Drug Resistance in Cancer Cells

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Editors

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Foreword by Susan E. Bates

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Foreword

It was estimated that in 2008, 1,437,180 patients would receive a new cancer diagnosis and 565,650 individuals would die of cancer (Jemal et al. 2008). Since the vast majority of patients dying of cancer will have had anticancer therapy, both conventional chemotherapy and novel targeted therapy, it can be concluded that these patients are dying with drug resistant cancer. The term multidrug resistance is also apt – in that these patients die after having undergone multiple rounds of different and structurally unrelated cancer therapies. However, for some, the concept of multidrug resistance is a worn out idea, stemming from disappointment with the drug resistance reversal strategies that were carried out in the 1990s using pump inhibitors to block drug resistance mediated by P-glycoprotein, product of the *MDR-1* gene. However, if one takes the larger definition – multidrug resistance as simultaneous resistance to multiple structurally unrelated anticancer therapies – its existence cannot be denied. The purpose of this book is to explore new concepts related to drug resistance in cancer, including resistance to the new molecularly targeted agents. Perhaps new terminology is needed for resistance that occurs following therapy with the targeted agents: Novel Targeted Agent Resistance (NTR). Alternatively, we can return to the original definition of multidrug resistance as simply the resistance to multiple agents that occurs in the course of normal cancer progression. This resistance is likely to be mediated by many factors.

Figure 1 presents a schematic that is meant to represent the complexity of our current understanding of drug resistance. At this, the schematic is still an oversimplification. The multifactorial nature of drug resistance is unquestioned now, but there was a time when a major goal of cancer investigation was to identify a single mechanism of resistance. Today, we can think of drug resistance in two classes: target-specific and target-nonspecific. These are very closely aligned with the older terms: acquired and intrinsic.

Target-specific mechanisms relate to the development of resistance mechanisms that are specific to the drug target. These resistance mechanisms are often acquired – whether due to a new event or to selection of pre-existing events. An example of this type of resistance is found in the subset of breast cancers demonstrating loss of estrogen receptor following emergence of tamoxifen resistance (Clarke et al. 2003). Another excellent example is the development of BcrAbl kinase domain mutations such as the T315I, which renders chronic myelogenous leukemia (CML) cells resistant to

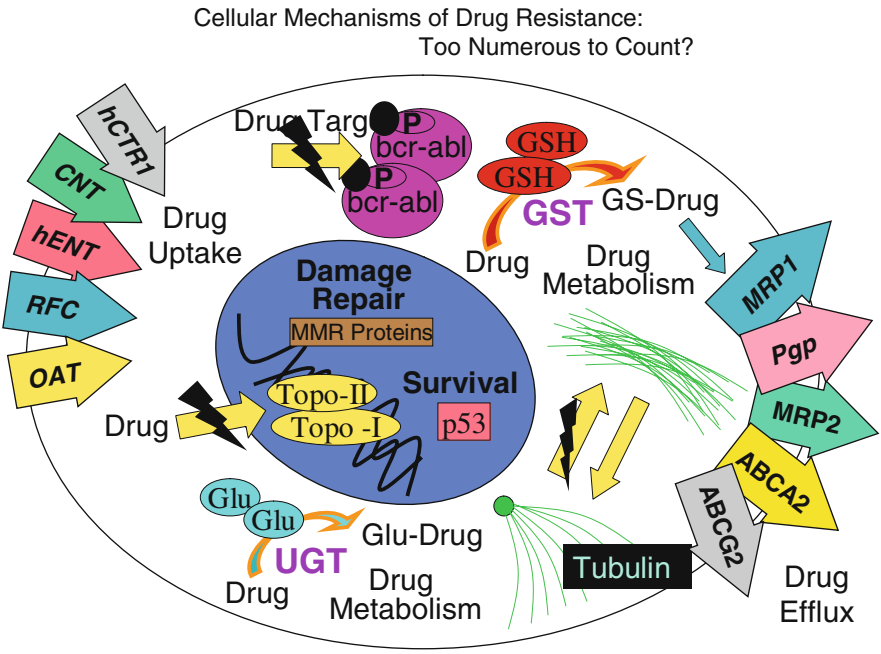

Fig. 1 Cellular mechanisms of drug resistance: too numerous to count? Six categories of resistance mechanisms are depicted: Drug Uptake, Drug Efflux, Drug Metabolism, Drug Target, Damage Repair, and Survival. The broken arrows indicate impaired drug binding to target such as might occur with an acquired mutation in Bcr-Abl, reduced levels of topoisomerase, or altered isotype composition in tubulin

imatinib (Gorre et al. 2001). Similarly, the T790M EGFR mutation renders NSCLC resistant to gefitinib (Pao et al. 2005). Turning to in vitro model systems, increased expression and a markedly increased phosphorylation of EGFR has been observed in trastuzumab resistant breast cancer cells (Ritter et al. 2007). The converse has also been demonstrated – upregulation of HER2 following suppression of EGFR with cetuximab suggests an alternate proliferative pathway for cancer maintenance (Wheeler et al. 2008). These target-specific mechanisms are difficult to predict in advance, and a single "fix" cannot be identified that would circumvent this type of resistance.

Target-nonspecific mechanisms, on the other hand, are often intrinsic or constitutive – that is, they are present a priori and may be expropriated to promote drug resistance. They include those general mechanisms that were identified beginning in studies of resistance to "cytotoxic" agents, and they are often upregulated or preferentially selected after exposure to anticancer agents. These include p21-, p27-, or p53-induced cell cycle arrest; reduction in cell proliferation through cell adhesion signaling; upregulation of Bcl-2 or other pro-survival molecules; upregulation of enzymes that increase metabolic inactivation, such as glucuronidation or glutathione

conjugation; upregulation of drug efflux transporters; and downregulation of drug influx transporters (Mellor and Callaghan 2008).

However, it would be a mistake to characterize every drug failure as drug resistance. Consider a clear and obvious example, the use of trastuzumab in HER2+ breast cancer. Early clinical trials in breast cancer demonstrated the activity of trastuzumab in the subset of patients whose cancers expressed high levels of HER2. Enrichment of patients with overexpressing cancers demonstrated a clinical benefit that may have been missed had the drug been developed in the entire patient population. The response rate in patients with breast cancer staining 2+ for HER2 was 6%, while it was 18% in patients with breast cancers staining 3+ for HER2 (Cobleigh et al. 1999). A later study suggested that immunohistochemical assessment incorrectly classified some of the patients. Among patients found to have cancers positive by fluorescence in situ hybridization (FISH), which documents amplification of HER2, responses were noted in 20%, relative to the absence of responses noted in FISH-negative tumors (Baselga 2001). Beyond the use of biomarkers to indicate that tumors are either HER2 positive or not, genomic analysis demonstrated that gene expression profiles can be used to cluster invasive breast cancers into six different subtypes, with HER2 positive tumors representing one distinct subtype (Sorlie et al. 2001). This suggests that there is not only HER2 overexpression but also that distinctive downstream signaling events result from activation of HER2. Tumors of this subtype are dependent on HER2 signaling for maintenance of the malignant phenotype. The remaining subtypes are equally distinctive but do not cluster with cells exhibiting HER2 overexpression. Do we then classify the cancers without HER2 expression as being "drug resistant?" In these tumors, the target is absent. Herceptin will not work in these tumors, and only confusion would result if investigators began to study mechanisms of resistance to Herceptin in such tumors.

Thus, we need to be careful about defining a tumor as drug resistant. Are the 60–70% of renal cell cancers that do not achieve a partial remission with sunitinib drug resistant (Motzer et al. 2007)? Sorafenib, which at its FDA-approved dose appears to be a less potent TKI in comparison to sunitinib, achieves partial remissions in less than 10% of cases (Escudier et al. 2007). Are these tumors drug resistant? Similarly, gefitinib was found to be more tolerable than erlotinib, perhaps because a lower effective dose was selected for development (Lorusso 2003; Soulieres et al. 2004). As with the sorafenib/sutent pairing, it would be hard to argue that a tumor responding to erlotinib and not to gefitinib was indeed drug resistant. A strong argument could be made for a definition of drug resistance in the era of novel-targeted agents that requires presence of the target and effective drug concentrations.

While an "effective drug concentration" may be related to a drug and its potency, another aspect that should be considered is interpatient variability in drug activation, absorption (for oral drugs), drug metabolism, and drug excretion. The expanding field of pharmacogenomics has already lent considerable insights into the potential impact of single nucleotide polymorphisms in altering drug clearance. As one example, tamoxifen is hydroxylated by cytochrome P450 (CYP) 2D6 to the potent

metabolites 4-hydroxytamoxifen (4OHtam) and 4-hydroxy-*N*-demethyltamoxifen (4OHNDtam). Carriers of CYP2D6 alleles with reduced enzyme activity metabolize tamoxifen more poorly and have lower levels of active metabolites. These alleles have been associated with a poorer outcome to tamoxifen therapy (Goetz et al. 2007). Inhibitors of CYP 2D6 have been shown to have similar effects. Together with the presence of other tamoxifen metabolizing pathways, these findings further increase the complexity of "personalized medicine".

Low drug concentrations due to impaired activation or low plasma levels due to rapid clearance generate *pharmacologic drug resistance*, a problem deserving intensive study in oncology. Trough levels of imatinib above 1002 ng/ml have been associated with better and deeper responses to treatment in CML (Picard et al. 2007). The genotypes involved in mediating differences in imatinib levels have not been worked out in detail, some have postulated a role for the multidrug transporter ABCB1. Similarly, a polymorphic variant in the multidrug transporter ABCG2 that results in impaired transport has been related to increased gefitinib levels and toxicity (Cusatis et al. 2006; Li et al. 2007). These studies to date have been carried out on a small scale, and the impact of this variant on efficacy is not known. However, it will not come as a great surprise to find greater efficacy in patients carrying the variant.

Finally, just as in the early 1990s, an overly simplistic view of multidrug resistance led to numerous ill-conceived trials attempting to overcome drug resistance (see Sakacs et al. in this book), so it would be overly simplistic to now consider resistance to gefitinib or to imatinib or to any other targeted agent as due solely to a single mechanism (Engelman and Janne 2008). Rather, it will be important to evaluate tumor samples for multiple mechanisms of drug resistance. Just as the field has set as its goal personalized approaches to cancer therapy (hoping to avoid intrinsic drug resistance by selecting appropriate therapies at the start), so we should develop strategies to identify individual mechanisms, or sets of mechanisms, of drug resistance.

Table 1 lists some examples of multiple mechanisms of drug resistance that have been identified in the setting of both traditional agents and novel targeted agents. This list for each drug is not exhaustive – rather the emphasis is on unique mechanisms that have been identified. General pro-survival mechanisms such as cell cycle arrest allowing time for repair of damage have not been included here.

Since it will not be possible to clinically address every possible mechanism of drug resistance for a given agent, the personalized medicine of the future will need to order the resistance mechanisms in terms of importance. For example, the secondary BCR-ABL mutation T315I, which renders CML cells more than 20-fold resistant to imatinib, is all that is needed to provoke clinical imatinib failure (Apperley 2007). However, BcrAbl mutations that render CML cells only 2–3-fold resistant to imatinib will need to coexist with another mechanism of resistance, in order to provoke clinical failure.

In medicine, we attempt to distill a group of patient symptoms into a single diagnosis, and skilled diagnosticians are prized. Yet, in considering drug resistance as a diagnosis, we must function in a counterintuitive fashion. The phenotype is simple –

Table 1 Multiple mechanisms of drug resistance

Imatinib in CML:
BCR-ABL kinase domain mutation
BCR-ABL gene amplification
Interindividual variation: Trough levels <1000 ng/ml
ABCB1/Pgp
ABCG2/BCRP
Tamoxifen in Breast Cancer:
Estrogen Receptor loss
Low Cytochrome 2A6 expression and failure to generate active metabolite
Gefitinib in NSCLC:
Lower potency
Lack of activating EGFR mutation
Secondary EGFR T790M mutation
Increased PI3K pathway and downstream survival signaling
MET amplification
ABCG2/BCRP
Cisplatin:
Metallothionines
Decreased uptake transporters
Increased DNA repair
Increased glutathione conjugation
Doxorubicin:
Decreased topoisomerase II levels
Increased oxygen radical scavenging
Increased glutathione conjugation
Increased ABCB1/Pgp expression

the drug fails to provide clinical benefit. But the underlying pathways to drug resistance are manifold and complex and very likely dependent on cell context. Rather than having a form of "stage migration" as one newly identified drug resistance mechanism subverts another in our collective imaginations, it will be important to begin to catalogue them all and to determine how to identify those operating in a particular tumor type. This book is an effort to describe in one place the array of drug resistance mechanisms confronting clinical oncologists today.

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Chapter 1
Multidrug Resistance Mediated by MDR-ABC Transporters

Gergely Szakács, Kenneth Kin Wah To, Orsolya Polgár, Robert W. Robey, and Susan E. Bates

Abstract Cancer cells are constantly being selected for survival and proliferation. During this process, tumor cells often co-opt basic physiological mechanisms to protect themselves from toxic chemotherapy. Of these mechanisms, the one that is most commonly encountered in laboratory studies is the increased cellular efflux of a broad class of cytotoxic drugs that is mediated by a family of energy-dependent transporters, known as ATP-binding cassette (ABC) proteins. Although 48 human ABC transporters have been described, only a handful are likely to be involved in drug resistance. These include P-glycoprotein (Pgp), the multidrug resistance associated protein (MRP), and the breast cancer resistance protein (BCRP), also termed ABCG2. In this chapter, we will provide an overview of the general structural and mechanistic features of the human ABC transporters thought to be involved in multidrug resistance in cancer cells. These transporters can no longer be understood only as candidate drug targets for overcoming multidrug resistance. Rather, these transporters are involved in multiple normal tissues in protection from drugs and xenobiotics. These include the blood brain barrier, oral bioavailability of drugs and xenobiotics through expression of transporters in the GI tract, drug disposition, and fetal protection through expression at the maternal–fetal barrier. We describe emerging information related to their potential role in drug and xenobiotic resistance and argue that studies of this drug resistance mechanism should continue in the laboratory and in the clinic.

Keywords ABC transporter · P-glycoprotein · ABCG2 · Drug resistance · Drug transporters

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Introduction

Cancer cells are constantly being selected for survival and proliferation. During this process, tumor cells often co-opt basic physiological mechanisms to protect themselves from toxic chemotherapy. Drug resistance can be the result of a variety of cellular and pharmacological mechanisms that are detailed in the chapters of this volume. Of these mechanisms, the one that is most commonly encountered in the laboratory is the increased efflux of a broad class of cytotoxic drugs that is mediated by a family of energy-dependent transporters, known as ATP-binding cassette (ABC) transporters. ABC transporters translocate a wide range of structurally unrelated compounds across biological membranes, including most of the routinely used agents of the current chemotherapy regimens. While several ABC transporters, particularly in the ABCC family, have been shown to transport certain drug substrates, three in particular have been studied for their role in drug disposition and in multidrug resistance. The three major multidrug resistance (MDR) proteins include members of the ABCB (ABCB1/MDR1/P-glycoprotein), the ABCC (ABCC1/MRP1), and the ABCG (ABCG2/MXR/BCRP) subfamilies. In this chapter, we will provide an overview of the general structural and mechanistic features of the human MDR-ABC transporters. We will also describe emerging information related to their potential role in drug and xenobiotic resistance and argue that studies of this drug resistance mechanism should continue in the laboratory and in the clinic.

Mechanistic Aspects of ABC Transporters

ABC transporters are present in all living organisms from bacteria to humans and are of great importance to various cellular functions. Forty-eight ABC transporters are identified in the human genome, grouped into seven subfamilies (ABCA–ABCG) based on phylogenetic analysis (Dean et al. 2001). Several human ABC transporter genes have been associated with inheritable diseases, including cystic fibrosis (ABCC7), Dubin–Johnson syndrome (ABCC2), adrenoleukodystrophy (ABCD1), and sitosterolemia (ABCG5/ABCG8) (Borst and Elferink 2002). ABC transporters are large membrane-bound proteins built of two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). The NBDs contain the conserved Walker A and B motifs, found in all ATP-binding proteins, as well as the C signature motif that distinguishes the superfamily from other ATPases. A subset of these ABC proteins has been shown to be drug transporters. The drug translocation pathway is formed by the transmembrane domains, which typically contain 12 (2×6) membrane-spanning alpha helices (TMs). This structural organization is either encoded in a single gene (a full transporter) in the case of P-glycoprotein (ABCB1) or MRP (ABCC1) or is achieved through the homo-, or heterodimerization of two half-transporters, containing a single TMD and NBD, in the case of the breast cancer resistance protein (ABCG2). Unfortunately, crystallization has, so far, not been accomplished for these transporters to a resolution sufficient for proper insight into how the pumps might actually

work. Crystallization of membrane-embedded proteins remains a very difficult task. Currently, crystal structures of approximately 160 transmembrane proteins are available to a resolution greater than 4 Å, while the number of available structures for soluble proteins is in the thousands. There are seven ABC transporter structures solved, all found in bacteria (Locher et al. 2002, Dawson and Locher 2006, Hvorup et al. 2007, Hollenstein et al. 2007, Pinkett et al. 2007, Ward et al. 2007, Oldham et al. 2007). Of these, Sav1866 from *Staphylococcus aureus* likely resembles the most the three major human multidrug resistance transporters. Indeed, all three have already been modeled using the Sav1866 structure as a template and tested to some extent using data derived from mutagenesis (O'Mara and Tieleman 2007, DeGorter et al. 2008, Hazai and Bikadi 2008).

As noted above, Pgp, MRP1, and ABCG2 have several common structural features; on the other hand, they differ significantly in some aspects. Pgp and MRP1 are so-called full transporters, composed of a tandem repeat of transmembrane and nucleotide-binding domains (TMD–NBD–TMD–NBD). ABCG2 has only one NBD and a single TMD, and it requires dimerization in order to be functional. There is compelling evidence supporting homodimerization of ABCG2 (Ozvegy et al. 2001, Henriksen et al. 2005, Kage et al. 2002). In addition to being a half-transporter, the domain organization of ABCG2 is reversed when compared to Pgp, MRP1, or Sav1866, with an N-terminal NBD and a C-terminal TMD. The importance of this reverse orientation, if any, has yet to be determined, nevertheless, it makes it even harder to predict the structure of ABCG2 in the absence of a crystal structure.

In addition to a Pgp-like core, MRP1 has an extra transmembrane domain with five membrane-spanning alpha helices, termed TMD_0 connected to TMD_1 via an intracellular loop (L_0), present in several other ABCC subfamily members. TMD_0 could not be modeled based on Sav1866 which has only 12 TMs. Interestingly, deletion of TMD_0 did not prevent the transport function and proper membrane localization of MRP1 (Bakos et al. 2000). However, certain mutations introduced to TMD_0 had structural and functional consequences (Yang et al. 2002).

There are no such striking differences between the ATP-binding sites of the three transporters, although the two sites demonstrate less symmetry in MRP1 than in Pgp. Upon binding and hydrolysis of ATP, conformational changes are presumed to occur that are transmitted to the TMDs, ultimately resulting in the translocation of the substrate from one side of the membrane to the other. Based on the observations from multiple bacterial NBD structures, the two ATP-binding sites are thought to form a "sandwich dimer", in which the Walker A and B motifs from one subunit bind ATP together with the C signature sequence of the other subunit (Hollenstein et al. 2007)

MDR-ABC Transporters

Based on a great deal of clinical and experimental work, it has been established that the MDR-ABC proteins recognize and transport a very wide range of drug substrates. This is relevant to their presumed normal role in human physiology in

regulating the GI absorption and normal tissue exposure of xenobiotics. Although recognized substrates are mostly hydrophobic compounds, MDR pumps can also extrude amphipathic anions and cations. As discussed in detail below, ABCB1 preferentially extrudes large hydrophobic molecules, while ABCC1 and ABCG2 can transport both hydrophobic drugs and large anionic compounds, e.g., drug conjugates. This "promiscuous" character and the additional overlapping substrate recognition by the three major classes of the MDR-ABC transporters provide an amazing network of drug resistance capacity in cancer cells (Sarkadi et al. 2006). These transporters were initially described in multidrug-resistant cancer cell lines (Juliano and Ling 1976, Cole et al. 1992, Doyle et al. 1998, Allikmets et al. 1998, Miyake et al. 1999); therefore, the first substrates identified were chemotherapeutic agents. Later, a wide variety of transported compounds unrelated to cancer therapy have been recognized, leading to an emerging role of these pumps in protection against xenobiotics and most recently in pharmacokinetics and pharmacogenomics. Although Pgp, the first of the three transporters, was identified more than 30 years ago, we have yet a long way to go before fully understanding the exact nature of their mechanism of action, and the role these pumps play in normal physiology and clinical multidrug resistance.

Transported Substrates

The list of compounds transported by Pgp, MRP1, and ABCG2 is rapidly expanding and demonstrates significant overlaps. Many of the substrates are structurally dissimilar, while certain trends for favored compounds for each transporter can be established (Table 1.1)

Pgp, similarly to ABCG2, preferentially transports large, hydrophobic, positively charged molecules. Its substrates include a wide variety of clinically relevant compounds, including anticancer drugs, human immunodeficiency virus (HIV)-protease inhibitors, immunosuppressive agents, antiarrhythmics, antihistamines, H-2 receptor blockers, antiemetics, steroids, cardiac glycosides, antibiotics, antihelmintics, antidepressants, antiepileptics, and analgesics. Among the chemotherapeutic agents transported by Pgp are anthracyclines (daunorubicin, doxorubicin, epirubicin), anthracenes (bisantrene, mitoxantrone), vinca alkaloids (vinblastine, vincristine, vinorelbine, vindesine), camptothecins (topotecan), taxanes (paclitaxel, docetaxel), and epipodophyllotoxins (etoposide, teniposide). Steroid hormones, phospholipids, and cytokines are among the potential physiological substrates of Pgp. Besides effluxing mostly neutral hydrophobic compounds, MRP1 is capable of transporting organic anions, such as molecules conjugated to glutathione (GSH), glucuronate, and sulfate. Among these conjugates are the compounds considered as endogenous substrates of the protein, e.g., GSH conjugates of leukotriene C4, leukotriene D4, leukotriene E4, prostaglandin A2, glucoronide conjugates of estradiol, and sulfate conjugates of estrone and dehydroepiandrosterone. Interestingly, GSH itself is a substrate for the pump. MRP1 also transports drugs currently used in the clinic, such as HIV-protease inhibitors,

Table 1.1 Clinically relevant substrates of the MDR-ABC transporters (Szakacs et al. 2006)

		ABCB1	ABCC1	ABCG2
Vinca alkaloids	Vinblastine	■	■	
	Vincristine	■	■	
Anthracyclines	Daunorubicin	■	■	■
	Doxorubicin	■	■	■
	Epirubicin	■	■	■
Epipodophyllotoxins	Etoposide	■	■	
	Teniposide	■		
Taxanes	Docetaxel	■		
	Paclitaxel	■		
Kinase inhibitors	Gleevec	■	■	■
	Flavopiridol			■
Campthotecins	Irinotecan (CPT-11)	■	■	■
	SN-38	■	■	■
	Topotecan			■
Thiopurines	6-Mercaptopurine			
	6-Thioguanine			
	5-FU			
Other	Bisantrene	■		■
	Cisplatin		■	
	Arsenite		■	
	Colchicine	■	■	
	Estramustine			
	Methotrexate	■	■	■
	Mitoxantrone	■	■	■
	Saquinivir	■		
	PMEA			
	Actinomycin-D	■		
	AZT			■
	Digoxin	■		

antibiotics, antiandrogens, and chemotherapeutic agents. The latter group includes anthracyclines (doxorubicin, daunorubicin), vinca alkaloids (vinblastine, vincristine), epipodophyllotoxins (etoposide, teniposide), camptothecins (topotecan, irinotecan), and methotrexate (Bakos and Homolya 2007). The manner in which MRP1 handles its substrates is rather complex. Transport of certain hydrophobic compounds, such as vincristine, was shown to be stimulated by GSH. In addition, vincristine transport was suggested to be dependent on physiological concentrations of GSH (Loe et al. 1998). These observations have led to the assumption that the two molecules are cotransported, but the question is complicated by the fact that efflux of some compounds, such as daunorubicin, is stimulated by GSH, while they themselves have no effect on GSH transport (Renes et al. 1999). In contrast, verapamil, which is not a substrate of MRP1, stimulates GSH transport. Cotransport could be envisioned with

two separate substrate-binding sites or with a single larger site that can accommodate the two molecules at the same time. The existence of an allosteric site that modulates the affinity of the substrate site is also possible. A third possibility is that some compounds alter the membrane environment which in turn has an effect on the affinity for certain substrates (Borst et al. 2006). Of the three multidrug resistance transporters, *ABCG2* is the most recently described, nevertheless, it also has a long list of established substrates comparable to that of Pgp and MRP1. *ABCG2*, as mentioned above, primarily transports large, hydrophobic, positively charged molecules, among which are flavonoids, porphyrins, antibiotics, HMG-CoA reductase inhibitors, antivirals, H-2 receptor blockers, antihypertensive agents, vitamins, and folic acid. A substantial number of chemotherapeutic agents are also effluxed by *ABCG2*, including mitoxantrone, flavopiridol, campthotecins (e.g., topotecan, irinotecan, SN-38), indolocarbazoles, antifolates, tyrosine kinase inhibitors (e.g., imatinib, gefitinib, erlotonib, nelotinib).

Besides the anticancer and other clinically important drugs, it is worth mentioning that several fluorescent compounds are transported by Pgp, MRP1, and *ABCG2*, such as Hoechst 33342 (Pgp, *ABCG2*), rhodamine 123 (Pgp), calcein (MRP1), calcein-AM (Pgp). These molecules proved to be excellent tools in studying these transporters in the laboratory.

Gene Regulation

P-glycoprotein is constitutively expressed in many normal tissues including the adrenal gland, liver, kidney, intestinal mucosa, muscle, and the endothelial cells of the blood–brain barrier and blood–testes barrier (Cordon-Cardo et al. 1990). The human *MDR1* gene is located on chromosome 7, band p21–21.1, and it extends over more than 100 kb containing 28 exons. The transcriptional regulation of *MDR1* has been extensively studied. The *MDR1* gene, as is true for all of the human multidrug transporters examined to date, has a "TATA-less" promoter. Activation of *MDR1* occurs rapidly in response to a variety of cellular stresses and stimuli ranging from DNA injury to serum starvation, linking Pgp to a general anti-apoptotic mechanism. Interestingly, signals from these stimuli were shown to converge on a common region of the *MDR1* promoter referred to as the "*MDR1* enhanceosome" (Jin and Scotto 1998). This region contains an inverted CCAAT and a GC-rich element, which serve as binding sites for the trimeric transcription factor NF-Y and the Sp family of GC-binding transcription factors. Upon DNA binding, these transcription factors recruit histone-acetyl transferase PCAF to the *MDR1* promoter, resulting in regional histone modification followed by further chromatin remodeling and subsequent gene activation (Hu et al. 2000). In addition, the *MDR1* promoter has been reported as susceptible to mulitple other regulatory mechanisms: the orphan nuclear receptor (SXR/PXR), steroid receptor, and the xenobiotic receptor may directly bind and activate the promoter (Cerveny et al. 2007, Song et al. 2004, Synold et al. 2001). Similarly, HIF-1α activates the *MDR1* promoter through a

consensus-binding sequence (5'-GCGTG-3') (Comerford et al. 2002). GC-binding protein Sp1 may be involved in the hypoxic response. Mutant p53 proteins have been shown to transactivate the *MDR1* promoter (Sampath et al. 2001). It has been shown that random chromosomal rearrangements could trap *MDR1* in close proximity to a constitutively active heterologous promoter, resulting in aberrant activation of the *MDR1* gene in drug-resistant cell lines and in a subset of drug refractory acute lymphocytic leukemias (Huff et al. 2006).

MDR1 expression can also be regulated at the epigenetic level. Methylation of the *MDR1* promoter has been shown to silence basal *MDR1* expression in cell line models and clinical samples (El-Osta et al. 2002, Harikrishnan et al. 2005). It has been suggested that the upregulation of *MDR1* expression in cancer cells following chemotherapy treatment may in some settings be attributable to a loss of *MDR1* promoter methylation (Nakayama et al. 1998, Tada et al. 2000). In related studies, drug-induced upregulation of *MDR1* was associated with increase in histone H3 acteylation and induction of methylated H3K4 within discrete regions of the *MDR1* locus (Baker et al. 2005). More recently, the posttranscriptional regulation of *MDR1* has been reported. In a drug-resistant variant of K562 cells, the presence of an additional 5' untranslated fragment in the *MDR1* mRNA was shown to greatly improve the translational efficiency of the mRNA, leading to overexpression of Pgp (Gomez-Martinez et al. 2007). Another study demonstrated that two microRNAs miR-27a and miR-451 are involved in the activation of *MDR1* expression and the multidrug-resistance phenotype (Zhu et al. 2008). MicroRNAs are known to have the ability to reduce protein expression by causing translational inhibition or degradation of the target mRNA. The effects of the microRNAs appear to be indirect and are likely mediated through inhibiting expression of some transcriptional factors involved in suppressing *MDR1* activation.

The human *MRP1* gene maps to chromosome 16p13.11–p13.12 and comprises 31 exons, spanning 200 kb. *MRP1* was found to be amplified in various anti-tumor drug-resistant cell lines (Cole et al. 1992, Paul et al. 1996), but its increased transcription can also occur without gene amplification. The basal promoter activity was localized to a GC-rich region of the *MRP1* gene between nucleotides –91 to +103. The binding of Sp1 to some putative binding sites in this GC-rich region was shown to be important for optimal *MRP1* transcriptional activity (Zhu and Center 1994, 1996). Although the *MRP1* promoter does not contain a p53 consensus sequence, it has been shown that wild type p53 may repress *MRP1* by reducing the binding of Sp1 (Wang and Beck 1998). A putative AP1 site at the *MRP1* promoter that can be bound by a complex containing c-*jun* and *jun*D is also important for *MRP1* transcription (Kurz et al. 2001). Decreased expression of *MRP1* has been reported in cells transfected with mutant c-*jun* (Cripe et al. 2002).

ABCG2 is constitutively expressed in many normal tissues, including liver, placenta, brain, hematopoietic stem cells, and putatively other types of stem cells. The human *ABCG2* gene is located on chromosome 4q21–4q22, and it extends over 66 kb, containing 16 exons and 15 introns. The molecular mechanisms regulating *ABCG2* expression are not well understood. The *ABCG2* promoter lacks a consensus TATA box found on many protein-encoding genes and instead contains numerous

Sp1, AP1, AP2 sites and a CCAAT box, presumably important for initiation of transcription. A basal *ABCG2* promoter has been identified at about 312 bp directly upstream from the transcriptional start site (Bailey-Dell et al. 2001). *ABCG2* has a putative CpG island located within its promoter. To date, most studies examining the regulation of *ABCG2* under physiological conditions are focused at the transcriptional level. Two functional *cis* elements in the *ABCG2* promoter, namely the estrogen and hypoxia (Krishnamurthy et al. 2004) response elements, and a peroxisome proliferator-activated receptor γ (PPARγ) response element, upstream of the *ABCG2* gene (Szatmari et al. 2006), have been identified and validated by electrophoretic mobility shift analysis. Ebert and colleagues proposed that aryl hydrocarbon receptor (AhR) upregulates *ABCG2* expression, but the responsive element has not been identified (Ebert et al. 2005). Conflicting data have been reported for the effect of the sex hormones estrogen, progesterone, and testosterone on *ABCG2* expression (Imai et al. 2005, Yasuda et al. 2005). In particular, the effect of estradiol on the *ABCG2* expression appears cell dependent and controversial (Imai et al. 2005). *ABCG2* expression is upregulated in the mammary gland during lactation (Jonker et al. 2005).

Overexpression of the *ABCG2* gene is frequently observed in cancer cell lines selected with a number of chemotherapeutic drugs (Doyle et al. 1998, Maliepaard et al. 1999, Miyake et al. 1999, Robey et al. 2001, Volk et al. 2002). Gene amplification and chromosome translocation have been shown to play a role for the increased expression of *ABCG2* (Knutsen et al. 2000). The use of alternative 5' promoters at the *ABCG2* gene due to differential expression of splice variants at the 5'-untranslated region (5'UTR) of *ABCG2* mRNA in drug-selected cells may offer another novel mechanism of *ABCG2* upregulation (Nakanishi et al. 2006), a finding similar to observations in *MDR-1* where rearrangement of the 5' region of *MDR-1* resulted in capture of that gene by another promoter (Huff et al. 2005).

ABCG2 has been shown to be under control of a number of epigenetic mechanisms. DNA methylation can repress *ABCG2* expression in human renal carcinoma (To et al. 2006) and multiple myeloma cell lines (Turner et al. 2006). Recently, we reported that overexpression of *ABCG2* in resistant cells was correlated with increased binding of a set of permissive histone modification marks, RNA polymerase II, and a chromatin remodeling factor Brg-1, but decreased association of a repressive histone mark, class I HDACs and Sp1 with the proximal *ABCG2* promoter (To et al. 2008). Enhanced mRNA stability and protein translation are also involved in the overexpression of *ABCG2* in drug-resistant cancer cells. *ABCG2* mRNA is less stable in a number of sensitive parental cancer cell lines than in their drug-selected and *ABCG2*-overexpressed resistant counterparts (To et al. 2008). Interestingly, the 3'-untranslated region (3'UTR) of the *ABCG2* mRNA was found to be longer in the parental cells than in the resistant cells. We identified a microRNA binding site (hsa-miR-519c), in the portion of 3'UTR missing from *ABCG2* mRNA in the resistant cells. In resistant S1M180 cells, hsa-miR-519c cannot bind to *ABCG2* mRNA because of the shorter 3'UTR and thus mRNA

degradation and/or repression on protein translation are relieved, contributing to overexpression of *ABCG2* (To et al. 2008) .

Significance of MDR-ABC Transporters in Cancer

Effective treatment of metastatic cancer requires the use of toxic chemotherapy. In most cases, multiple drugs are used, since resistance to single agents occurs almost universally. In vitro, the three MDR-ABC transporters efflux a broad range of chemotherapeutics used clinically for first- and second-line treatment of cancer. In that setting, inhibitors can sensitize drug-resistant cell lines to known substrates (see Table 1.2), yet despite the wealth of information collected in the laboratories, translation of this knowledge from the bench to the bedside has proved to be unexpectedly difficult. The clinical relevance of Pgp is proven by correlative studies evaluating the effect of Pgp expression on patient survival. It remains to be seen whether clinical trials designed to evaluate transporter inhibition will translate into improved patient survival.

Table 1.2 Inhibitors of MDR-ABC transporters (Adapted from, Szakacs et al. 2006)

		ABCB1	ABCC1	ABCG2
1st generation	Amiodarone	■		
	Cyclosporin A	■	■	
	Quinidine	■	■	
	Quinine	■	■	
	Verapamil	■	■	
	Nifedipine	■		
2nd generation	Dexniguldipine	■		
	PSC833	■		
	VX-710 (Biricodar)	■	■	■
3rd generation	GF120918 (Elacridar)	■		■
	LY475776	■	■	
	LY335979 (Zosuquidar)	■		
	XR-9576 (Tariquidar)	■		■
	V-104	■	■	
	R101933 (Laniquidar)	■		
	Disulfiram	■		
	CBT-1	■	■	
Other	Flavonoids		■	
	FTC			■
	Progesteron			
	MK571		■	
	Pluronic L61	■		■
	Campothecin	■		

Association of MDR-ABC Transporters with Treatment Failure

Anticancer drugs fail to kill cancer cells for a variety of reasons. To discern the contribution of individual factors, systemic studies using large cohorts of patients must be performed. To prove the association of a biomarker with poor prognosis, the studies must be evaluated in a strictly standardized fashion. Unfortunately, the scientific community working on MDR-ABC transporters has failed to define and embrace universally accepted guidelines for clinical validation. To date, differences in assay protocols result in confusing data that are often impossible to compare across institutions. As a result, the role of ABC transporters in clinical anticancer resistance has been difficult to assess. Evidence linking Pgp expression with poor clinical outcome is conclusive for breast cancer, sarcoma, and certain types of leukemia, suggesting that Pgp contributes to treatment failure of these malignancies (Szakacs et al. 2006). Importantly, over one-third of leukemic samples are found positive for Pgp expression. Pgp expression in patients with acute myelogenous leukemia (AML) has consistently been associated with reduced chemotherapy response rates and poor survival, and it was found to be an independent prognostic variable for induction failure in adult AML (Pallis and Russell 2004, van der Holt et al. 2005).

The prognostic significance of the other two MDR-ABC transporters in clinical MDR is still unknown. MRP1 is not a significant factor in drug resistance in AML (Leith et al. 1999), and its prognostic implication in chronic lymphocytic and promyelocytic leukemia, non-small cell lung cancer (NSCLC), and breast cancer remains controversial (Michieli et al. 2000, Linenberger 2005, Filipits et al. 2005). Even less is known clinically about ABCG2, as most of the studies have been performed on drug-selected cell lines. According to the analysis of blast cells from acute leukemia patients, higher ABCG2 expression is associated with poor response to chemotherapy (Benderra et al. 2004, Steinbach et al. 2002, Suvannasankha et al. 2004, Suvannasankha, Minderman et al. 2004, Uggla et al. 2005). Like adult stem cells, cancer stem cells express high levels of ABC transporters, including Pgp and ABCG2. According to the cancer stem cell model, this population of drug-resistant pluripotent cells defies treatment and serves as an unrestricted reservoir for drug-resistant tumor relapse (Dean et al. 2005).

Overcoming MDR with Inhibitors

The activity of MDR-ABC transporters can be readily inhibited by a plethora of compounds (see Table 1.2). Due to the promiscuity of the transporters, it has been relatively easy to find high affinity substrates that block transport. In vitro, sensitization of resistant cells is achievable through the prevention of the efflux of cytotoxic drugs (Dano 1973), suggesting that inhibitors would prove effective in a therapeutic setting (Tsuruo et al. 1981). Emerging data from knockout mice studies (loss of mdr1a and mdr1b does not result in an obvious phenotype) were also

interpreted to suggest that pharmacological modulation of human Pgp is a feasible strategy to treat multidrug-resistant cancer. Over the years, the pharmaceutical industry has invested considerably into clinical trials. The first phase I and II clinical trials tested the clinical potential of "first-generation" Pgp inhibitors, including verapamil, quinine, and cyclosporine A, which were already FDA approved for other medical purposes. In general, these compounds were ineffective or toxic at the doses required to attenuate Pgp function. Accordingly, most of the trials conducted with first-generation inhibitors failed to show improvement of the outcome, and toxic side effects were common (see Table 1.3) (Daenen et al. 2004). Thus, the ultimate goal of the trials was not met, and it became evident that inhibition of ABC transporters had far-reaching pharmacokinetic consequences influencing drug clearance and metabolism. Despite these failures, the few positive outcomes suggested that successful Pgp modulation is feasible (Wattel et al. 1999). The hope of conquering MDR through the inhibition of ABC transporter-mediated drug efflux encouraged further development of second-generation inhibitors. Much interest was focused on cyclosporine A (CsA), which proved to be beneficial in a randomized phase III clinical trial conducted with poor-risk acute myeloid leukemia patients treated with cytarabine and daunorubicin (List et al. 2001). Second-generation inhibitors were synthesized around first-generation pharmacophores. The primary aim was to increase the affinity to Pgp while reducing dose-limiting toxicities. PSC-833 (Valspodar), a second-generation cyclosporine D analog lacking immunosuppressive effects, has been tested most frequently in clinical trials (see Table 1.3). While PSC-833 is a high-affinity Pgp inhibitor, devoid of immunosuppressive effects, its use was associated with unwanted pharmacokinetic interactions. As a result of the inhibition of "physiological Pgp" in pharmacological barriers, PSC-833 decreased the systemic clearance of anticancer drugs, thus increasing exposure to normal and malignant cells. Pgp inhibitors, with a drug-binding site similar to that of cytochrome 3A (CYP3A), also interfered with CYP3A-mediated intestinal or liver metabolism, further reducing drug clearance. Taken together, these factors increased the severity and/or incidence of adverse effects associated with the anticancer therapy. To accommodate expected elevations in systemic drug exposure, the chemotherapy regimens were modified empirically. As a result, some patients were undertreated, while others were overdosed. Related to these problems, a phase III trial using PSC-833 in previously untreated AML patients over age 60 was closed early due to excessive mortality during induction in the experimental arm (Baer et al. 2002) (see Table 1.3). A subsequent dose-escalation trial of 410 AML patients under age 60 revealed an overall survival advantage in an unplanned subset of patients younger than 45 years old when given PSC833 with variable doses of daunorubicin, etoposide, and fixed doses of cytarabine (Kolitz et al. 2004). That apparent benefit has not been duplicated, nor is it likely to be, as development of PSC-833 has been discontinued. Similarly, active development of another second-generation inhibitor showing initial promise (VX-710, Biricodar) has been curtailed (Goldman 2003).

Third-generation inhibitors are products of combinatorial chemistry programs designed specifically for the development of compounds showing high transporter

Table 1.3 Characteristics and results of complete and ongoing phase III clinical trials with ABC transporter inhibitors (Adapted from, Szakacs et al. 2006)

	Modulator	Cancer type	Anticancer drugs	Outcome	References
1st generation	Quinidine	Breast	Epirubicin	No benefit	Wishart et al. (1994)
	Quinine	High-risk MDS	Mitoxantrone, cytarabine	Improved O.S. in Pgp + patients	Wattel et al. (1998) and Wattel et al. (1999)
	Quinine	Poor-risk acute leukemia	Mitoxantrone, Ara-C	No benefit	Solary et al. (1996)
	Quinine	De novo AML	Idarubicine, cytarabine, mitoxantrone	Significant improvement of the CR rate in Pgp-positive patients. No O.S. advantage	Solary et al. (2003)
	Verapamil	NSCLC	Vindesine, Ifosfamide	Improved O.S.	Millward et al. (1993)
	Verapamil	SCLC	CAVE	No benefit	Milroy (1993)
	Verapamil	Myeloma	VAD	No benefit	Dalton et al. (1995)
	Verapamil	Breast	Vindesine, 5FU	Improved O.S & R.R.	Belpomme et al. (2000)
	Megestrol acetate	SCLC	CAV/EP	No benefit	Wood et al. (1998)
	Cyclosporine	Relapsed and refractory AML	ADE (cytarabine, daunorubicin, etoposide)	No benefit	Liu Yin et al. (2001)
	Cyclosporine	Poor-risk AML, RAEB-t	Daunorubicin, cytarabine	Improved O.S. in CsA group	List et al. (2001)
	Cyclosporine	Myeloma	VAD	No benefit	Sonneveld et al. (2001)
2nd generation	PSC-833	AML	Daunorubicin, Ara-C, etoposide	No benefit	van der Holt et al. (2005)
	PSC-833	AML	Mitoxantrone, etoposide, Ara-C	No benefit	Sonneveld et al. (2001)
	PSC-833	Untreated AML	Daunorubicin, etoposide, cytarabine	Term. early 2° toxicity	Baer et al. (2002)
	PSC-833	Untreated AML	Daunorubicin, etoposide, cytarabine	No O.S. advantage age > 45, survival benefit $<$ age 45	Kolitz et al. (2004)
	PSC-833	Ovarian	Carboplatin, paclitaxel	No benefit	Joly (2002)
	PSC-833	Refractory AML, high-risk MDS	Mitoxantrone, etoposide, cytarabine	No benefit	Greenberg et al. (2004)
	PSC-833	NSCLC	Carboplatin, paclitaxel	Term. early 2° toxicity	Novartis
	PSC-833	AML	IL-2	Results pending	Cancer.gov
3rd generation	LY335979	AML, MDS	Daunarubicin, Ara-C	Results pending	Cancer.gov

affinity and low PK interaction. Clinical trials conducted with Pgp inhibitors indicated that the "ultimate inhibitor" was to be efficient, devoid of unrelated pharmacological effects such as pharmacokinetic interactions with the concomitantly administered drugs or CYP interaction. Indeed, inhibition of CYP3A, responsible for many adverse PK effects with previous generation inhibitors, has generally been avoided with the third-generation of inhibitors, including laniquidar (R101933), OC144-093 (ONT-093), zosuquidar (LY335979), elacridar (GF-120918), CBT-1, and tariquidar (XR9576) (Rumpold et al. 2005). Tariquidar (XR9576) has the added benefit of extended Pgp inhibition, as a single intravenous dose inhibited efflux of rhodamine from CD56+ cells (biomarker lymphoid cells that express Pgp) for at least 48 hours (Stewart et al. 2000). In 2002, phase III clinical trials began using tariquidar as an adjunctive treatment in combination with first-line chemotherapy for patients with non-small-cell lung cancer (NSCLC). Despite the promising characteristics mentioned above, the studies were stopped early because of toxicities associated with the cytotoxic drugs. This study also illustrates a defect in experimental design, since there is no strong evidence to suggest that NSCLC expresses Pgp to a significant extent. Also, the combination chemotherapy was administered at a dose higher than the maximum tolerated dose in combination trials (Fox and Bates 2007). Following the review of the aborted trials, the National Cancer Institute has commenced further exploratory phase I/II and phase III studies with tariquidar. Zosuquidar (LY335979) has recently been evaluated in patients with AML. Preliminary analysis indicates that zosuquidar may be safely given without chemotherapy dose reductions; trial endpoints have not yet been analyzed (Szakacs et al. 2006).

Emerging Role of MDR-ABC Transporters in Resistance Against Targeted Agents

The era of novel drug targets was supposed to leave chemotherapy behind, and with it, multidrug resistance. It was thought that the targeted agents, and especially those with vascular targets, would be so specific that resistance would not develop. While the introduction of targeted agents has had a marked impact on outcomes in patients, the disappointing fact is that most of these therapies improve progression-free survival but induce no cures. As a highly effective therapy for chronic myelogenous leukemia (CML), imatinib confers a complete hematologic response in 95% of patients and a complete cytogenetic response in 74% of patients – but, by 5 years, 20% of patients will have experienced disease progression (Moen et al. 2007). Several mechanisms mediating imatinib resistance have been identified (such as the amplification of the *bcr-abl* gene, the overexpression of the Bcr-Abl protein, and compensatory activation of the Src kinases); the most frequent mechanism seems to be the appearance of point mutations in the kinase domain of Abl. However, mutations are found in only 45% of patients with refractory CML, and only 26% in early chronic phase (Apperley 2007). Furthermore, when these mutations have been studied in vitro for their ability to confer resistance to imatinib, many were found

to confer only limited resistance (Apperley 2007). For example, while the most commonly reported mutation, T315I, confers a 23-fold resistance (as compared to the wild type), the third most commonly reported mutation confers only 2-fold resistance in biological assays. Furthermore, increasing the imatinib dose results in major cytogenetic response and complete hematologic response in 27% and 55% of patients, respectively (Kantarjian et al. 2007). These observations, together with data showing that though levels of imatinib are correlated with depth of response, are consistent with the notion that exposure to imatinib is important (Picard et al. 2007). These results, together with laboratory studies showing unequivocally that imatinib is a substrate for transport by both Pgp and ABCG2 (Rumpold et al. 2005, Brendel et al. 2007), suggest a hypothesis wherein the drug transporters, Pgp and ABCG2, could be important in drug resistance in CML. Oral absorption occurs because imatinib inhibits the transporters at the high local concentrations present in the GI tract. At the lower concentrations surrounding the tumor, however, a drug transporter could reduce intracellular concentrations just enough to allow a weak mutation to confer drug resistance. Unfortunately, systematic studies evaluating MDR1 expression in CML have not been performed.

Conclusion

The exclusive focus on mutations in imatinib resistance reflects a recent bias that drug transporters are not important in clinical oncology. Rather, the story should show that drug resistance is complex, and that a single mechanism is unlikely to be exclusive, even in targeted therapy. A large number of studies have identified Pgp as a negative prognostic factor, particularly in acute myelogenous leukemia; such studies have not been done in CML. For any cancer, studies with ABCG2 have only just been initiated. The question is not whether ABC transporters can confer drug resistance, but rather in what disease and whether that drug resistance can be blocked in the clinic. The strategy of Pgp inhibition failed primarily because of the inadequate trial designs and the lack of proper target validation. An analysis of the literature clearly demonstrated serious flaws in the phase III clinical trial designs (Szakacs et al. 2006). Numerous studies were carried out with inferior agents, and few studies were actually been performed with the more potent, nontoxic agents that we now have available. Results from improved phase III trials using third-generation inhibitors will be pivotal in determining whether inhibition of Pgp, or other ABC transporters, can result in improved patient survival. The improved clinical trials should be based on a standardized system used to determine whether a tumor expresses the ABC transporter of interest. Using a proper, quantitative laboratory diagnostic method, an adequate transporter expression and/or function should be measured as a criterion for trial enrollment (the beneficial effect of transporter inhibition will likely be confined to patients “positive” for the transporter target). An improvement of therapy outcome is expected only if the chemotherapeutic regimens involve transported substrates. Additionally, chemotherapy–inhibitor

combinations should be used at concentrations previously proven safe and effective in phase I/II trials, taking into account potential PK interactions with either the parental drug compound or its metabolites. Drug pharmacokinetics and early signs of hepatic, neurological, or bone marrow toxicity should be monitored closely. To ensure abrogation of the MDR phenotype, surrogate assays should be performed to assess the effect of the inhibitor in each patient. This may either be done ex vivo, by using flow cytometry to measure Pgp function in CD56+ cells taken from patients treated with inhibitors, or in vivo using 99mTc-sestamibi (Agrawal et al. 2003) or other imaging modalities to directly image accumulation of Pgp substrates within tumors.

With an ever-expanding list of drugs in the anticancer armamentarium, there is more reason than ever to develop methods of detecting Pgp-mediated or transporter-mediated drug resistance. Sensitive imaging modalities are needed to teach us the scope of the problem. In 2008, roughly 40% of the patients diagnosed with cancer in the United States are destined to die of their disease. Virtually, all of these deaths represent a failure of systemic therapy. We no longer think of resistance as solely due to Pgp or any other drug transporter as we may have in the 1980s; nor should its presence be ignored. We know that every other physiologic cell survival mechanism can be conscripted to cause drug resistance in cancer – such as DNA repair or anti-apoptotic mechanisms – and there is no reason to think that drug transporters would not as well be exploited by cancer cells.

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