisms. However, cancer is a heterogeneous disease that can exhibit different characteristics from patient to patient or even within a single patient. Spatial and temporal heterogeneity is a result of continuous adaptation to selective pressures through sequential genetic changes that ultimately convert a normal cell into intractable cancer. Thus, cancer cells are moving targets, as individual cells in a tumor mass constantly adapt to local environmental challenges. In the context of this pre-existing diversity, chemotherapy exerts a strong selective pressure favoring the growth of variants that are less susceptible to treatment. In the case of targeted therapies, mechanisms of resistance might be limited to the specific drugs whose action is dependent on a given cancer-specific target.

Combination of drugs with multiple targets might prevent treatment failure due to drug resistance, but at a cost of increased side effects caused by long-term multiple-drug treatments.⁴ Combination treatments can also lose efficacy due to cellular mechanisms that induce resistance to multiple cytotoxic agents. Of these mechanisms, the one that is most commonly encountered in the laboratory is the increased efflux of a broad class of hydrophobic cytotoxic drugs that is mediated by ATP-binding cassette (ABC) transporters.⁵ Multidrug resistance (MDR) conferred by ABC transporters, including ABCB1 [MDR1/P-glycoprotein (P-gp)], ABCC1 (MRP1), and ABCG2 (BCRP/MXR), represents a significant clinical challenge for drug design and development.

2.1. ABC Transporters That Confer MDR

Biological membranes represent a significant permeation barrier and thus play a critical role in the protection of pharmacokinetic compartments. Conversely, the activity of a drug ultimately depends on the ability of the compound to reach its target, which might reside in a well-protected pharmacological sanctuary. It is widely accepted that drug permeation across membrane barriers is regulated by the basic physical characteristics of the drugs as well as their interactions with membrane transporters.^{6–8} In cancer therapy, the ultimate membrane barrier is the plasma membrane of the cancer cell. ABC transporters are active components of this barrier, and on the basis of their overlapping substrate recognition patterns, they act as a shield for drug-resistant cancer cells.⁵ Functional ABC transporters are large integral membrane proteins

containing two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). The molecular mechanism of transport is fueled by the energy of ATP hydrolysis, which results in a series of conformational changes sweeping through the molecule from the cytoplasmic ATP-binding units to the TMD helices forming the transmembrane pore. ATP binding and hydrolysis regulates the association and disassociation of the NBD dimers, which is, in turn, coupled to a change in substrate binding affinity and transport.⁹ ABC transporters recognize an extremely large variety of toxicologically relevant compounds, including (but not limited to) anticancer drugs, human immunodeficiency virus (HIV) protease inhibitors, antibiotics, antidepressants, antiepileptics, and analgesics.¹⁰

In the case of P-gp (ABCB1), which mostly exports hydrophobic compounds, the molecular explanation of this promiscuous behavior is that substrates are recognized in the context of the plasma membrane. Thus, P-gp was suggested to act as a “hydrophobic vacuum cleaner” of the plasma membrane, preventing the cellular entry of xenobiotics.¹¹ Overexpression of P-gp has been observed in drug-resistant cell lines generated through exposure to increasing concentrations of cytotoxic drugs, suggesting that preemptive transport is surprisingly efficient in keeping cytotoxic drugs below a cell-killing threshold. P-gp expression is well-characterized in hematological malignancies, sarcomas, breast cancer, and other solid cancers and is frequently correlated with poor clinical response to chemotherapy.¹²

MRP1 (ABCC1) was discovered in 1992.¹³ In addition to the canonical (TMD-NBD)₂ organization, MRP1 (ABCC1) contains an additional N-terminal domain, TMD0, composed of five transmembrane helices.¹⁴ In contrast to P-gp, the two nucleotide binding domains, NBD1 and NBD2, are non-equivalent with respect to their ability to bind and hydrolyze ATP: NBD1 binds ATP with a higher affinity than NBD2, but is defective for ATP hydrolysis in contrast to NBD2. MRP1 has a marked preference for negatively charged substrates, namely, organic anions, including diverse secondary metabolites such as glutathione (GSH), glucuronate, and sulfate conjugates. MRP1 recognizes and exports several forms of glutathione (GSH), including the oxidized form glutathione disulfide (GSSG), reduced glutathione (GSH/GS⁻), and glutathione conjugates (GS-X). The MRP1-preferred endogenous substrate is the glutathione-conjugate leukotriene C4, suggesting a physiological role in inflammation. In addition to conjugates, some hydrophobic P-glycoprotein substrates, such as vinblastine and vincristine, are also transported by MRP1 by symport with GSH.¹⁵ The expression of MRP1 is ubiquitous, with high levels in lung, kidney, testes, and placenta. MRP1 is generally located at the blood–tissue barriers, suggesting that the transporter contributes to protection of sanctuary sites in the body. MRP1 mostly localizes at the basolateral membrane in polarized cells, in contrast with the apical membrane localization of P-gp and ABCG2. The ability of MRP1 to transport both GSH and GSSG suggests its possible contribution to maintain the cell redox state. In the clinical setting, even low levels of MRP1 expression can have prognostic relevance. Allen et al. showed that the relatively low levels of MRP1 expression found in most untreated tumors could substantially affect their basal sensitivity to antineoplastic drugs.¹⁶ Although MRP1 is not considered a primary actor in MDR, its relevance in oncology is supported by studies linking its expression to unfavorable prognosis in ovarian, lung, breast, renal, prostate, leukemia, and colorectal

cancers.^{17–21} In particular, the association of MRP1 expression with poor clinical outcome was convincingly demonstrated in a prospective trial based on a high-powered statistical analysis of a large primary neuroblastoma patient group.²² Homozygous deletion of the MRP1 gene in primary murine neuroblastoma tumors results in increased sensitivity to MRP1 substrate drugs, suggesting that inhibition of MRP1-mediated drug efflux might be a viable strategy for therapy improvement.²³

The ABCG2 transporter was discovered in three different laboratories and was named ABCP, based on its abundance in placenta;²⁴ breast cancer resistance protein (BCRP), based on the expression in resistant breast cancer cells;²⁵ and mitoxantrone resistance protein (MXR), based on its ability to confer resistance to mitoxantrone.²⁶ ABCG2 is a “half-transporter”, containing a single NBD and TMD. Similar to bacterial ABC half-transporters, which were crystallized as homodimers,²⁷ ABCG2 displays dimerization motifs²⁸ and indeed needs to dimerize to be functional.²⁹ The physiological functions of ABCG2 are diverse. Its expression in normal tissues is relatively high in placental syncytiotrophoblasts, brain microvessels, and endothelial tissue, as well as in the kidney, small intestine, liver, testes, ovary, and colon. The steroid dependence of its expression, upregulation by progesterone in the placenta, and downregulation by either 17 β -estradiol or dexamethasone in breast cancer cells suggests a role in steroid transport, in agreement with the observed ABCG2-dependent transport of sulfated estrogens.^{30–32} Recently, ABCG2 was shown to efflux urate, and mutations in ABCG2 have been identified to be responsible for at least 10% of all gout cases.³³ Although the direct ABCG2-mediated efflux of GSH has not been shown, a recent study suggests that overexpression of human ABCG2 in yeast results in increased extracellular GSH accumulation, in line with the correlation of ABCG2 expression with extracellular GSH levels in human cancer cell lines.³⁴ Similarly to ABCB1, ABCG2 is located at the apical membrane of polarized cells, such as the blood–brain barrier.³⁵ ABCG2 is also highly expressed in stem cell membranes and was shown to be responsible for the Hoechst 33342-dim phenotype of side-population (SP) cells.³⁶ Its likely role is to protect critical cells against xenobiotics or endogenous catabolites, such as heme and porphyrins,³⁷ which can be toxic under unfavorable hypoxic conditions. These intrinsically resistant stem cells might contribute to tumor resistance to chemotherapy.³⁸ Expression of ABCG2 mRNA, but not always protein, has also been detected in human embryonic stem cells.^{39–41} ABCG2 is able to transport various types of drug substrates, including many anticancer chemotherapeutics.^{31,42–45} Studies aimed at correlating the expression of ABCG2 in cancer cells and its effects on clinical outcome have produced controversial results.⁴⁶ ABCG2 is prognostic in adult and pediatric acute myeloid leukemia (AML).^{47–49} It is expressed in many types of solid tumors and is commonly highly expressed in tumors of the digestive tract, endometrium, and melanoma.⁵⁰ It remains to be seen whether the association of ABCG2 expression in solid tumors with adverse treatment outcome is directly related to drug efflux or whether ABCG2 merely serves as a marker for other mediators of poor-risk cancers such as the presence of cell subpopulations with “stem-like” properties.⁴⁷

2.2. Efforts to Overcome MDR

Genetic or phenotypic alterations that are related to treatment response can serve as biomarkers for the stratification of patients and can also reveal targets for chemotherapeutic

intervention. P-gp has long been recognized as a drug target (Figure 2). Because attempts to develop drugs that bypass P-

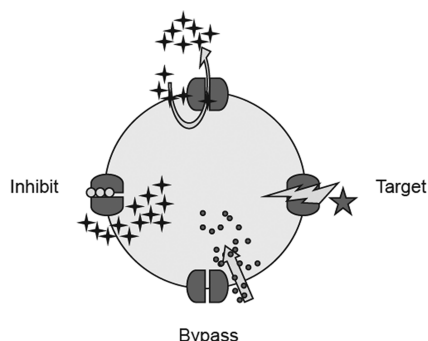


Figure 2. Efforts to overcome transporter-mediated MDR. ABC transporters protect MDR cells by keeping the concentration of cytotoxic drugs below a cell-killing threshold. Concomitantly administered inhibitors block the transporter, thus preventing the efflux of the cytotoxic compounds.¹⁸⁴ Another strategy for improving therapy response is to design new classes of anticancer agents that bypass the multidrug transporters. Selective toxicity of MDR-selective compounds is specifically tied to the activity of multidrug transporters, suggesting a fatal weakness that can be exploited by a new modality for tackling multidrug-resistant cancer. Adapted with permission from ref 5. Copyright 2006 Nature Publishing Group.

gp-mediated extrusion have led to limited success,⁵¹ the pharmaceutical industry has concentrated on strategies to circumvent the reduced drug accumulation conferred by these polyspecific efflux transporters.⁵² In fact, efflux pumps were believed to be key to the understanding and eventual defeat of multidrug-resistant cancer.³ Given the ability of P-gp to protect cells in tissue culture (and the ease of inhibiting drug efflux), it seemed reasonable to expect that this same effect would also occur in vivo. Seduced by the prospect of easy success, the MDR field has become disenchanted perhaps too easily as clinical trials conducted with P-gp inhibitors continued to produce negative results.⁵³ In 2010, a multicenter randomized trial failed to demonstrate any benefit of the third-generation MDR inhibitor zosuquidar in AML or high-risk myelodysplastic syndrome (MDS), strengthening the emerging consensus that P-gp should be taken off the list of druggable targets.⁵⁴ In our view, the negative results of the clinical trials are over-interpreted. In addition to the pharmacokinetic limitations of the tested inhibitors, inadequate trial design, unwanted drug–drug interactions occurring at physiological sites expressing P-gp, and the cross-inhibition of cytochrome P450 could have contributed to the failure of the studies.^{12,55} The physiological role of ABC transporters that confer MDR is linked to the general “chemoimmunity” network that protects our body against the accumulation of foreign chemical agents, such as the cytotoxic drugs commonly found in chemotherapy regimens.¹⁰ Inhibition of P-gp in cancer cells resulted in the altered distribution of coadministered cytotoxic compounds. Indeed, because selective modulation of P-gp in cancer cells (and not at physiologic sites) will be difficult to achieve, attempts to circumvent MDR will need to consider the profound effects on the pharmacokinetics and distribution of concomitantly administered drugs. As such, inhibitors are losing their appeal for drug development, and today, only a few open studies remain listed on the NIH’s clinical trials home page (www.clinicaltrials.gov). In our opinion, the verdict on inhibitors is

still out; perhaps, we should lower our expectations about the magnitude of the potential benefit and wait for the results of trials conducted on selected patients with confirmed expression of functional ABC transporters in their tumors.⁵⁶ It is possible that the failure to improve treatment outcome with P-gp inhibitors was, at least in part, attributable to the contribution of MRP1 and/or ABCG2 to MDR. The substrate specificity of the three MDR ABC transporters shows substantial overlap, so inhibition of P-gp alone might not necessarily prevent drug efflux. Interestingly, inhibitors appear much more restricted, with very few acting on all three transporters.⁵⁷ Although affinity for multiple drug transporters might extend the functionality of inhibitors to MRP1- or ABCG2-expressing tumors showing MDR, the scope of possible side effects might limit their clinical use.

MDR transporters are enjoying a renaissance, as their role in shaping the interindividual differences in drug efficacy and toxicity is increasingly recognized.⁵⁸ Although inhibitors have generally proved underwhelming in trials aimed at sensitizing multidrug-resistant malignancies to chemotherapy, the same compounds have proved effective at inhibiting P-gp expressed in pharmacologically relevant barriers. For example, tariquidar, a third-generation inhibitor, has been shown to inhibit the function of P-gp at the blood–brain barrier. Tariquidar was measured by increased penetration of radiolabeled P-gp substrates using positron emission tomography (PET), albeit at higher doses of inhibitor than used in cancer clinical trials.⁵⁹

2.3. Collateral Sensitivity: Strength into Weakness

The concept that cancer cells, in adapting to a cytotoxin, also acquire inherent sensitivity to alternative cytotoxins is attractive from a therapeutic viewpoint.⁶⁰ Our review of the literature identified several compounds that were reported to be preferentially toxic against P-gp-expressing cells. The paradoxical hypersensitivity (collateral sensitivity) of otherwise multidrug-resistant cells suggests that this well-studied drug resistance mechanism can be exploited as a weakness by compounds whose activity is potentiated, rather than diminished, by the activity of transporters that confer MDR. It is important to emphasize that the concept of MDR targeting based on collateral sensitivity is substantially different from the strategy of transporter inhibition. Small-molecule transporter inhibitors do not exhibit intrinsic toxicity (no limiting toxicities have been reported); they are coadministered with cytotoxic drugs to prevent drug efflux and to reverse resistance, resulting in sensitivity equivalent to that of a cell without transporter expression.

Much work remains to ascertain whether the development of resistance to a single drug, or drug regimen, consistently results in cellular alterations that render the cell susceptible to an MDR-selective agent. In the following sections, we discuss the general mechanisms underlying collateral sensitivity and focus on compounds reported to elicit increased toxicity in cells overexpressing one of the three major multidrug transporters.

3. MDR-SELECTIVE COMPOUNDS TARGETING CELLS OVEREXPRESSING P-GP

3.1. Serendipitous Findings

The paradoxical hypersensitivity of P-gp-expressing multidrug-resistant cells was initially perceived as a curious anomaly.⁶¹ Most compounds were identified in studies that were undertaken with the intent of characterizing the extent of drug resistance in multidrug-resistant cells. The first of these

was reported by Gupta, who assessed the sensitivity of CHO cells selected for resistance to vinblastine (Vin^R) and taxol/paclitaxel (Tax^{R-2}).⁶² Both cell lines showed cross-resistance or equal sensitivity (compared with parental cells) to 10 microtubule inhibitors, as might be expected given the mechanism of action of taxol and vinblastine. The cells were then assessed against 37 other cytotoxins of varying mechanisms, of which nine were found to elicit collateral sensitivity, with two being DNA-damaging agents (cisplatin, bleomycin) and six being antimetabolites (vidarabine, acicivir, cytarabine, 5-fluorouracil, tegafur, tiazofurin). In a similar study, Jensen et al. characterized the activity of 19 cytotoxins against daunorubicin-resistant H69/DAU4 human lung small-cell carcinoma (expressing P-gp), etoposide-resistant H69/VP (with alteration in topoisomerase II activity), and teniposide-resistant OC-NYH/VM human lung small-cell carcinoma cells.⁶³ The mechanism of resistance in the latter cell line was not well understood at the time but has subsequently been shown to be due to overexpression of MRP1.⁶⁴ Among the 19 drugs tested, the multidrug-resistant cell lines showed collateral sensitivity to cytarabine (antimetabolite) and carmustine (DNA damaging). The same authors went on to identify cross-resistance and collateral sensitivity patterns in a set of multidrug-resistant human small-cell lung cancer cell lines. Resistance to alkylating agents (cisplatin and carmustine); topoisomerase inhibitors (topotecan and camptothecin); or other cytotoxins such as daunorubicin, etoposide, and vinblastine was partly due to the upregulation of ABC transporters—although, in each case, there were undoubtedly other alterations to the cells.⁶⁵ Clonogenic assays were used to assess the activity of 20 cytotoxins against these cells. All seven multidrug-resistant cell lines demonstrated collateral sensitivity to at least one cytotoxin. Clinically, the most interesting observation was the inverse relationship between taxol and cisplatin, where cells cross-resistant to taxol showed collateral sensitivity to cisplatin, and vice versa. This has important implications because platinum and taxanes are used in combination in the clinic.⁶⁶ Rickardson et al. assessed the activity of the library of pharmacologically active compounds (LOPAC, 1266 compounds), at a single dose of 10 μ M, against RPMI 8226 human myeloma cells and the doxorubicin-resistant subline 8226/Dox40.⁶⁷ Thirty-three compounds were found to selectively kill the 8226/Dox cells, including a group of compounds whose structural similarity clustered with the glucocorticoid betamethasone (beclomethasone, budesonide, dexamethasone, triamcinolone, hydrocortisone), the most active being dexamethasone with ~30-fold selective killing. Glucocorticoid steroids bind to the glucocorticoid receptor (GR), at the cell surface, to elicit downstream intracellular signaling. Dose–response killing curves of each hit, with and without addition of the GR antagonist RU-486, demonstrated reversal of selectivity that is consistent with the collateral sensitivity being elicited by GR binding. Several other multidrug-resistant cell line pairs were tested with the glucocorticoids, with only one (the teniposide-resistant CCRF-CEM subline CEM/VM1) showing collateral sensitivity. Microarray analysis of RPMI 8226 and 8226/Dox gene expression revealed increased expression of the GR NR3C1 in the doxorubicin-resistant cell line. Glucocorticoids are known to induce apoptosis in hematological malignancies (and therefore in the myeloma cells used in this study).⁶⁸ It has been reported that dexamethasone-resistant cells downregulate the expression of GRs to diminish apoptotic signaling.⁶⁹ In this

instance, the upregulation of a GR in the resistant cells is responsible for their hypersensitivity to glucocorticoids. The caveat here is that the acquisition of the collateral sensitivity mechanism is specific to hematologic-derived cell lines.

Another example of a compound whose MDR-selective activity was found to be restricted to a cell line is the orphan drug tiopronin, the condensation product of glycine and thiolactic acid. As with many ad hoc observations of this kind, tiopronin was assessed for P-gp substrate activity but was unexpectedly found to selectively kill multidrug-resistant KB-V1 cells.⁷⁰ MCF7 VP-16 cells that overexpress MRP1 also showed strong hypersensitivity to tiopronin (cf. Figure 13). However, unlike the P-gp-specific agents described in this review, selectivity toward P-gp-expressing cells could not be reversed by tariquidar, and P-gp-transfected cells and a number of other resistant P-gp-expressing cells were not hypersensitive to tiopronin. These data suggested that a molecular alteration in multidrug-resistant cells, not related to P-gp expression, was responsible for the hypersensitivity of cells to tiopronin. Tiopronin contains a thiol group, and synthetic analogues of tiopronin prepared with a methylated thiol (thioether), or the thiol replaced altogether, showed no selective activity, demonstrating that the thiol is critical for tiopronin activity.⁷⁰ Analogues of tiopronin were also generated by replacing the glycine with alanine, valine, serine, or phenylalanine; all retained selective activity, emphasizing that the thiol was indeed critical for activity. Given that reactive oxygen species (ROS) have been implicated in selective killing (vide infra), a range of other thiol-containing and thiol-reactive compounds were tested to confirm that simply the presence of a thiol was however not sufficient for selective toxicity.

Taken together, the above examples demonstrate that multidrug-resistant cells may indeed exhibit collateral sensitivity to selected compounds. One limitation of these findings is that the contribution of MDR pumps, versus other acquired cellular alterations, was not (and perhaps could not be) delineated. That ABC transporters (in particular P-gp) conferring MDR may confer sensitivity, rather than resistance, to cancer cells was to be proven with systematic studies.

3.2. NCI-60 Cell Panel: Data Mining in the Database of the Developmental Therapeutics Program (DTP) of the National Cancer Institute

The U.S. National Cancer Institute (NCI) 60 anticancer drug screen was developed in the late 1980s as an *in vitro* “disease-oriented” screening model aiding anticancer drug discovery.⁷¹ Although the diversity of mechanisms dictating chemosensitivity of real tumors greatly surpasses that of the NCI-60 cancer cell lines, representing nine distinct tumor types, the screen successfully identified compounds targeting particular tumor types (the most notable success was the development of the proteasome inhibitor bortezomib (Velcade; PS-341), which was approved by the U.S. Food and Drug Administration (FDA) in March 2003 for use in the treatment of myeloma). While the relevance of cell line-based approaches in drug resistance research is continuously debated,^{72,73} the NCI-60 screen produced a vast data set containing patterns of drug action generated with standard anticancer drugs and tens of thousands of candidate anticancer agents. Unexpectedly, the screening data was found to reflect drug action and mechanisms of drug resistance or sensitivity. It was early recognized that P-gp may leverage toxicity profiles, and a multidrug-resistant cell line expressing high levels of P-gp was intentionally included in

the cell panel to delineate the importance of MDR in drug discovery and development.⁷¹ Early laboratory investigations of the cells suggested a correlation of P-gp expression with drug resistance.⁷⁴ The development of the 'omic' technologies and integration of multiple forms of system-wide information with drug-sensitivity profiles revealed more of the genomic basis of anticancer drug response.⁷⁵ The comprehensive molecular characterization of ABC transporters across the NCI-60 panel identified the transporters that contribute to in vitro drug resistance.⁷⁶ Drug-transporter pairs could be identified by linking ABC transporter function to resistance against specific compounds, and correlating the expression patterns of ABC transporters with the growth inhibitory profiles of candidate anticancer drugs tested against the cells. Given the well-known role of P-gp in MDR, it was expected that the activity pattern of confirmed substrates (e.g., geldanamycin, paclitaxel, doxorubicin and vinblastine) would show an inverse correlation to P-gp expression (Figure 3, lower panel). Extended data mining identified additional P-gp substrates, as well as several other ABC transporters and their respective substrates.⁷⁶ Unexpectedly, the toxicity of a thiosemicarbazone (NSC73306, cf. Figure 6) showed positive correlation with the expression of P-gp. These data suggested that NSC73306 could inhibit the growth

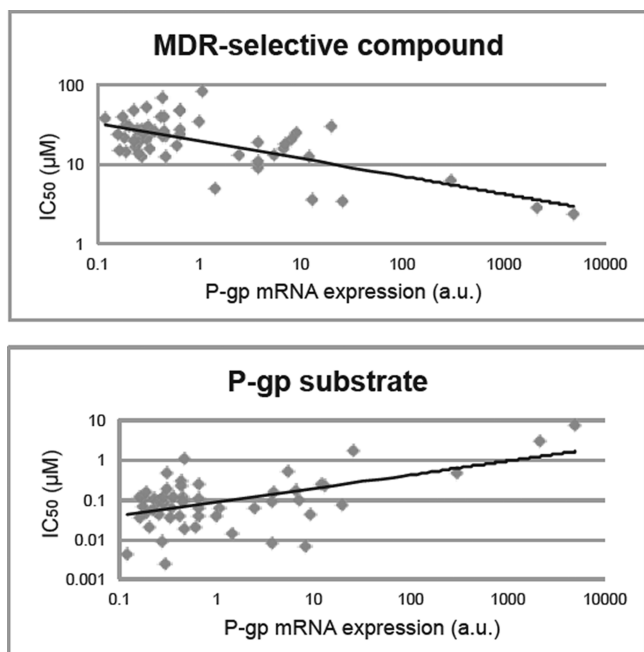


Figure 3. Correlation of drug-sensitivity patterns and gene-expression profiles in the NCI-60 cell tumor cell panel reveals putative mechanisms of drug resistance (lower panel) and collateral sensitivity (upper panel). The NCI-60 cell panel encompasses wide P-gp expression levels, which provides an opportunity to relate P-gp levels to drug activity. The toxicity of a drug can decrease if the compound is extruded from the cells by P-gp. Consequently, the IC_{50} values of transported substrates and the P-gp expression levels across the 60 cells are expected to be positively correlated (lower panel). Analysis of positively correlated compound-gene sets was shown to provide an unbiased method for identifying substrates and discovering molecular features defining substrate specificities.^{76,185} Unexpectedly, some drugs show increased toxicity in cells expressing P-gp (upper panel). The negative correlation between IC_{50} values and P-gp expression suggests that compounds can inhibit the growth of cancer cells more strongly if P-gp is overexpressed.⁷⁹

of cancer cells more effectively if P-gp was overexpressed in the cells (Figure 3, upper panel). The positive correlation between P-gp expression and drug efficacy suggested that the toxicity of certain compounds may be potentiated, rather than antagonized, by P-gp. Because NSC73306 was identified based on the correlation of its toxicity to P-gp expression within the NCI-60 panel, it was hypothesized that its toxicity would proportionally increase with functional P-gp.

Experiments conducted with the KB-3-1/KB-V1 (parent/multidrug-resistant) cell pair (not included in the NCI-60 panel) provided evidence that P-gp may indeed render the cells more sensitive. The causal relation between P-gp and the collateral sensitivity of multidrug-resistant cells could be further verified using a panel of nearly isogenic cell lines selected with increasing concentrations of either colchicine (KB-8-5 and KB-8-5-11) or vinblastine (KB-V1).⁷⁷ The contribution of P-gp to the resistance and collateral sensitivity could be assessed because the increasing drug resistance of these cells is due to increasing levels of P-gp expression encompassing the spectrum of clinically relevant expression levels. As expected, it was found that the toxicity of NSC73306 increased in the KB gradient cell lines in proportion to P-gp function.⁷⁸ Preferential toxicity of NSC73306 was observed even in the KB-8-5 human epidermoid cell line that expresses P-gp at modest levels typical of human tumors. Further experiments conducted with P-gp inhibitors and knock-down constructs showed that the potentiation of toxicity requires functional P-gp. Finally, it was shown that MDR-selective toxicity of NSC73306 pertains to cells with intrinsic or acquired MDR. Taken together, these results suggested that increased sensitivity to NSC73306 is due to the function of P-gp, and not to other, nonspecific, properties of multidrug-resistant cells.

To evaluate the potential of P-gp targeting and to expand the scope of MDR-selective compounds, the Developmental Therapeutics Program (DTP) data set was further investigated, and an in-depth analysis based on the correlation of activity profiles and P-gp expression identified a set of 64 additional candidate MDR-selective agents.⁷⁹ The MDR-selective compounds identified by the extended data mining efforts showed striking structural coherence, highlighting features that may be responsible for MDR-selective toxicity (Figure 4). Twenty-two of the 35 molecules that were available for testing showed preferential growth inhibition in the P-gp-overexpressing KB-V1 cell line. Four compounds (NSC10580, NSC168468, NSC292408, and NSC713048) were tested in additional in vitro models including drug-selected and P-gp-transfected cell line pairs (Figure 5). All four compounds showed elevated toxicity in P-gp-expressing cells relative to their parental lines. For each drug, inhibition of P-gp rendered the multidrug-resistant cells less sensitive to the compounds, thus confirming that functional P-gp is required for the increased toxicity of the identified MDR-selective agents. A search for structural analogues of the confirmed MDR-selective compounds, based on Tanimoto coefficients (with a threshold distance of 0.6), led to the identification of 15 additional MDR-selective compounds and also set the stage for preliminary structure-activity relationship (SAR) studies.⁷⁹ Taken together, these results demonstrated that the MDR-selective activity of NSC73306 is not unique, and represents a robust modality for targeting MDR.

Interestingly, MDR-selective compounds with similar scaffolds have been identified through serendipitous findings as well. In a search for anticancer metal drugs, Heffeter and co-

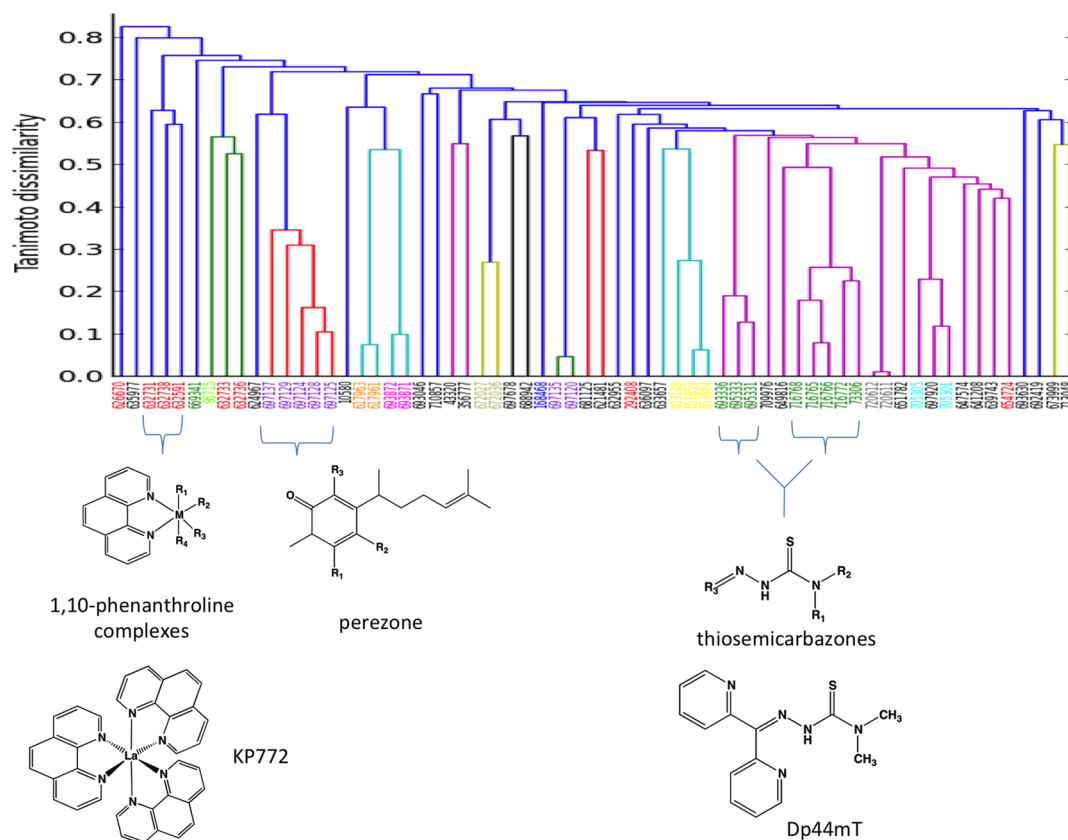


Figure 4. MDR-selective compounds identified by correlating toxicity profiles and P-gp mRNA expression patterns in the NCI-60 cell panel. Compounds whose toxicity profiles show high correlation to P-gp expression were clustered on the basis of structural features (2D Tanimoto dissimilarity scores were clustered using the average linkage algorithm). Molecular scaffolds associated with MDR-selective toxicity include thiosemicarbazones, 1,10-phenanthrolines, and natural-product-derived sesquiterpenic benzoquinones (adapted from Türk et al.).⁷⁹ The structures of KP772 and Dp44mT, which were identified independently to exhibit MDR-selective toxicity, are shown in the respective clusters.^{80–83}

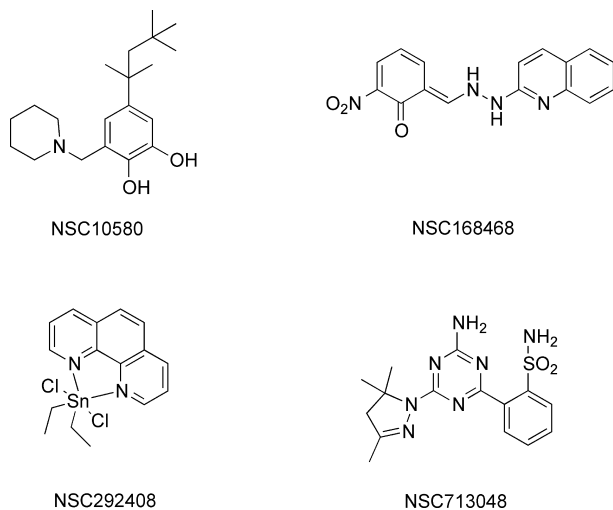


Figure 5. Structures of NSC10580, NSC168468, NSC292408, and NSC713048.

workers have independently discovered that the 1,10-phenanthroline ligand is associated with MDR-selective toxicity. The 1,10-phenanthroline lanthanum complex KP772 (tris-[(1,10-phenanthroline)lanthanum(III)] thiocyanate) was originally developed based on its promising *in vivo* anticancer properties, but it was unexpectedly found to be more toxic to multidrug-resistant cells overexpressing P-gp, MRP1, or

ABCG2 (Figure 4).⁸⁰ In a series of elegant experiments, the authors demonstrated that collateral sensitivity to KP772 is indeed mediated by transporter activity, as hypersensitivity was abrogated by transporter inhibition and long-term KP772 treatment led to a complete loss of drug resistance. Importantly, KP772 induced apoptosis in MDR cells without preferential accumulation or evidence of direct transporter interaction. KP772 induces apoptosis by targeting DNA synthesis through the inhibition of ribonucleotide reductase,⁸¹ which is also one of the primary targets of the class of iron chelators, exemplified by triapine.⁸² A di-2-pyridylketone thiosemicarbazone (di-2-pyridylketone 4,4'-dimethyl-3-thiosemicarbazone (Dp44mT)) was found to be equally, or even more, effective in suppressing the proliferation of etoposide-resistant breast cancer cells (MCF-7/VP) and vinblastine-resistant epidermoid carcinoma (KB-V1), when compared with wild-type chemo-sensitive cells (Figure 4).⁸³ Whether inhibition of the ribonucleotide reductase can be tied to the preferential toxicity of KP772 and Dp44mT in P-gp-expressing MDR cells is unknown, and should be a matter for future studies.

4. STRUCTURE–ACTIVITY STUDIES WITH MDR-SELECTIVE COMPOUNDS

4.1. Isatin- β -thiosemicarbazones and Analogues

The bioinformatic identification of NSC73306 described above precipitated further investigation into its mechanism of action

and interest in the prospect of preclinical development. Seven of the 60 compounds with the strongest predicted MDR1-selective activity contained an isatin- β -thiosemicarbazone moiety (NSC73306, NSC658339, NSC716765, NSC716766, NSC716768, NSC716771 and NSC716772) (Figure 6).⁷⁹ This

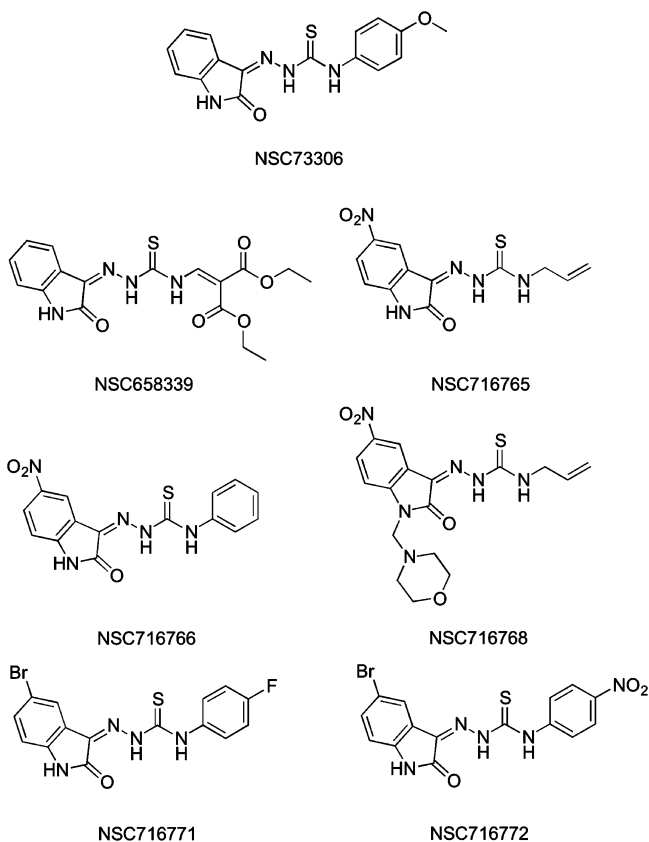


Figure 6. Isatin- β -thiosemicarbazones identified as MDR-selective compounds in the Developmental Therapeutics Program data set.

strong structural commonality reinforced the MDR1-selective potency of NSC73306. However, there were two aspects of NSC73306 that needed to be resolved. First, thiosemicarbazones are notoriously insoluble,⁸⁴ and NSC73306 is not an exception. Second, improvement in both absolute activity against multidrug-resistant cells and selectivity of killing multidrug-resistant cells over parental cells is needed. Several rounds of SAR work were performed to gain insight into the structural features of isatin- β -thiosemicarbazones needed for their MDR-selective activity profile, with the intention of identifying more selective compounds and potential sites of

substitution with hydrophilic functional groups (or amenable to hydrochloride salt formation, etc.).

The synthesis of isatin- β -thiosemicarbazones can be achieved in a number of different ways.^{85,86} The SAR work described is best demonstrated in the context of the synthesis of NSC73306. 4-Methoxyphenyl isothiocyanate is reacted with hydrazine to produce 4-(4'-methoxyphenyl)thiosemicarbazide and a simple condensation reaction between the thiosemicarbazide and isatin produces 1-isatin-4-(4'-methoxyphenyl)-thiosemicarbazone (NSC73306).⁸⁷ It should be noted that, in situations where a particularly electronegative substituent is located on the phenyl ring (such as 4-fluorophenyl isothiocyanate), a dimerization occurs upon reaction with hydrazine, resulting in two thiocyanate groups linked by the hydrazine. This dimerization can be avoided (in our experience) by reacting a Boc-protected form of hydrazine (*tert*-butyl carbamate) with the isothiocyanate. This produces a Boc-protected thiosemicarbazide that can be deprotected using standard conditions.⁸⁷

A diverse range of isatin- β -thiosemicarbazones were initially synthesized to identify general regions of the molecule that were essential for selective activity (Figure 7). This involved testing each of the components of NSC73306 (isatin, thiosemicarbazide, etc.) by incorporating halogen substitutions on both the isatin and phenyl rings, removal of the phenyl ring, and addition/removal of the functional groups on the isatin moiety. A number of electron-withdrawing substituents (fluoro, bromo, nitro) at the 5-position of the isatin or the 4-position (*para*) of the phenyl ring resulted in compounds with improved MDR1 selectivity (with the exception of a sulfonic acid group, which abrogated activity, probably as a result of the net negative charge conferred). However, any gross structural changes that deviated from the 4-phenyl isatin- β -thiosemicarbazone core produced compounds with either no cytotoxic activity or no MDR-selective cytotoxicity. These changes included removal of the lactam group (H-bond donor and H-bond acceptor) of the isatin, removal of the phenyl ring, or introduction of charge. An analogue of NSC73306, with greater steric bulk on the isatin group (a naphthyl analogue), was in fact converted to a P-gp substrate, demonstrating the small structural changes that can allow P-gp to recognize this molecule. Similarly, a number of bioactive thiosemicarbazones (triapine, MAIQ) were also tested and found to be P-gp substrates (rather than MDR-selective compounds). Paralleling the loss of MDR-selective toxicity that accompanies the removal of the N4 phenyl ring of 73306, addition of a phenyl ring to the N4 position of triapine produced a molecule no longer recognized by P-gp.⁸⁷

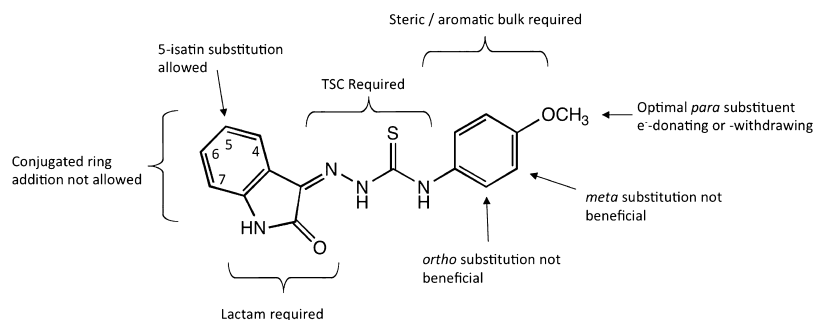


Figure 7. Summary of structure-activity relationships of thiosemicarbazone derivatives targeting MDR cells overexpressing P-gp.

4.2. Desmosdumotin B Analogues

The natural product desmosdumotin B is a flavonoid that was first identified from the root bark of *Desmos dumosus*. Nakagawa-Goto et al. subsequently reported a multistep total synthesis for the compound starting with 2,4,6-trihydroxyacetophenone and assessed its cytotoxic activity against a number of cells including KB and the vincristine-resistant derivative KB-VIN.⁸⁸ Desmosdumotin B was preferentially toxic toward KB-VIN cells, demonstrating at least 20-fold selective toxicity as compared to parental KB cells. The identification of desmosdumotin B's selective killing of KB-VIN cells led to multiple follow-up structure–activity studies (Figure 8). As

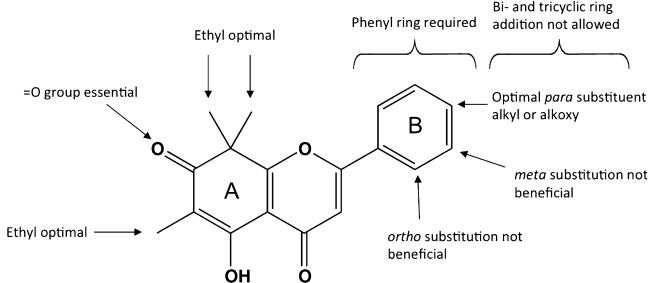


Figure 8. Summary of structure–activity relationships derived from desmosdumotin derivatives targeting MDR cells overexpressing P-gp.

defined by the authors, desmosdumotin B contains an A ring within the bicyclic flavone system and a B phenyl ring substituted at the 2 position. (Note: The authors varied the lettering associated with these rings in various publications; we will use the ring notation from the first report for all SAR discussion of subsequent studies, as shown in Figure 8.) These two rings are primarily amenable to structure–activity studies. The first approach examined the effects of substitutions and changes on the B phenyl ring.⁸⁹ A range of alkyl substitutions resulted in virtually no effect on the 20-fold selectivity, although a 2,4,6-trimethyl substitution or replacement with a naphthylene group reduced selectivity dramatically (not a single analogue suffered from cross-resistance). Substitution of the methyl groups of the A ring with ethyl groups resulted in a 2 orders-of-magnitude improvement in selectivity, 200- to 460-fold, depending on the B-ring substitutions. The most selective compound (6,6,8-triethyl-desmosdumotin, TEDB, 460-fold selective) contained a 4-ethyl substitution on the phenyl ring. Curiously, propyl groups (6,6,8-triethyl-desmosdumotin) on the A ring showed equivalent killing of the parent and multidrug-resistant lines (i.e., loss of selectivity). The active analogues showed very low cytotoxicity against KB and other parental lines (activity values were reported in micrograms per milliliter). KB-VIN cells were cotreated with desmosdumotin analogues and the P-gp inhibitor verapamil, resulting in a loss of cell killing (the effect on parental cells was not shown), suggesting that inhibition of P-gp attenuates selective toxicity. Although not subsequently published, the authors did note that not all multidrug-resistant cell lines are hypersensitive to the desmosdumotin B analogues, suggesting that specific characteristics of the KB-VIN cells contribute to the MDR-selective toxicity.

Subsequent SAR work has been reported using 6,6,8-triethyl-desmosdumotin (TEDB) as the lead with structural alterations of the B phenyl ring.⁸⁹ A large number (>50) of analogues were synthesized with a mono- or multisubstituted B

ring. The additions of 4'-methyl and 4'-ethyl substituents increased selectivity 460- and 320-fold, respectively; however, all other substituents at the 2', 3', 4', or 5' positions on the B ring resulted in lowering of the selectivity (~10-fold, on average), mainly through loss of cytotoxicity toward the KB-VIN cells. Most multisubstituted analogues also showed a reduced selectivity (from 1.4- to 20-fold), although 3'-methyl-4'-methoxy-TEDB (273-fold), 3'-fluoro-4'-methoxy-TEDB (250-fold), and 3',5'-dimethyl-TEDB (100-fold) substitutions produced strong selectivity and reinforced the sense that increased selectivity and toxicity toward KB-VIN cells were conferred by small hydrophobic groups on the B ring. Next, the investigators reported the synthesis and testing of a series of nine analogues, wherein the B phenyl ring was replaced with heteroaromatic and alkyl ring systems, to examine the effects of aromaticity and bulk on selective killing.⁹⁰ The replacement of the phenyl ring with other ring systems reduced selectivity by an order of magnitude. The most selective compounds were the 2-(furan-3'-yl)-TEDB (>12-fold selective) and 2-(thiophen-2'-yl)-TEDB (16-fold selective), suggesting that retention of aromaticity in the ring system assists selectivity. These two compounds showed approximately equivalent selectivities toward HepG2-VIN cells.

The investigators also followed up on their early SAR work with desmosdumotin B analogues, showing that replacement of the B phenyl ring with bulky dicyclic systems such as a naphthyl group produced compounds that were cytotoxic but not MDR1-selective.⁸⁹ A range of bicyclic and tricyclic replacements of the B ring in TEDB were assessed, generally showing increased cytotoxicity but lacking selective cytotoxicity.⁹¹ Interestingly, the authors found that the new analogues with potent cytotoxicity elicited microtubule aggregation; however, the lead TEDB (up to 40 μ M) did not demonstrate any effect on microtubules.

The more active TEDBs, 4'-methyl-TEDB and 4'-ethyl-TEDB, were tested against Hep3B human hepatoma cells and the vincristine-resistant subline Hep3B-VIN cells, to determine whether the MDR-potentiated activity of desmosdumotin B analogues persisted across diverse MDR cell models.⁹² Selective toxicity persisted but was significantly lower in the hepatoma cells as compared to the parental lines (4'-methyl-TEDB was 460 times more toxic to KB-VIN cells and only 30-fold more toxic to Hep3B-VIN cells). The reasons for the lower selectivity were not explored, and the correlation of P-gp expression with selective toxicity was not assessed.

Assessment of the possible underlying mechanism of the MDR-selective activity of the desmosdumotin B class of compounds has been attempted. Initial identification of desmosdumotin B showed that co-incubation of verapamil reversed the collateral sensitivity of KB-VIN cells, suggesting that functional P-gp was necessary for activity.⁸⁹ It was shown using a calcein-AM efflux assay that, whereas the P-gp inhibitors cyclosporin A and verapamil elicit complete P-gp inhibition, this was not true for TEDB, 4'-methyl-TEDB, or 4'-ethyl-TEDB at high concentrations (~30 μ M). Co-incubation of the three analogues with verapamil appeared to potentiate inhibition and hinder inhibition by cyclosporin A, suggesting that the desmosdumotin compounds, although not inhibitors, can interact with P-gp. To gain further insight into the mechanism, Kuo et al. explored the activity of TEDB (note that, in their article, the authors referred to TEDB as "KNG-I-322" and, quite remarkably, did not categorically identify the compound by structure or any other name; hence, they might

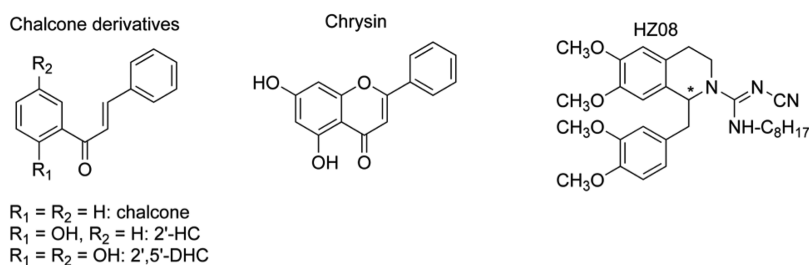


Figure 9. Pro-oxidant compounds targeting MRP1-overexpressing cancer cells. Chalcone derivatives, chrysin, and HZ08 increase the toxicity of pro-oxidants such as cisplatin, doxorubicin, and curcumin in MRP1-overexpressing cancer cells by triggering cellular GSH depletion through MRP1, which induces mitochondrial dysfunction (for 2',5'-DHC and chrysin) or cell cycle arrest and apoptosis signaling (for HZ08).

have employed another analogue) against Hep3B and Hep3B-VIN cells.⁹³ Incubation of Hep3B-VIN cells with TEDB, up to 10 μM , did not affect P-gp expression or inhibit its function. However, P-gp ATPase activity, measured using a luminescent assay kit, demonstrated a P-gp stimulation by TEDB (at 1, 10, and 100 μM) equivalent to verapamil (10 μM). Hep3B-VIN cells transfected with siRNA against P-gp demonstrated losses of P-gp protein and sensitivity to TEDB, reinforcing the notion that elevated sensitivity of Hep3B-VIN cells is indeed linked to functional P-gp. TEDB was shown to induce apoptosis and cleave caspase-3. Consistent with the potentiating effect of P-gp, this apoptosis was hindered by co-incubation with verapamil. One effect of ATP depletion is inhibition of mTOR signaling, leading to reduced phosphorylation of downstream targets such as p70S6J and 4E-BP.⁹⁴ TEDB inhibited phosphorylation of these targets only in the Hep3B-VIN cells, and this was reversed in cells pretreated with siRNA against P-gp. In a similar fashion, the endoplasmic reticulum (ER) chaperone GRP78 protein (but not its mRNA) was downregulated by TEDB.

This work collectively demonstrates that the desmosdumotin B class of small molecules selectively kills two vincristine-selected P-gp-expressing cell lines. It is not clear whether the vincristine-selected KB cells are identical to the HeLa sublines used in most studies,⁷⁷ and the level of P-gp expression in relation to the widely characterized KB-V1 cells is also unknown. Nevertheless, silencing of P-gp by siRNA has been shown to offset this hypersensitivity, suggesting that the selective killing is tied to P-gp in a similar fashion to the isatin- β -thiosemicarbazones. Clearly, further characterization of these compounds is warranted to assess how closely the mechanism of action is tied to P-gp expression. Reversal of selectivity with a strong P-gp inhibitor such as CsA, tariquidar, or elacridar would aid in demonstrating the necessity of functional P-gp for hypersensitivity, and other MDR cell lines selected with other cytotoxins, or transfected with MDR1, would aid in understanding the mode of activity.

5. COMPOUNDS TARGETING MULTIDRUG-RESISTANT CELLS OVEREXPRESSING MRP1

MRP1-mediated resistance can be overcome by coadministration of cytotoxic MRP1-substrate drugs and MRP1 inhibitors. A number of MRP1 modulators whose mechanism of inhibition is poorly characterized have been reported.^{95,96} In many cases, including the reference inhibitor leukotriene antagonist MK571, studies suggest that the mechanism of action relies on competitive inhibition.^{97,98} Although MK571 was able to completely reverse vincristine resistance of MRP1-overexpress-

ing cells, the required concentrations were too high to be used in vivo.⁹⁹ Other MRP1 inhibitors, such as probenecid and agents targeting glutathione-S-transferase (GST), have primary pharmacological activities and cannot be used for clinical studies. A screen dedicated to improving the treatment of neuroblastoma and other MRP1-overexpressing drug-refractory tumors revealed pyrazolopyrimidines as a prominent structural class of potent MRP1 inhibitors. Reversan, the lead compound of this class, increased the efficacy of both vincristine and etoposide in murine models of neuroblastoma, without showing side effects related to primary toxicity or increased exposure to the chemotherapeutic drug.²³

In addition to transporter inhibition, MRP1-mediated resistance can be overcome by MDR-selective compounds because the expression of MRP1 has also been shown to elicit collateral sensitivity of multidrug-resistant cells.

5.1. Small Molecules Preferentially Targeting MRP1-Overexpressing Multidrug-Resistant Cells by Promoting GSH Efflux

GSH is a tripeptide constituting the major low-molecular-weight thiol compound in animals.¹⁰⁰ GSH serves many important cellular roles as a redox regulator, cofactor, substrate, and antioxidant.¹⁰¹ Cancer cells are in permanent oxidative stress, which is often compensated by upregulation of the GSH synthesis pathway. A decrease in intracellular GSH levels by an active efflux mediated by MRP1 has been associated with apoptosis.^{102–106} This observation suggested that modulation of intracellular GSH levels through MRP1 might be a powerful approach to cancer therapy.¹⁰⁷ Indeed, GSH-depleting compounds were shown to increase the toxicity of pro-oxidant drugs such as cisplatin,¹⁰⁸ doxorubicin¹⁰⁹ and curcumin¹¹⁰ in MRP1-overexpressing cancer cells. GSH depletion was induced by selected flavonoids such as 5,7-dihydroxyflavone (chrysin) and 2',5'-dihydroxychalcone (DHC)^{108,110,111} or by other compounds such as HZ08¹¹² (Figure 9) promoting cell-cycle arrest and apoptosis signaling. In addition, as documented above for P-gp, there exist some compounds with inherent cytotoxic activity that are able to exploit the collateral sensitivity elicited by MRP1.

5.1.1. Verapamil and Derivatives. Verapamil, a well-known inhibitor of P-glycoprotein, does not reverse the resistance mediated by MRP1.¹¹³ Verapamil is not transported by MRP1, but has been found to stimulate MRP1-mediated glutathione export.^{114,115} As a result, verapamil induces a large (up to 90%) depletion of intracellular GSH, resulting from a rapid extrusion (half-maximal effect of less than 30 min) in MRP1-transfected Baby Hamster Kidney 21 (BHK-21) cells and triggering selective apoptosis of the cells. This effect was indeed dependent on MRP1, because it was not observed under

the same conditions with the parental BHK-21 cell line. MRP1 function was directly implicated in this phenomenon, because the transfected BHK-21 cells expressing an inactive mutant of MRP1 (containing the K1333L mutation within the Walker A motif of nucleotide-binding domain 2) did not show hypersensitivity.¹¹⁶ This finding was validated in H69AR cancer cells that overexpress MRP1 due to continuous selection of the small-cell lung carcinoma NCI-H69 line with increasing concentrations of doxorubicin.¹¹⁷ Both *R*- and *S*-enantiomers of verapamil were shown to strongly bind to MRP1, but in different ways.¹¹⁸ Interestingly, only the *S*-isomer was responsible for GSH efflux stimulation and concomitant apoptosis of the MRP1-overexpressing cells. In contrast, the *R*-isomer sensitized cell growth to vincristine by inhibiting drug transport. Therefore, the two isomers probably bind to MRP1 at distinct binding pockets and induce different conformational changes.

A structure–activity relationship study was performed with various verapamil derivatives containing iodine substitutions, in an attempt to develop more selective analogues (Figure 10).¹¹⁹

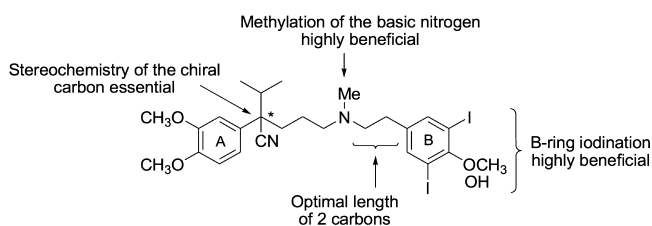


Figure 10. Structure–activity relationships of verapamil derivatives targeting MRP1-overexpressing cells. Verapamil stimulates MRP1-mediated GSH export, which triggers selective apoptosis of MRP1-overexpressing cells. Iodinated derivatives were designed to increase verapamil potency.

Iodination greatly enhanced verapamil effectiveness, lending support to a possible hydrophobic binding pocket for verapamil interaction within MRP1 (addition of an iodo group increases the log *P* value by approximately 1).¹²⁰ The methyl group on the central nitrogen atom played an important role, and modifications of the linker length also appeared critical. The best compound, a di-iodinated derivative, was 10-fold more potent than verapamil, with a half-maximal effective concentration (EC_{50}) value for cytotoxicity of 1.1 μM for MRP1-transfected BHK-21 cells compared to 54.7 μM for parental BHK-21 cells, giving a selectivity ratio (SR) of 50. Unfortunately, the known cardiotoxicity of verapamil limits its *in vivo* use at concentrations that elicit MRP1-selective toxicity. Therefore, additional MRP1 ligands, devoid of primary pharmacological activity, were screened to identify activators of GSH transport that could be used as part of a new therapeutic approach to trigger apoptosis of cancer cells overexpressing MRP1.

5.1.2. Xanthenes. Xanthone derivatives were identified as specific “killers” of the MRP1-overexpressing H69AR cells.¹²¹ The most efficient analogue, 1,3-dihydroxy-6-methoxyxanthone (Figure 11), was found to be as effective as racemic verapamil ($EC_{50} = 11 \mu\text{M}$, SR > 9). It contains hydroxyl groups at positions 1 and 3, indicating the requirement for H-bond-donor ability. A methoxy group was mandatory at position 6, whereas either H or methoxy was allowed at vicinal position 5. Interestingly, extended SAR studies of xanthenes demonstrated that the compounds’ ability to trigger MRP1-mediated GSH

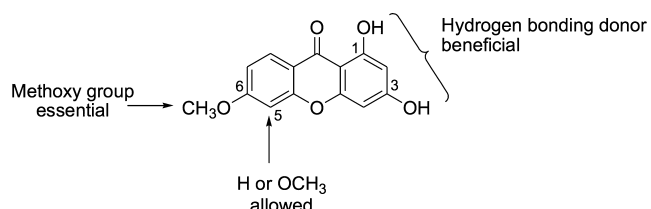


Figure 11. Structure–activity relationships of substituted xanthenes. Xanthone derivatives trigger selective death of MRP1-overexpressing cells through MRP1-mediated GSH export with a marked dependency on their structure.

efflux is not directly linked to MDR-selective toxicity, because several compounds induced a strong GSH efflux but were not selectively cytotoxic. These results suggested that GSH efflux is necessary, but not sufficient, for the selective induction of apoptosis in MRP1-expressing cells.

5.1.3. Flavonoids. The flavonoid compound apigenin (5,7,4'-trihydroxyflavone, cf. Figure 12) was identified in

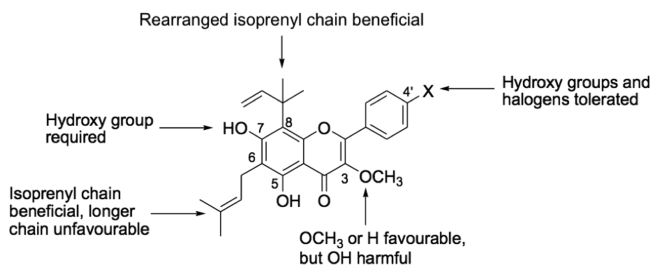


Figure 12. Structure–activity relationships of substituted flavones. A number of flavone derivatives trigger selective death of MRP1-overexpressing cells through MRP1-mediated GSH export in relation to their structures. Substitution at position 3 appears to be critical.

another screen as a specific killer of drug-selected H69AR cells and MRP1-transfected HeLa cells.¹¹⁷ Flavonoids are naturally derived compounds that display both anti- and pro-oxidant properties. Flavonoids have been used in cancer chemoprevention and chemotherapy,¹²² and some were also described to decrease cellular glutathione levels. Inspired by the success of the SAR studies conducted with verapamil, various flavonoid derivatives, such as flavones and flavonols, were submitted to screening.

The best natural compound identified in the first screen was chrysin (5,7-diOH-flavone), with an SR value of >20 and an EC_{50} value of 4.9 μM . A slightly higher activity was observed for 3-*O*-methylgalangin (SR > 24, $EC_{50} = 4.0 \mu\text{M}$), showing the positive effect of a methoxy group at position 3 (Figure 12). Prenyl chains were tolerated at positions 6 and 8, as well as halogens or hydroxyl at position 4'. Again, as seen with xanthenes,¹²¹ some flavonoids [such as the flavonol galangin (3,5,7,4'-tetraOH-flavone)] exhibited a strong induction of the MRP1-mediated GSH extrusion activity without actually inducing cell death. Structure–activity relationships for cell death induction show that (i) the core of MRP1-selective toxic compounds is deprived of steric substituents and (ii) the absence of a hydroxyl group at position 3, a characteristic of flavonols, is an absolute requirement for toxic activity (submitted). The MDR-selective effect of the compounds was indeed MRP1-specific, because these flavonoids did not trigger the death of cells overexpressing either P-gp or ABCG2. In fact, plant flavonoids, including silymarin, hesperetin, quercetin, and

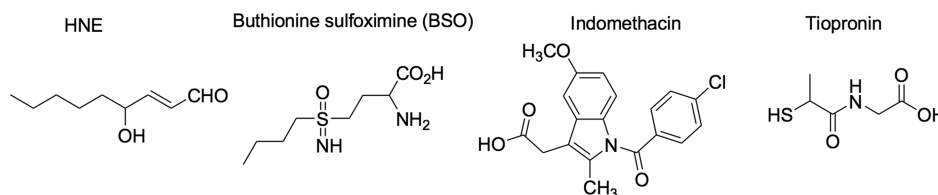


Figure 13. Structures of additional compounds displaying a selective cytotoxicity in MRP1-overexpressing cells. HNE, BSO, indomethacin, and tiopronin selectively sensitize MRP1-overexpressing cells through either an induced GSH depletion (HNE, BSO, and indomethacin) or currently unknown mechanisms (tiopronin).

daidzein, have been shown to increase the intracellular accumulation of mitoxantrone in ABCG2-expressing cells.¹²³ The inhibitory effect of naturally occurring flavonoids on ABCG2 has been correlated to their positive effects on the pharmacokinetics of anticancer drugs.¹²⁴ Another proposed explanation for a lack of inducing cell death by some of these compounds might involve their ability to evoke an adaptive glutathione-synthesis response through the *nrf2* signaling pathway.¹¹¹

5.1.4. Additional Compounds Targeting MRP1-Expressing Cells through Induced GSH Depletion. These compounds include 4-hydroxy-2-nonenal (HNE), buthionine sulfoximine (BSO) and indomethacin (Figure 13). HNE, a highly reactive and cytotoxic product of lipid peroxidation, is eliminated from the cells after conjugation to glutathione by glutathione-S-transferases (GSTs) and efflux of the resulting HNE–glutathione conjugate (HNE–SG). The effects of phase II (conjugation) and phase III (efflux) metabolism on HNE-induced cellular toxicity, GSH depletion, and HNE–protein adduct formation were examined in MCF7 cells.¹²⁵ Coexpression of subunit M1 of GST and MRP1 resulted in a 2.3-fold higher sensitivity to HNE cytotoxicity, as opposed to the expected protection conveyed by the detoxifying system. Interestingly, the expression of GST-M1 or MRP1 alone resulted in only a slight sensitization to HNE (1.3- or 1.4-fold, respectively), whereas HNE induced a greater-than-80% GSH depletion in MRP1-expressing cells, as also observed for xanthenes¹²¹ and flavonoid derivatives. Coexpression of GST-M1 and MRP1 strongly enhanced the formation of HNE–protein adducts, suggesting that these two enzymes might act synergistically to enhance both HNE–protein adduct formation and HNE-induced cytotoxicity. This cytotoxicity is facilitated by GSH depletion mediated by both GST-M1 through conjugation and MRP1 through efflux.

BSO is a well-known inhibitor of γ -glutamylcysteine ligase (γ -GCL), the enzyme that completes the first and rate-limiting step of glutathione synthesis. MRP1-overexpressing cell lines are highly sensitive to BSO;^{117,126,127} for example, H69AR and HeLa-MRP1 were found to be 300- and 22.2-fold more sensitive to BSO than parental NCI-H69 and HeLa cell lines, respectively.¹¹⁷ BSO treatment results in a strong and gradual intracellular GSH depletion within 24 h. Cells can adapt to progressive GSH depletion.¹²⁸ One might wonder whether hypersensitivity to BSO of MRP1-overexpressing cells could be related to their basal GSH level, which is lower than in parental cells, as probably resulting from enhanced GSH utilization and GSH efflux by MRP1.^{127–129} Similarly, the effect of BSO on the cytotoxic activity of chlorambucil or doxorubicin was greater in MRP1-overexpressing cells than in parental ones. It is interesting to note that hypersensitivity to BSO, alone and in combination with melphalan, was also observed in neuroblastoma cell cultures, which are known to overexpress MRP1

in response to *N*-myc oncogene amplification.¹³⁰ Conversely, *Mrp1*(–/–) mice were found to be resistant to the GSH-depleting activity of intraperitoneally injected BSO compared with wild-type mice.¹²⁷

Human GLC4-Adr cells, selected for adriamycin resistance and MRP1 overexpression, were shown to be highly sensitive to indomethacin (Figure 13) when compared to the GLC4 parental line. However, the 31% decrease observed in cellular GSH level is unlikely to be the primary cause of selective indomethacin-induced apoptosis.¹³¹

5.2. GSH-Independent Collateral Sensitivity of MRP1-Expressing Multidrug-Resistant Cells

Two compounds were found to specifically sensitize MRP1-expressing cells without inducing cellular GSH depletion. The orphan drug tiopronin (Figure 13), known to target some P-gp-expressing cells, was also reported to induce collateral sensitivity of multidrug-resistant cell lines overexpressing MRP1.⁷⁰ A relative sensitivity of 43-fold was observed for the MRP1-overexpressing VP-16 cell line in comparison with parental MCF7 cells. BSO did not potentiate tiopronin-triggered collateral sensitivity, suggesting that the effect of tiopronin is GSH-independent. Finally, the antitumor activity of 6-(7-nitro-2,1,3-benzoxadiazol-4-ylthio)-hexanol (NBDHEX) was described on both MRP1-overexpressing H69AR and parental H69 cells.¹³² Interestingly, NBDHEX, which is not an MRP1 substrate, triggered two types of cell death. In the MRP1-positive H69AR cells, cell death was mediated by caspase-dependent apoptosis, with *c*-Jun NH₂-terminal kinase and *c*-Jun activation, whereas the MRP1-negative parental H69 cells exhibited a necrotic phenotype with glutathione oxidation and activation of p38 (MAPK). Apoptosis of H69AR cells might be related to lower expression of the antiapoptotic protein Bcl-2 in these cells.

6. COMPOUNDS TARGETING ABCG2-EXPRESSING MULTIDRUG-RESISTANT CELLS

Despite the wide substrate specificity of ABCG2, relatively few specific inhibitors, active at submicromolar concentrations, have been reported. Fumitremorgin C (FTC) is a selective ABCG2 inhibitor, but with neurotoxic effects preventing any clinical use.¹³³ Less toxic and more potent analogues were developed, including Ko143, which was shown to significantly increase the oral availability of topotecan in mice.¹³⁴ Recently, chromones have been identified as selective and less toxic ABCG2 inhibitors.¹³⁵ Although minimal structural modifications of the P-gp inhibitors tariquidar and elacridar can result in a dramatic shift in favor of ABCG2 inhibition,^{136,137} clinical trials attempting to reverse ABCG2-mediated MDR have not been initiated.

Compounds that interact with, and selectively kill, ABCG2-overexpressing cells are only now being investigated. Multidrug-

resistant cells overexpressing ABCG2 gradually lose transporter expression following the withdrawal of selective pressure, as similarly observed with P-gp.¹³⁸ This observation suggests that the expression of ABCG2 also carries a fitness cost that might be exploited by ABCG2-selective compounds.

6.1. Serendipitous Findings

Loss of drug efficacy is also noted in patients with rheumatoid arthritis (RA) receiving long-term treatment by disease-modifying antirheumatic drugs (DMARDs). In vitro mechanisms conferring DMARD resistance to human T lymphocytes [CEM (T) cells] include the overexpression of ABCG2. Intriguingly, ABCG2-overexpressing CEM cells show significant collateral sensitivity to dexamethasone, discussed earlier as a P-gp-targeting compound.¹³⁹ An ABCG2 inhibitor, NP-1250, was recently reported to induce caspase-independent collateral sensitivity in MCF7/MX, ABCG2-overexpressing mitoxantrone-selected breast cancer cells.¹⁴⁰

It remains to be established whether collateral sensitivity in the above examples is indeed tied to ABCG2 expression and function. As noted earlier, the experiments should be extended to additional cell lines overexpressing ABCG2, to rule out the contribution of cell-specific targets different from ABCG2.

6.2. Mining the DTP Database for ABCG2-Related Compounds

Studies attempting to correlate the mRNA expression profile of ABCG2 with the DTP drug activity patterns failed to identify substrates or ABCG2-potentiating compounds.⁷⁶ The lack of meaningful correlations could be explained by the statistical limitations of the correlative approach, caused by the limited range of ABCG2 expression in the NCI-60 panel, as well as the uncertainty about the relationship between mRNA and protein expression. In addition, it seemed that the commonly observed overlapping substrate specificity of ABCG2 and P-gp could not be discerned by the bioinformatic analysis.¹⁴¹ In a subsequent study, Deeken et al. used a flow cytometry assay to measure ABCG2 efflux function in the NCI-60 cell lines and correlated the pattern of activity, rather than mere expression profiles, with the screening data. This strategy identified 70 putative ABCG2 substrates (compounds showing a significant inverse correlation), as well as compounds to which ABCG2 expression seemed to confer greater sensitivity.¹⁴² Although known cytotoxic substrates of ABCG2 such as mitoxantrone or topotecan were missed, several novel substrates and transporter-interacting compounds were identified. Compounds whose toxicity showed a direct correlation with ABCG2 function were further analyzed for their ability to interact with the transporter and to induce collateral sensitivity of ABCG2-transfected HEK293 cells by comparison to HEK293 control cells. Only two compounds were found to selectively target ABCG2-overexpressing cells, namely, NSC103054 and NSC174939, both displaying rather low selectivity ratios of 3 and 2.5, respectively (Figure 14). Further experiments demonstrated that NSC103054, a dibromo derivative of estradiol, strongly prevented photoaffinity labeling by IAAP and inhibited ABCG2-mediated pheophorbide a efflux, suggesting that NSC103054 directly binds to ABCG2. Whether selective killing is tied to ABCG2 function (as seen with MDR-selective DTP compounds targeting P-gp) awaits confirmation with independent experiments using ABCG2-silencing specific inhibitors and additional MDR cell models with ABCG2 overexpression.

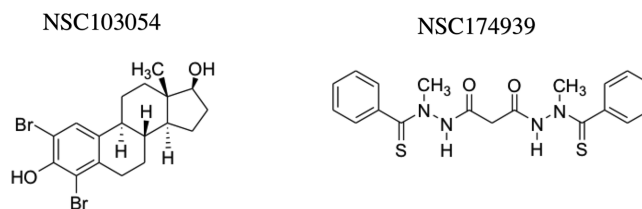


Figure 14. MDR-selective compounds identified by correlating toxicity profiles and ABCG2 function pattern in the NCI-60 cell panel.¹⁴²

6.3. Photodestruction of ABCG2-Rich Extracellular Vesicles

In more recent publications, a novel photodynamic therapy strategy for overcoming MDR by selectively killing ABCG2-overexpressing cells was investigated.¹⁴³ The strategy is based on a specific form of MDR in which ABCG2-rich extracellular vesicles (EVs) that form between neighboring cancer cells highly concentrate various chemotherapeutics in an ABCG2-dependent manner, thereby sequestering them away from their intracellular targets. Overexpression of ABCG2 in the EVs is correlated with cellular resistance against a wide range of anticancer drugs, including topotecan and imidazoacridinones. The vesicles are believed to be responsible for drug sequestration, which prevents access of the compounds to their cellular targets. In addition to anticancer drugs, ABCG2 sequesters imidazoacridinones (Figure 15). These compounds

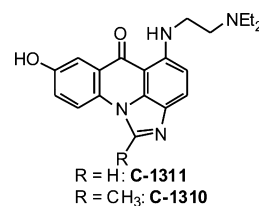


Figure 15. Photosensitive imidazoacridinones produce reactive oxygen species (ROS) upon illumination, causing damage to extracellular vesicles overexpressing ABCG2.

are photosensitive drugs that produce reactive oxygen species (ROS) upon illumination, causing damage to the EVs and ultimately leading to cell death. Multidrug-resistant cells that are devoid of EVs contain an increased number of lysosomes that were shown to accumulate imidazoacridinones. Upon photosensitization, these cells were also preferentially killed through ROS-dependent lysosomal rupture. The combination of targeted lysis of imidazoacridinone-loaded EVs and lysosomes elicited a synergistic cytotoxic effect resulting in MDR reversal. The exclusive accumulation in EVs enhanced the selectivity of the cytotoxic effect exerted by photodynamic therapy to multidrug-resistant cells, without harming normal cells.¹⁴⁴

7. COMMON MECHANISTIC FEATURES: TARGETING THE FITNESS COST OF RESISTANCE

The acquisition of new phenotypic traits comes at a cost to the cancer cell, as the ability to respond to specific perturbations can be affected when certain regulatory circuits have been rendered defective by mutation.¹⁴⁵ In that context (acquired resistance to anticancer agents), the overexpression of ABC transporters that confer MDR might prove to be “synthetically lethal” in the presence of MDR-selective compounds. To ensure stable overexpression of the efflux pumps in vitro, multidrug-resistant cells have to be maintained under

continuous selective pressure. A common laboratory finding is the spontaneous decrease of expression of ABC transporters conferring MDR in cells selected to be resistant to substrate drugs once that selection pressure is removed, suggesting that the elevated expression of the transporters carries a fitness cost. The compounds discussed in this review exploit this fitness cost and the ensuing paradoxical hypersensitivity that is invariably associated with the acquisition of the resistant trait. The mechanisms of action of MDR-selective compounds could be as diverse as are the mechanisms that support anticancer drug resistance. In the next subsection, we list three examples to demonstrate that a solid understanding of the genetic changes underlying resistance allows rational selection of MDR-selective compounds.

7.1. Collateral Sensitivity of Multidrug-Resistant Cells Might Not Be Linked to Transporter Expression

Austocystin D was identified from a natural product extract from the *Aspergillus* isolate UGM218.¹⁴⁶ The cytotoxic activity of the isolated fraction was greater toward MIP101 human colon carcinoma cells overexpressing P-gp than SW620 human colon carcinoma cells with low P-gp expression. Whereas the crude fraction showed ~100-fold selectivity, austocystin D, which was found to be the small molecule responsible for selective toxicity, displayed a ~900-fold increased toxicity against MIP101 cells, and a selectivity of ~20-fold was observed in drug-selected cells.¹⁴⁶ Marks et al. examined the cytotoxicity of austocystin D toward a panel of cancer cell lines and demonstrated that the expression of P-gp was not necessary for cells to show increased sensitivity.¹⁴⁷ Subsequent work revealed that the selective cytotoxic action of austocystin D arises from its selective activation by cytochrome P450 (CYP) enzymes in MIP101 cells.

Another example is cisplatin resistance, which has been associated with a wide range of cellular changes (referred to as pleiotropic resistance mechanisms), including reduced accumulation, deactivation by glutathione, and DNA-damage repair processes.¹⁴⁸ Given the wide range of alterations that can occur, it is perhaps unsurprising that a number of reports show collateral sensitivity of cisplatin-resistant cells to a range of other small molecules. Cell lines selected with increasing concentrations of cisplatin are found to be cross-resistant to other platin drugs (such as carboplatin), but at the same time, they can show significant collateral sensitivity to SN-38 (7-ethyl-10-hydroxycamptothecin), an active metabolite of the topoisomerase I inhibitor camptothecin.^{149,150} It was found that cisplatin-resistant cells can acquire increasing reliance on the DNA repair function of topoisomerase I, which renders them hypersensitive to topoisomerase I inhibition. Finally, small-molecule Bcl-2 inhibitors are of particular interest, as their enhanced toxicity against multidrug-resistant cells has been recapitulated in vivo. Resistant cells often resort to the repression of apoptotic pathways by upregulating Bcl-2. Das et al. reported a series of SAR campaigns centered on the small-molecule Bcl-2 inhibitor ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA 14-1). Assessment of cytotoxicity toward JURKAT B-cell lymphoma cells led to the identification of the metabolically stable ethyl 2-amino-6-(3',5'-dimethoxyphenyl)-4-(2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (CXL017).¹⁵¹ CXL017 was 20-fold more potent than the model compound against JURKAT cells. CXL017 was then assessed for activity against two multidrug-resistant cell lines compared with their partner parent lines: the

camptothecin-resistant CCRF-CEM/C2 T-lymphoblast (derived from CCRF-CEM) and the mitoxantrone-resistant HL-60/MX-2 promyelocytic leukemia (derived from HL-60) cell lines. Both cell lines demonstrated collateral sensitivity (2–4-fold) to CXL017 despite the overexpression of Bcl-2.¹⁵¹ Das et al. demonstrated that the in vitro selective toxicity evoked by CXL017 can translate to analogous activity in vivo.¹⁵² The basis for the enhanced toxicity of CXL017 in MX2 cells and xenografts appears to be due to overexpression and therefore reliance on the antiapoptotic protein Mcl-1. In MX2 cells, the pro-apoptotic proteins (such as Bak, Bax, and Bim) are held “at bay” by Mcl-1, whose inhibition by CXL017 results in a greater degree of cell death than is possible in the parent cells, leading to collateral sensitivity.

Taken together, these examples illustrate the link of collateral sensitivity to the complex genetic or transcriptional alterations that occur in parallel during the acquisition of the MDR phenotype. Selective toxicity of SN-38 against cisplatin-resistant cells and the increased toxicity of CXL017 against multidrug-resistant cells were identified based on the known mechanism of action of the compounds. In each case, collateral sensitivity was due to the addition of multidrug-resistant cells to a specific compensatory pathway, rather than the overexpression of transporters. The acquisition of MDR resulted in increased reliance on a single protein/pathway, and that reliance was exploited with inhibitors to elicit collateral sensitivity in those cells. In other examples, the elevated expression of transporters that confer MDR merely coincides with a trait that is responsible for collateral sensitivity. For example, the collateral sensitivity of resistant cells expressing P-gp to austocystin D is, in fact, due to the concomitant overexpression of the drug-metabolizing CYP enzyme. It has to be noted that coordinated upregulation of multidrug transporters and CYP enzymes is well characterized,¹⁵³ and a logical extension of the observations related to austocystin D is that additional small molecules activated by CYP 3A4 should demonstrate a similar activity profile toward multidrug-resistant cells.

7.2. Role of Transporters in the Collateral Sensitivity of Multidrug-Resistant Cells

In the case of MDR-selective compounds that were identified accidentally, as well as the compounds targeting MRP1-expressing cells, the elevated expression of transporters that confer MDR is *necessary but not sufficient* to convey collateral sensitivity to cells. As detailed above, the ability of compounds to reduce intracellular GSH levels does not necessarily induce selective toxicity. Similarly, the elevated toxicity of most MDR-selective compounds described in section 3.1 was found to be restricted to a few cell lines overexpressing P-gp, suggesting that additional factors related to the selection of resistant clones contribute to the collateral sensitivity of the cells. The previous section demonstrated some of the pathways identified as being responsible for collateral sensitivity. However, the toxicity of a subset of compounds, particularly those of the MDR-selective compounds identified in the DTP database, is specifically enhanced by the activity of P-gp. In that case, the activity of P-gp seems to be both *necessary and sufficient* to define collateral sensitivity. That P-gp is necessary is evidenced by the loss of collateral sensitivity in the presence of specific efflux inhibitors or upon genetic silencing of the transporter; that P-gp is sufficient is demonstrated by the finding that the P-gp-potentiated toxicity of these MDR-selective compounds persists in several cell lines with intrinsic or acquired MDR. How efflux

transporters convey collateral sensitivity to multidrug-resistant cells has not been identified. In the following subsections, we offer a conceptual framework to summarize possible mechanisms (Figure 16).

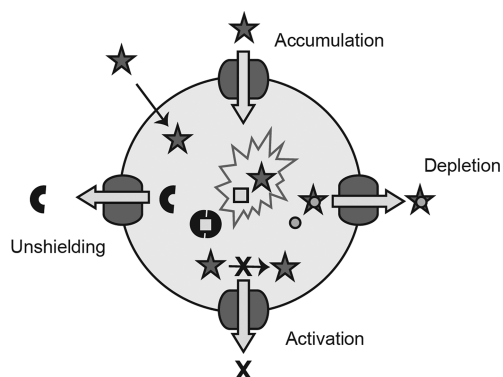


Figure 16. Possible mechanism of action of MDR-selective agents. MDR transporters might change the intracellular milieu, unshielding MDR cells by exposing the molecular targets (squares) of the MDR-selective compounds (stars). Alternatively, MDR-selective compounds might initiate a yet unknown, transporter-mediated signaling pathway, or the transporters might simply increase their intracellular accumulation. It is also possible that MDR transporters efflux an endogenous molecule (X), thus increasing the activation of the compounds. Finally, MDR-selective compounds might modulate the transport/substrate specificity of the transporters, which would result in the export and/or cellular depletion of essential endogenous molecules (circles), such as glutathione.

7.2.1. Transporters as Targets. Theoretically, the most straightforward mechanism of potentiation would rely on a transporters' ability to promote, rather than reduce, the accumulation of compounds in multidrug-resistant cells. This seems unlikely because, in contrast to several prokaryotic ABC transporters that act as importers, eukaryotic ABC proteins that are involved in substrate transport are invariably exporters, pumping their substrates from the cytoplasmic side to the extracellular space or an intracellular lumen. The orientation of extracellular vesicles directs the efflux activity of ABCG2 and actually results in an increase of the vesicular concentration of its substrates. This process can be exploited by photodynamic therapy. Instead of mediating direct influx, transporters that confer MDR might facilitate the accumulation of MDR-selective agents indirectly, for example, by altering plasma membrane properties. For example, selective toxicity of Triton X was suggested to rely on the altered biophysical properties of P-gp-expressing cell membranes.¹⁵⁴ Similarly, preferential toxicity of the NK-lysin-derived cationic peptide NK-2 was explained by the elevated net negative charge of the P-gp-overexpressing multidrug-resistant cell membrane analyzed in the study.¹⁵⁵

Many MDR-selective agents are not inhibitors, and many do not appear to have any interaction with the transporters. Modulation of the transporter through direct interaction was shown to be necessary for conveying selective toxicity in the case of MRP1-targeting agents.¹¹⁸ In agreement with this notion, verapamil enhances the photolabeling of MRP1 by iodo-aryl-azido-GSH (IAA-GSH).^{117,156} However, apigenin, which induces the same apparent effects as verapamil (i.e., strong intracellular GSH depletion and selective cell death in MRP1-overexpressing cells), does not stimulate photolabeling

of MRP1. ABC multidrug transporters display complex mechanistic features and can harbor several independent substrate-binding sites. In the case of MRP1, GSH might be effluxed along with another substrate, or it might stimulate substrate efflux without being transported.¹⁵⁷ The verapamil-induced increase in IAA-GSH labeling of MRP1 suggests that verapamil increases the affinity of the transporter toward GSH, increasing GSH export. A distinction between the GSH-binding site (G site) and substrate- (such as daunorubicin-) binding site (D site) within MRP1 was proposed to explain the ability of some compounds (named class I) to inhibit the MRP1-mediated efflux of daunorubicin, whereas some others (named class II) modulate the MRP1-mediated efflux of GSH.¹⁵⁸ In the presence of class I modulators, such as verapamil, the G and D sites might disengage, leading to a rapid turnover of GSH at the G site. Verapamil could be engaged in a futile cycle, and this might explain the strong and rapid GSH efflux observed.¹¹⁴ In a similar fashion, MDR-selective compounds targeting P-gp-expressing cells might modulate the transport/substrate specificity of P-gp,^{159,160} which would result in the export, and eventual cellular depletion, of essential endogenous molecules. Alternatively, MDR-selective compounds might modulate P-gp without influencing transport. P-gp was shown to regulate cell fate by inhibiting caspase-dependent apoptosis¹⁶¹ or by reducing ceramide levels through either the reduction of inner leaflet sphingomyelin pools or the modulation of the glycosphingomyelin pathway.^{162,163}

7.2.2. Unshielding. Efflux pumps can be exploited for selective killing of multidrug-resistant cells by combining an apoptosis-inducing agent that is not recognized as a transported substrate with an antiapoptotic compound that is effluxed from the multidrug-resistant cells. This concept was validated in vitro, because transporter-naïve cells were shown to be selectively rescued by the caspase inhibitor Z-DEVD-fmk, in contrast to P-gp (or MRP1)-expressing cells that effluxed Z-DEVD-fmk and therefore succumbed to flavopiridol (which is not a P-gp substrate). Thus, caspase inhibitors protected normal cells, whereas multidrug-resistant cells were unshielded by the transporters.¹⁶⁴ Using the analogy of caspase inhibitors, it might be hypothesized that the activity of MDR transporters selectively sensitizes multidrug-resistant cells by exposing the molecular target of MDR-selective compounds.

7.2.3. Activation. A general anticancer strategy relies on the use of prodrugs that become toxic upon intratumoral activation. Although agents that are selectively toxic to ABC transporter-expressing cells are not, in general, direct substrates of the transporter, it is possible for transporter activity to result in the selective activation of drugs. Many drugs, such as esters of toxic compounds, and certain nucleoside analogues require activation by intracellular enzymes. These activating pathways could, in theory, be under the control of small molecules that are substrates for ABC transporters. Similarly, ABC transporters could contribute to the selective activation of certain metal-based drugs whose toxicity is increased by reduction. As observed for several cobalt complexes, "activation by reduction" is believed to increase the intracellular activity of the metal drugs and also contributes to the selective transport and release of cytotoxic ligands.¹⁶⁵

7.2.4. Depletion. The physiological substrate of ABC transporters conferring MDR is not known. The paradoxical vulnerability of multidrug-resistant cells can be linked to the efflux and selective depletion of critical endogenous substrates such as ATP, GSH, and metals.

Depletion of ATP. Pharmacological depletion of tumoral ATP levels was initially suggested because of the characteristically increased metabolism and the consequent vulnerability of cancer cells.¹⁶⁶ Selective blocking of ATP-generating pathways, and, in particular, inhibition of glycolysis, was considered to be a viable strategy because of cancer cells' reliance on aerobic glycolysis rather than oxidative phosphorylation (known as the Warburg effect). In the context of MDR, inhibition of glycolysis was shown to result in ATP depletion and apoptosis in multidrug-resistant cells, suggesting that deprivation of the cellular energy supply might be an effective way to overcome MDR.¹⁶⁷ Furthermore, the antimetabolite D-glucose analogue 2-deoxy-D-glucose (2-DG) inhibits the glycolysis pathway and has been shown to selectively kill multidrug-resistant cells.^{168,169} 2-DG showed corresponding 2- to 14-fold selectivity in KB cell lines of increasing MDR. Subsequent research has shown that increased resistance of the KB cells was accompanied by a gradual loss of the glucose uptake transporter (GLUT-1), the pharmacologic target of 2-DG. Thus, lower 2-DG concentrations were needed to inhibit GLUT1, and the multidrug-resistant cells were therefore more sensitive. Although 2-DG inhibits glycolysis, and therefore reduces ATP levels, this has not been demonstrated to preferentially occur in multidrug-resistant cell lines. Rotenone, an inhibitor of the mitochondrial electron chain transport, has also been shown to selectively kill multidrug-resistant hamster ovary cells.¹⁷⁰ Batrakova et al. demonstrated that poly(ethylene oxide)–poly(propylene oxide) block copolymer (Pluronic) shows a 250-fold selective killing toward MCF-7/ADR cells compared with parent MCF-7 cells, and this extended to other P-gp-expressing cells.¹⁷¹ This sensitivity of P-gp-expressing cells correlated with a strong depletion of cellular ATP. The latter was not linked to P-gp ATPase (which was inhibited by Pluronic), but rather by the polymer being trafficked to the mitochondria, where it inhibited the respiratory chain and elevated ROS levels, selectively in the multidrug-resistant cells.¹⁷² Paradoxically, the activity of P-gp might sensitize cells by contributing to the depletion of intracellular ATP levels and, thus, might be directly responsible for the collateral sensitivity of multidrug-resistant cells. Enhanced activity of P-gp might lead to a greater consumption and the ultimate depletion of ATP, especially *in vitro*, where the concentration of the transported substrate such as verapamil remains constant in the medium. Thus, whereas P-gp might efficiently keep the intracellular levels of verapamil low at a high energetic cost, the concentration of verapamil in the lipid bilayer will remain constant. As a result, P-gp will be engaged in endless futile transport with a single substrate molecule possibly responsible for the consumption of a many-fold-greater amount of ATP. This mechanism was suggested to underlie the hypersensitivity of P-gp-expressing CHRC5 Chinese hamster ovary cells to verapamil. Inhibition of P-gp reversed hypersensitivity of the MDR line, but did not affect parental AUXB1 cells.¹⁷³ Whether energy depletion is a viable strategy for targeting multidrug-resistant cells selectively remains to be proven in relevant *in vivo* models.

Depletion of Glutathione. As detailed above, the collateral sensitivity of MRP1-overexpressing cells is mainly due to a dramatic GSH depletion in these cells. MRP1-overexpressing cells have characteristically lower intracellular GSH levels as compared to parental cells. Intracellular GSH is further decreased by compounds preferentially targeting MRP1-expressing multidrug-resistant cells. This effect is mediated

either by inducing MRP1-mediated GSH transport, as illustrated by verapamil,¹¹⁶ or by inhibiting GSH synthesis (exemplified with BSO). Alternatively, cell death triggered by GSH depletion through stimulation of MRP1-induced GSH efflux by verapamil and derivatives, xanthenes and flavonoids, might be mediated by MRP1 itself, through the putative efflux of a vital unidentified endogenous compound in cotransport with GSH.¹¹⁴ Selective depletion of intracellular GSH levels in MRP1-expressing cells results in oxidative stress or apoptosis.¹⁷⁴ The rapid and large GSH depletion observed with MRP1-specific compounds was similar to that described for puromycin,¹²⁸ diphenyleioidonium-¹⁷⁵ and anti-Fas/APO-1 antibody.¹⁷⁶ For the latter, a rapid decrease of reduced GSH was observed, which preceded an irreversible commitment to cell death. Enhanced cellular GSH release with a concomitant decrease of intracellular GSH appeared to be necessary for the progression of apoptosis. Indeed, an increase in GSH synthesis, compensating MRP1-mediated GSH efflux, rendered the cells less susceptible to apoptosis.^{104,105} Activation of caspase 3 results in the direct inhibition of γ -glutamyl cysteine ligase, which prevents replenishment of intracellular GSH.¹⁷⁷ While the role of GSH in regulating apoptotic cell death is unclear, these results highlight the importance of GSH in controlling key regulatory events during cell death.

Depletion of Metal Ions. The structural coherence of the MDR-selective compounds identified in the DTP data set implied a shared mode of action pertaining to structurally related compound subsets.⁷⁹ In particular, there was a significant enrichment of metal chelators. This suggested that metal-ion interaction could be key to the cytotoxicity of at least a subset of the MDR-selective compounds. P-gp overexpression could potentiate the toxicity of chelators if multidrug-resistant cells are deprived of essential metals as a result of the efflux activity. Unfortunately, convincing evidence linking the activity of P-gp to cellular metal depletion is lacking. Furthermore, metal chelation alone is not sufficient for P-gp-potentiated activity, as evidenced by a series of anticancer chelators that are devoid of MDR-selective toxicity.

Depletion of ATP leads to oxidative stress (characterized by ROS) through increased oxidative phosphorylation. Consistent with this mechanism, an increase in ROS upon using inhibitors of the mitochondrial electron-transport chain was shown to synergize with the MDR-selective toxicity of verapamil.¹⁷⁰ Similarly, the anticancer activity of metal complexes is largely based on reduction–oxidation (redox) cycling, including Fenton-like reactions in which metals switching between oxidation states catalyze ROS production.¹⁷⁸ Furthermore, metal complexes inhibit the antioxidant defense network by interfering with the thioredoxin and glutathione systems.¹⁶⁵ Changes in cellular redox homeostasis, through GSH oxidation or MRP1-mediated GSH efflux, contribute to the initiation or propagation of the apoptotic cascade. It can be hypothesized that the activity of transporters results in an imbalance of the redox homeostasis, ultimately leading to the collateral sensitivity of multidrug-resistant cells to MDR-selective compounds. It should be noted that not every agent known to deplete cells of ATP or GSH exhibits bona fide MDR-selective activity, suggesting that these attributes might be necessary but not sufficient for the selective elimination of MDR cells. Detailed metabolomic studies assessing the effect of efflux activity and MDR-selective compounds on P-gp-expressing cells and their sensitive counterparts are needed to test these hypotheses.

8. PERSPECTIVES

As we have seen, the Achilles heel of MDR cells can be readily targeted by compounds exploiting the fitness cost of ABC transporter overexpression. So, is resistance “useless”?¹⁴⁸ The broadening concept of MDR-targeted therapy has been lacking the crucial support of a proof-of-concept *in vivo* study. The MDR field has been misled by the straightforward interpretations of *in vitro* data obtained with transporter inhibitors, so caution is warranted in the interpretation of the results. Despite the obvious advantages of cell culture systems (e.g., availability of a wide range of human tumor cell lines, flexibility of culture conditions, ease of biochemical characterization), cell line data do not recapitulate clinical features and do not predict the therapeutic index. As a result of a continuous selection for growth in an endless supply of nutrients or space to grow, cell lines exhibit characteristic changes in expression patterns that distinguish them from corresponding clinical samples.¹⁷⁹ In addition, cell lines are not limited by cell-to-cell contacts that exert a major influence on tumor cells and drug penetration in cancerous tissue. Thus, *in vitro* data on drug resistance or collateral sensitivity might not accurately represent the clinical efficacy of compounds. Nevertheless, the implications of the studies on collateral sensitivity for the development of more precise and improved chemotherapy of cancer are profound. Recognition that cancer therapy will be effective insofar as it takes advantage of the special features of cancer cells leads to the idea that the development of drug resistance itself provides a target for improved treatment of drug-resistant cancer.¹⁸⁰ Although there is some controversy about whether expression of multidrug transporters such as ABCB1, ABCC1, and ABCG2 is necessary for the development of MDR, there should be no debate about whether such expression is sufficient for drug resistance, nor that such expression occurs in at least 50% of human cancers some time during treatment.¹⁸¹ Thus, whereas inhibition of ABC transporters might or might not sensitize cells to further chemotherapy (owing to expression of other resistance mechanisms), there can be no argument that such expression can selectively sensitize multidrug-resistant cells to the agents discussed in this review.

Is exploiting the collateral sensitivity of multidrug-resistant cells clinically feasible? First, it would require the determination of the MDR mechanisms in a specific patient's tumor. This could be achieved by molecular pathology of tumor samples or, if it occurs during the course of therapy, by direct *in vivo* imaging of transporter function. Based on the result, an MDR-selective regimen could be added to the next rounds of chemotherapy, to kill multidrug-resistant cells that express the transporter. Another approach could be to automatically add a sensitizer to all chemotherapy regimens of cancers likely to express ABC transporters, some time during the course of treatment. In the Darwinian environment of a cancer, the fitter chemosensitive cells proliferate at the expense of the less-fit chemoresistant cells. Mathematical models suggest that, by maintaining a stable population of therapy-sensitive cells, it is possible to suppress the growth of resistant phenotypes through intratumoral competition. The “adaptive therapy” model predicts that a continuously modulated treatment protocol, adjusted to therapy-induced resistance, would maintain a stable tumor burden in which the proliferation of the less-fit but chemoresistant subpopulations is suppressed by the chemosensitive majority.^{182,183} In that paradigm, MDR-selective compounds could be used to maintain a stable

population of therapy-sensitive cells, to suppress growth of resistant phenotypes through intratumoral competition.

The clinical application of MDR-selective therapy is not without significant impediments. Most studies suggest that ABC transporter expression must be at a moderately high level to solicit the differential sensitivity phenomenon; thus, it might be that only a minority of multidrug-resistant cells are susceptible to this treatment. Because MDR-selective compounds do not inhibit efflux, a profound effect on drug absorption, distribution, metabolism, excretion, and toxicity (ADME-Tox) is unlikely. However, there are many normal cell types that express ABC transporters at significant levels (e.g., bone marrow stem cells, brain capillary epithelial cells, epithelial barrier cells), and these might be subject to toxicity from the agents that induce collateral sensitivity. Preliminary toxicology studies suggest that normal tissues expressing ABC transporters are not differentially sensitive to these agents, raising the possibility that it is the combination of the malignantly transformed state with the expression of the ABC transporters that leads to toxicity. Before clinical trials of MDR-selective compounds can be contemplated, more detailed preclinical studies are needed to determine the best way to deliver these drugs and to establish the proof of concept that MDR-selective compounds can kill transporter-expressing cells *in vivo* to eliminate, prevent, or reverse transporter-mediated drug resistance.

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Notes

The authors declare no competing financial interest.

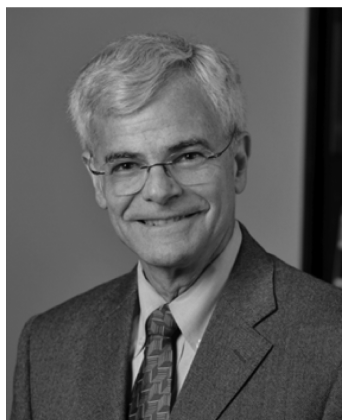
Biographies



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Michael M. Gottesman is the Deputy Director for Intramural Research at the National Institutes of Health and Chief of the Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute. He obtained his M.D. from Harvard Medical School, completed his internship and residency in medicine at the Peter Bent Brigham Hospital in Boston, and received his postdoctoral research training in molecular genetics with Martin Gellert at the NIH. He became Chief of the Molecular Cell Genetics Section of the Laboratory of Molecular Biology in 1980 and Chief of the Laboratory of Cell Biology in 1990. Since 1993, he has been the NIH Deputy Director for Intramural Research. Dr. Gottesman developed somatic cell genetic systems for the analysis of drug resistance in cancer cells. Those studies led to the cloning of the human MDR1 gene and the elucidation of its function in normal tissues and in cancer cells. Recently, his laboratory has evaluated the role of other ABC transporters in multidrug resistance in cancer cells and has begun studying mechanisms of resistance to anticancer drugs. He received the ASPET Award in 1997 and was elected to the Institute of Medicine in 2003 and to the American Academy of Arts and Sciences in 2010.



Prof. Ahcène Boumendjel is the head of the Medicinal Chemistry of Natural Products group at the Department of Medicinal Chemistry of the Joseph Fourier University, Grenoble, France. He obtained his Ph.D. degree in 1991 from the University of Reims (France). He spent three years at the National Institutes of Health (NIH, Bethesda, MD) as a postdoctoral fellow, working under the guidance of Dr. S. Miller on the chemistry of sphingolipids. In 1994, he moved to Cambridge, MA, to work for a biotechnology company (Procept, Inc) on peptidomimetics as anti-HIV agents. In 1996, he was appointed as an Assistant Professor of Pharmacognosy at the Joseph Fourier University of Grenoble (France), and since 2008, he has been a full professor of Medicinal Chemistry. The research interests of Prof. Boumendjel deal with the discovery of drug candidates using natural products as hit sources. He has authored and coauthored more than 80 international publications and 6 international patents.



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Attilio Di Pietro is heading the group entitled “Drug resistance mechanism and modulation” at the Institute of Protein Biology and Chemistry (IBCP) of Lyon, France, supported by the National Center for Scientific Research (CNRS) and the University of Lyon. He earned a Doctorat d'Etat es-Sciences from the University of Lyon at Villeurbanne in 1981, on mitochondrial bioenergetics, and spent his postdoctoral training with Prof. André Goffeau at the University of Louvain-La-Neuve, Belgium, in 1982 and 1983 studying yeast plasma membrane transporters. He became independent in 1992 in studying membrane ATP-binding cassette (ABC) transporters involved in multidrug resistance, especially in cancer cells. He chaired a Gordon Research Conference on “Multidrug Efflux Systems” in Oxford, U.K., in August 2005 and has organized the annual French–Belgian meeting on ABC transporters since 2006. He has published around 130 papers in international journals and received several patents. He has presented approximately 65 invited talks to international meetings in the fields of drug discovery and mechanisms of cancer cell multidrug resistance. Since 2009, his group has been certified by the French National League against Cancer.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on April 23, 2014, with an error in Figure 13. The corrected version was reposted on April 24, 2014.