

Special Section on Transporters in Toxicity and Disease—Minireview

Inhibition of the Multidrug Resistance P-Glycoprotein: Time for a Change of Strategy?

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ABSTRACT

P-glycoprotein (P-gp) is a key player in the multidrug-resistant phenotype in cancer. The protein confers resistance by mediating the ATP-dependent efflux of an astonishing array of anticancer drugs. Its broad specificity has been the subject of numerous attempts to inhibit the protein and restore the efficacy of anticancer drugs. The general strategy has been to develop compounds that either compete with anticancer drugs for transport or act as direct inhibitors of P-gp. Despite considerable *in vitro* success, there are no compounds currently available to “block” P-gp-mediated resistance in the clinic. The failure may be attributed to toxicity, adverse drug

interaction, and numerous pharmacokinetic issues. This review provides a description of several alternative approaches to overcome the activity of P-gp in drug-resistant cells. These include 1) drugs that specifically target resistant cells, 2) novel nanotechnologies to provide high-dose, targeted delivery of anticancer drugs, 3) compounds that interfere with nongenomic transfer of resistance, and 4) approaches to reduce the expression of P-gp within tumors. Such approaches have been developed through the pursuit of greater understanding of resistance mediators such as P-gp, and they show considerable potential for further application.

Introduction

The “permeability glycoprotein” or P-glycoprotein (P-gp or ABCB1) was discovered in 1976 in rodent cells known to display reduced sensitivity to anticancer drugs (Juliano and Ling, 1976). It was soon demonstrated that selection of cultured cancer cell lines in chemotherapeutic drugs displayed a phenotype consistent with the presence of P-gp. Moreover, these drug-resistant cell lines displayed resistance to a large number of chemically, structurally, and functionally unrelated drugs; hence the moniker of “multidrug resistance” (MDR). By the 1980s, antibodies had been developed to P-gp, and it was revealed that the protein was expressed in many distinct types of cancer as well as numerous normal tissues (Kartner et al., 1985; Cordon-Cardo et al., 1989, 1990).

The overexpression of P-gp in cancer was either an inherent or acquired process: the former, a reflection of its physiologic expression, and the latter, generated by the presence of anticancer drugs. P-gp confers resistance by preventing sufficient accumulation of anticancer drugs within the cell, thereby avoiding their cytotoxic or apoptotic effects. This is achieved by its ability to mediate ATP-dependent drug translocation across the plasma membrane against considerable concentration gradients. P-gp is a member of the B-class of the eukaryotic

ATP binding cassette (ABC) superfamily of transporters. Its influence in conferring MDR was at one time considered the paramount factor in the phenotype (Steinbach and Legrand, 2007). However, the burgeoning biochemical characterization of cancer cells revealed that the protein is a member of a network of cellular factors or tissue features that produce drug resistance (Mellor and Callaghan, 2008). The influence of P-gp was apparently further diluted by the discovery of two other ABC proteins able to confer MDR, namely, MRP1 (ABCC1) and BCRP (ABCG2) (Cole et al., 1992; Doyle et al., 1998). It is worth noting that although all three mediate active drug extrusion, their substrate specificities and expression patterns in cancer are distinct but with some overlap. The present review will focus on the role of P-gp and attempts to overcome its unwanted influence in cancer.

The multiplicity of factors contributing to drug resistance and the inability to overcome the actions of P-gp and restore the sensitivity of chemotherapy have led to researchers questioning its very involvement in clinical resistance (Bradshaw and Arceci, 1998; Merino et al., 2004; Perez-Tomas, 2006). This clear overreaction should be tempered by the plethora of investigations that have described the association of P-gp with drug resistance and the positive relationship between expression and poor prognosis (Gottesman et al., 2002; Leonard et al., 2003; Modok et al., 2006; Shaffer et al., 2012). The present review will not further discuss the relative merit or influence of P-gp in drug resistance but concentrate on efforts to overcome its actions.

Originally, it was thought that the actions of P-gp were limited to conferring resistance to classic genotoxic anticancer drugs, such as

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ABBREVIATIONS: ABC, ATP binding cassette; CHO, Chinese hamster ovary; MDR, multidrug resistance; MP, microparticle; P450, cytochrome P450; PEG, polyethylene glycol; P-gp, P-glycoprotein; PKC, protein kinase C.

vinblastine, doxorubicin, and paclitaxel. The broad or polyspecificity of P-gp is legendary (or infamous), and the list of compounds known to interact with this transporter is well in excess of 300 (Wang et al., 2011; Chen et al., 2012). It is apparent that many of the much touted “new generation” anticancer compounds (e.g., kinase inhibitors) are also substrates for transport by P-gp (Hegedus et al., 2002; Wang and Fu, 2010). There is a clear need to generate compounds, or strategies, to overcome the actions of P-gp in 1) limiting the effectiveness of chemotherapy in cancer and 2) influencing the pharmacokinetic profile of a vast number of clinically prescribed drugs.

Overcoming Drug Resistance to Chemotherapy Caused by P-gp

The general strategy to overcome multidrug resistance has been to coadminister chemical inhibitors of P-gp with anticancer drugs. Inhibition of P-gp would thereby lead to increased accumulation of anticancer drug within the cell and produce cell cytotoxicity. Alternatively, addition of a P-gp substrate in conjunction with the anticancer drug would achieve a similar effect by competing for the transport process. The first inhibitor (or more correctly referred to as a P-gp modulator) identified was the L-type calcium channel blocker verapamil (Tsuruo et al., 1982, 1983). This drug was shown to circumvent MDR using a variety of cell cytotoxicity, transport, binding, and photolabeling assays (Cornwell et al., 1987; Safa, 1988). However, clinical trials with verapamil were beset by serious cardiac side effects, and the compound was removed as a viable option (Ozols et al., 1987; Dalton et al., 1989). Verapamil inhibits L-type calcium channels with picomolar affinity; however, the potency to block P-gp was in the micromolar range, a 10^6 higher concentration.

In the ensuing 30 years, three distinct generations of P-gp modulator have been produced, and unfortunately a clinically useable inhibitor remains elusive (McHugh and Callaghan, 2008; Crowley et al., 2010). The first generation of compound used drugs with established and unrelated pharmacological actions and relied on the polyspecificity of P-gp. The next generation employed chemically modified first generation inhibitors. Ideally, these compounds were devoid of the parent compound's activity while retaining (or improving) the potency of interaction with P-gp. The third generation produced inhibitors from de novo synthesis using a variety of combinatorial chemistry approaches and benefiting from the burgeoning database of structure-activity relationships for drug-P-gp interaction.

A large number of compounds have been examined, and several recurring themes have emerged from their preclinical and clinical characterization. The first two generations were beset with poor potency of the compounds and a number of “off-target effects” that resulted in problems with toxicity (Gottesman et al., 2002; McHugh and Callaghan, 2008).

The third generation of P-gp modulators was frequently associated with adverse drug reactions that necessitated a reduction in the dose of anticancer agents. Many of the adverse drug interactions are related to the overlapping substrate specificity between P-gp and the drug metabolizing enzyme cytochrome P450 (CYP3A4 isoform) (Wacher et al., 1995; Yu, 1999). The combined effects of these two “defender proteins” will influence the absorption, distribution, metabolism, and elimination of a large proportion of known medications. Therefore, the coadministration of two drugs (i.e., modulator and anticancer drug) that interact with both P-gp and CYP3A4 often generates unpredictable toxicity related to the emergence of unwanted pharmacokinetic parameters. Such issues with pharmacokinetic profiles have resulted in the apparent downfall of potent P-gp inhibitors, such as Tariquidar (Pusztai et al., 2005). The fall from grace of this leading P-gp inhibitor led to considerable pessimism regarding the validity of utilizing efflux

pump inhibition as a means to overcome drug resistance. However, more recent observations have demonstrated that the coadministration of Tariquidar with Vinorelbine (Abraham et al., 2009) did not produce a similar toxicity profile as demonstrated with doxorubicin/docetaxel (Pusztai et al., 2005). Moreover, a structure-activity investigation has produced derivatives of Tariquidar with near negligible ability to interact with CYP3A4, while retaining P-gp inhibition (Labrie et al., 2007). These observations provide an argument for the retention of this strategy.

The presence of “endogenous or physiological” P-gp has also become problematic to the chemical inhibition strategy. P-gp is expressed at barrier tissue to sanctuary sites (e.g., blood-brain barrier) and at secretory/absorptive tissues (e.g., gastrointestinal tract) (Cordon-Cardo et al., 1989, 1990). The protein acts as a cellular defender and is involved in establishing the overall pharmacokinetic profile for numerous drugs. Unfortunately, chemical inhibitors are not capable of discriminating between P-gp expressed at normal sites in the body and that found in cancerous tissue. Therefore, the strategy generates unwanted side effects at a number of nontumor sites in the body, for example, increased permeability at the blood-brain barrier. Increased penetration, or distribution, of genotoxic anticancer drugs into the brain is associated with a severe toxicity profile that necessitates dose reduction.

Novel Approaches to Overcoming the Actions of P-gp

The preceding sections indicated a significant role for P-gp in conferring MDR to genotoxic anticancer drugs and many novel, target specific cytostatic compounds. The controversy surrounding the quantitative extent of its role in solid tumors cannot overshadow its involvement. Moreover, the importance of chemotherapy as a primary, adjuvant, or palliative treatment in cancer remains considerable and thus justifies the need to overcome, or evade, the influence of P-gp.

Unfortunately, the strategy of developing potent and selective compounds to inhibit P-gp and overcome resistance has encountered a number of issues. The issues relate to toxicity, dose reduction of anticancer drugs, and perturbation of key barrier tissues. In recent years there is a general pessimism by the pharmaceutical sector and research funding bodies despite the consistent and demonstrable improvements in design of inhibitors. There are also numerous epiphenomena related to P-gp expression that offer the prospect of therapeutic intervention. In this review we briefly describe some of the novel approaches rooted in academic research to counter, or overcome, the phenomenon of P-gp-mediated drug resistance.

Collateral Sensitivity

The emergence of a drug-resistant phenotype may be considered the product of the influence of a number of environmental stress factors. Typically, this will involve altered patterns of gene expression to enable survival of the cancer cell. A large number of cultured cell lines have been rendered drug resistant by prolonged exposure to certain anticancer drugs, and they undoubtedly contain modified expression patterns for numerous resistance mediators or enablers. The advent of high-throughput proteomic analyses revealed that drug-resistant cells or tumors display considerably modified protein expression profiles (Righetti et al., 2005; Peng et al., 2010). For example, in a taxol-resistant A2780 ovarian cancer cell line, proteomic analysis revealed marked changes in stress response effectors, cell cycle, and apoptotic mediators and numerous alterations in pathways for bioenergetic metabolism (Cicchillitti et al., 2009). Any proteomic alterations that are specific to drug-resistant cancer cells may, therefore, provide a

target for potential therapeutic management. In acquiring a drug-resistant phenotype, cancer cells may unwittingly proffer targets for their own eradication.

Increased or even hypersensitivity of resistant cells to various drugs was first observed in bacterial cells in the early 1950s (Szybalski and Bryson, 1952). The phenomenon was referred to as collateral sensitivity and was promoted a decade later for improving the efficacy of anticancer drugs in combination chemotherapy (Paigen, 1962). In the 1980s a multitude of studies demonstrated improved efficacy of methotrexate (Herman et al., 1979), folate analogs (Diddens et al., 1983), and DNA alkylating drugs (Sladek et al., 1985) in the presence of collateral sensitizing compounds. Unfortunately, the majority of these studies were not able to provide a description of the underlying resistant phenotype. A study in 1985 (Gupta, 1985) demonstrated collateral sensitivity between a spectrum of anticancer drugs in Chinese hamster ovary (CHO) cells that were eventually shown to express high levels of P-gp. Subsequently, in 1987, Cano-Gauci and Riordan provided the first demonstration that P-gp modulators (i.e., calcium channel blockers) preferentially inhibited the growth of drug-resistant cell lines.

It appears that the phenomenon of collateral sensitivity is closely associated with all three of the multidrug ABC pumps expressed in cancer cells (Oguro et al., 1990; Jensen et al., 1992). The precise mechanism underlying collateral sensitivity remains unresolved, with many hypotheses presented. As efforts in the 1990s to purify functional P-gp began in earnest it was widely demonstrated that resistant cells were more susceptible to procedures (e.g., nitrogen cavitation, shear force) or compounds (e.g., surfactants and ionophores), leading to physical disruption of the plasma membrane (Bech-Hansen et al., 1976; Callaghan and Riordan, 1995). It was proposed that high expression of this large polytopic membrane protein conferred greater fragility to the host cell membrane. However, the vast majority of observations were gleaned from cell lines selected for drug resistance and therefore likely to display numerous functional alterations. In addition, the issue of whether the membrane biophysical changes were a cause or effect of the resistant phenotype remained unclear as did the relationship to collateral sensitivity.

Observations that metabolic inhibitors also imparted collateral sensitivity suggested that bioenergetic metabolism in resistant cells may provide another therapeutic target (Ferretti et al., 1993; Bentley et al., 1996; Goda et al., 2002). The metabolic adaptations observed in cancer cells (e.g., Warburg effect) are now considered a hallmark feature of oncogenic transformation (Gillies and Gatenby, 2007; Zhao et al., 2011). Multidrug efflux pumps such as P-gp mediate an ATP-dependent transport process and sustain a high rate of ATP hydrolysis in the presence of substrate. Cancer cells have modified their metabolic profile to ensure sufficient anabolism to generate biomass for proliferation. This is achieved by a dampening of mitochondrial oxidative phosphorylation, with a heavy reliance on glycolysis despite its lower yield of energy (Bui and Thompson, 2006; Gillies and Gatenby, 2007). Thus, resistant cancer cells, with ATP-consuming efflux pumps, may conceivably be more susceptible to conditions of low energy status.

A significant proportion of established collateral sensitivity agents is known to be a substrate for transport by P-gp or stimulate its rate of ATP hydrolysis. A study by Broxterman et al. (1988) demonstrated that the collateral sensitizing drug verapamil significantly depleted ATP levels in a drug-resistant cell line. It has been proposed that depletion of ATP necessitates increased rates of oxidative phosphorylation in drug-resistant cells and that this oxidative stress results in an increased production of reactive oxygen species (e.g., O_2^- , H_2O_2). Increased reactive oxygen species and

concomitant reductions in the antioxidant glutathione were associated with apoptosis in drug-resistant CHO cells (Karwatsky et al., 2003). Conversely, compounds that are known to inhibit ATPase activity of P-gp (e.g., Tariquidar and PSC833) can diminish the effectiveness of collateral sensitivity agents. In addition, the extent of collateral sensitivity is proportional to the expression levels of P-gp, which may also reflect the strain on ATP levels (Goldsborough et al., 2011). More systematic characterization of ATP levels and metabolic fuel utilization in drug-resistant cells is clearly warranted.

A number of compounds have been demonstrated to elicit collateral sensitivity and include Tiopronin (Goldsborough et al., 2011), Desmosumotin (Nakagawa-Goto et al., 2008), NSC73306 (Hall et al., 2011), Dp44mT (Whitnall et al., 2006), and sigma-2 receptor agonists (Niso et al., 2013). These compounds impart collateral sensitivity; however, their mechanism remains unclear to date. Although these compounds are too preliminary for clinical application, they provide proof-of-principle that targeting this metabolic “weakness” in drug-resistant cells may provide an important adjunct to conventional chemotherapy.

Cytotoxic Drug Delivery Using Particles or Polymers

Chemotherapy drugs, particularly the genotoxic class, are unfortunately associated with severe and dose-limiting side effects, particularly in proliferating tissues. Therefore, the emergence of a drug-resistant phenotype cannot simply be overcome with escalation of dose. As indicated in preceding sections, the strategy to overcome resistance by inhibiting the activity of efflux pumps, such as P-gp, is also fraught with danger. P-gp is expressed at numerous locations in healthy tissue, and its inhibition will effectively open sanctuary sites (e.g., central nervous system and testes) to cytotoxic anticancer drugs (Leslie et al., 2005; de Vries et al., 2007; Robillard et al., 2012; Ke et al., 2013).

The observation that encapsulating doxorubicin within liposomes could circumvent P-gp-mediated MDR (Thierry et al., 1989; Sadasivan et al., 1991) provided a degree of cautious optimism. Clearly, this novel drug delivery route was able to bypass the actions of P-gp, because liposomal doxorubicin would enter the cells after endocytic engulfment of the particles rather than diffusion through the bilayer. This observation was used to formulate the “vacuum cleaner” hypothesis for the mechanism of P-gp (Higgins and Gottesman, 1992; Ferte, 2000). Elegant studies with the fluorescent P-gp substrate calcein-AM were used to demonstrate that drugs do not need to “enter” the cytoplasm to become translocated by P-gp (Homolya et al., 1993). A mechanism of translocation whereby P-gp extracts drugs directly from the lipid milieu (Raviv et al., 1990; Homolya et al., 1993) gained general acceptance.

The liposomal delivery system may also provide a safe mechanism for significant dose escalation, because the volume of distribution for the drug will be modified by restricting diffusion out of the circulatory system. Clearance from the circulatory system was recognized early on as a limiting factor for the use of liposome-based systems. The primary issue is the ability of the reticuloendothelial system to trap and remove liposomes from the plasma, thereby considerably reducing residence time in the circulation (Oku and Namba, 1994). The incorporation of polymers, such as PEG or sialo-gangliosides, reduces trapping by the reticuloendothelial system (Oku and Namba, 1994; Gabizon and Martin, 1997). Tumors are widely accepted as a suitable target for liposomal encapsulation strategies because of their aberrant vasculature. In particular, the intratumor vasculature is leaky and thus enables high extravasation of the liposomes.

A considerable and ongoing level of interest in this strategy remains, although some refinement remains to improve particle stability and provide targeted delivery of the payload to the tumor.

Encapsulation strategies are now considerably more elaborate than the early liposomal systems. Modern delivery systems are referred to as nanoparticles or nanocarriers that feature drugs adsorbed, internalized, conjugated, or chelated to a platform (Hall et al., 2007; McNeil, 2009; Milane et al., 2011). The platforms include emulsions (e.g., PEG), liposomes, polymers, colloidal gold, and nanocrystals; moreover, they frequently contain mixtures of these. Nanoparticles improve the therapeutic index (LD_{50}/ED_{50}) by increasing bioavailability (fraction of dose reaching circulation) and reducing toxicity (Daum et al., 2012). Nanocarriers with encapsulation render the pharmacokinetic profile of a drug to be entirely dictated by properties of the platform. In addition, the high surface-to-volume ratios enable high encapsulation or surface adsorption of the payload drug.

Nanocarriers may be readily configured to contain 1) multiple payloads (e.g., drug and DNA/RNA), 2) molecules to enable tissue-specific targeting, and 3) "optical" agents for imaging and tracking. Strategies to target nanocarriers to tumors make use of the altered surface protein profile or expression levels for receptors in cancer cells. For example, folate derivatives (Yang et al., 2011), transferrin (Bellocq et al., 2003), or epidermal growth factor receptor (EGFR)-related molecules (El-Sayed et al., 2006; Magadala and Amiji, 2008) may be surface localized on nanocarriers to facilitate interaction with their respective receptor targets, which frequently display distinct expression patterns at the tumor site. The most commonly used techniques for modern imaging of nanocarriers are positron emission tomography or single photon emission computed tomography, which make use of a growing arsenal of radiolabeled compounds (Petersen et al., 2012). However, optical approaches such as fluorescence, infrared spectroscopy, and ultrasound are also widely used in experimental or clinical settings (Kozłowska et al., 2009). Imaging techniques provide measures of plasma clearance and tumor bioavailability: the latter a largely overlooked parameter in standard clinical trial design yet integral in generating an efficacious response. After internalization of the nanocarrier, the payload must be released to reach the specific intracellular target. Typically, internalized particles will enter the endosomal pathway that leads to lysosomal degradation (Riezman et al., 1997; Wattiaux et al., 2000), which may result in irretrievable transformation or sequestration of the payload. A number of strategies have been adopted, including the use of cell-penetrating peptides (Sarko et al., 2010; Choi et al., 2011) or platforms containing drug tethers that are destabilized by the acidic intratumoral microenvironment (Andreev et al., 2010; Gao et al., 2010; Du et al., 2013).

The use of nanocarriers to circumvent MDR by P-gp has adopted a number of guises. Lipid-dextran complexes as nanocarriers were shown to increase the potency of doxorubicin and suppress the expression of P-gp in resistant cells (Susa et al., 2010). The twofold effect of these nanocarriers on resistance was achieved using a payload of high doxorubicin dose and siRNA directed against the *mdr1* gene. Another study using a dual payload strategy (paclitaxel and lonidamine) also overcame the multidrug-resistant phenotype in cultured cells (Milane et al., 2011). Paclitaxel is a tubulin-disrupting anticancer drug, whereas lonidamine is a hexokinase II inhibitor that reduces glycolytic flux that produces cellular energetic stress. The platform consisted of a combination of polycaprolactone with PEG and contained an EGFR specific peptide. The latter was included to ensure targeting to drug-resistant MDR cells, which have been demonstrated to express elevated levels of EGFR.

Super-hi-magnetization nanocarriers produced with a highly magnetic Fe_3O_4 core and a poly-[*N*-(1-butyrac acid)]aniline shell have also been used to tackle P-gp-mediated MDR (Hua et al., 2011). The Fe_3O_4 core acted as a contrast agent to enable tracking of the nanocarriers using magnetic resonance imaging techniques. High doxorubicin loading was achieved (271 mg drug per mg nanocarrier) by virtue of conjugating the drug to the aqueous shell. The conjugated doxorubicin facilitated high-capacity delivery and markedly improved thermal stability of the drug. A recent investigation used platform comprising a copolymer of polystyrene oxide-polyethylene oxide to target drug-resistant cells expressing P-gp (Cambón et al., 2013a,b). Intriguingly the copolymer unimers were able to directly inhibit the activity of P-gp, and the iron oxide-titanium oxide core enabled magnetic resonance imaging tracking of the spherical nanocarriers. Doxorubicin was the primary payload and was bound to the shell via titanium oxide, which was labile in the acidic pH within a tumor microenvironment. These polymers were remarkably stable and delivered ~150% more doxorubicin than observed with drug alone.

This exciting area of research has limitless possibilities and provides the potential for high-dose delivery of anticancer drugs and P-gp inhibitors (chemical or nucleotide based). The delivery may be safely tracked using noninvasive procedures and specifically targeted to the tumor site.

Microparticles as Novel Therapeutic Targets for the Prevention of MDR

Microparticles (MPs), also known as microvesicles, are enclosed plasma membrane fragments of size 0.1–1 μm characterized with phosphatidylserine expression on their surface (Gyorgy et al., 2011). MPs are typically released by vesiculation from the plasma membrane of stimulated or preapoptotic eukaryotic cells and had long been deemed to be inert cell debris (Freyssinet, 2003; Freyssinet and Toti, 2010).

In the last decade however, increasing evidence suggests that MP plays an essential role in cell-to-cell communications, allowing for cell regulation and crosstalk without the need for direct cell-to-cell contact. MPs act as vectors disseminating different biologic information, depending on their donor cell membrane and cytoplasmic composition. It is clear that MPs are important intermediates in inflammation, coagulation, vascular homeostasis, chemotaxis, adhesiveness, and thrombogenicity of endothelial cells and cells of hematopoietic lineage (Morel et al., 2004; Hugel et al., 2005). MPs shed from cancer cells are associated with tumor angiogenesis, evasion of immunosurveillance, cell invasiveness, survival, apoptosis, chemoresistance, and the hypercoagulable state (Dvorak et al., 1983; Ginestra et al., 1997; Dolo et al., 1999; Angelucci et al., 2000; Kim et al., 2002; Morel et al., 2004; Bebawy et al., 2009; Jaiswal et al., 2012b). Moreover, higher concentrations of systemic MPs are linked to disease progression and development in cerebral malaria, sepsis, autoimmune disorders, atherosclerosis, HIV-1, diabetes, and cancer (Horstman and Ahn, 1999; Nomura et al., 2004, 2008; Combes et al., 2006; Bebawy et al., 2009; Burnier et al., 2009).

The emergence of MDR was long attributed to "genetic alterations" within the cancer cell after an initial chemotherapeutic insult resulting in alterations to the cellular apparatus for prosurvival mechanisms. Bebawy et al. (2009) first reported that MDR can also be acquired in a non-"genetic manner" through a process of cell-to-cell communication in the absence of drug exposure. MDR cancer cells were shown to spontaneously shed MPs from their surface. These MDR-MPs contain cargo from the donor-resistant cells, in particular, functional resistance

proteins, which when transferred to a drug-responsive recipient cancer cell confer MDR within 4 hours (Bebawy et al., 2009; Lu et al., 2013).

This phenomenon has since been shown to occur *in vivo* (Jaiswal et al., 2013), whereby these microparticles transfer resistance proteins deep within the tumor core within 24 hours of MP exposure. This process also serves to "retemplate" the transcriptional landscape of recipient cells and in doing so results in the selective dominance of deleterious traits within the cancer cell population (Jaiswal et al., 2012a). Furthermore, in addition to the transfer of resistance proteins mediating MDR, these microparticles effectively sequester anticancer drugs and reduce the available free drug concentration available to a tumor mass, hence constituting a parallel resistance pathway (Gong et al., 2012).

Modulation and/or inhibition of MP formation and release may provide a novel therapeutic strategy for the prevention of MDR (Roseblade et al., 2013). A range of inhibitors have demonstrated promising effects in diminishing MP production by targeting key players in the formation of MPs. These include compounds that modulate calpain activation, calcium channels, and other factors that indirectly stimulate MP production (Roseblade et al., 2013).

Other inhibitors such as ticlopidine, pantethine, cystamine, LMP-20, or ROCK inhibitors have also been shown to lower MP levels after treatment (Shouzu et al., 2004; Wassmer et al., 2005; Penet et al., 2008; Antonyak et al., 2012). Although some of these drugs are effective in reducing MP production in patients, they were not capable of bringing down MP levels similar to the healthy control involved in the same study (Nomura et al., 2005, 2007). Further investigations are required to identify more effective inhibitors of MP formation and production, because it would provide a promising treatment strategy in the management of tumor drug resistance.

Altered Expression of P-gp

The expression of P-gp in normal (i.e., noncancerous) tissues is regulated by the nuclear orphan receptors namely, the pregnane X, steroid and xenobiotic, and the constitutive androstane receptors (Geick et al., 2001; Synold et al., 2001; Xu et al., 2005). These receptors mediate induction of the protein in response to cellular needs, typically defined by the presence of potential substrates. In particular, these two receptor subtypes are targets for a number of clinically used medications as well as steroid-based metabolites. The receptors also induce expression of phase I (e.g., cytochrome P450) and phase II (e.g., glutathione transferase) metabolizing enzymes (Ekins and Erickson, 2002; Xu et al., 2005; Weiss and Haefeli, 2013), further reinforcing the functional link between the efflux pumps and biotransformation pathways.

Expression of P-gp in cancer tissue is a more complex, or perhaps a more convoluted, regulatory system. There have been a wide range of factors that regulate P-gp expression, including hypoxia (via hypoxia inducible factor 1α) (Comerford et al., 2002), metabolic acidosis (Thews et al., 2010), and metabolic perturbation such as glucose deprivation (Ledoux et al., 2003) and generation of reactive oxygen species (Wartenberg et al., 2005). Many of these factors derive from the hostile intratumor microenvironment and reflect cell or tissue stress. Moreover, the ability of anticancer drugs and radiotherapy to alter P-gp expression occurs via a cellular damage/stress response rather than a classic induction mechanism. It appears that P-gp is a key "first responder" to chemical or environmental insult on cancer tissue to remove potentially lethal compounds. After induction of cellular stress, the precise pathway to invoke induction of P-gp expression is complex and likely involves a multitude of cell signaling pathways (Callaghan et al.,

2008; Sui et al., 2012). It is for this reason that an ever increasing number of investigations have targeted specific kinases in signaling pathways with a view to modulating P-gp expression and, thus, the degree of chemoresistance.

The ability of kinases or signaling pathways to regulate P-gp function and/or expression warrants a discussion of posttranslational modifications for this transporter. The seminal manuscript that gave P-gp its name relied on detecting the protein in drug-resistant CHO cell membranes by virtue of its glycosylation status (Juliano and Ling, 1976). Further studies on its glycosylation status indicated that P-gp was synthesized as a 140-kDa precursor that was glycosylated over a 2- to 4-hour period to its fully mature form that migrated as a broad band at 180 kDa in SDS-PAGE (Richert et al., 1988; Yoshimura et al., 1989). Two investigations demonstrated that in the drug-resistant human KB cell line, P-gp was markedly stable, with a half-life greater than 24 hours (Richert et al., 1988; Yoshimura et al., 1989). In multidrug-resistant CHO cells, turnover of P-gp was considerably more rapid, with a stability half-life of 17 ± 3 hours (McClellan and Hill, 1993). More recently, it was demonstrated that expression of a P-gp-EGFP fusion protein (human P-gp isoform) in KB cells displayed a total half-life of 2.2 days and a surface stability of 3.7 days (Petritz et al., 2004). It remains unclear why the human and hamster versions of P-gp display distinct stabilities; moreover, this is not the only reported difference between the human and rodent isoforms.

It was subsequently demonstrated that the stability of P-gp was increased by 4- to 6-fold by serum deprivation or growing cells at high density (Muller et al., 1995), suggesting an involvement of growth factors in stability. Similarly, the expression of P-gp is cell cycle dependent, with greatest stability in the G0/G1 phase (Zhang and Ling, 2000).

What is the "trigger" to alter the stability of membrane-bound P-gp? The most likely candidate is the phosphorylation status, and the protein has of numerous PKC consensus motifs within in the linker region (Chen et al., 1986; Gros et al., 1986). Moreover, it has been demonstrated that P-gp is phosphorylated by PKC at several of these motifs within the linker region (Chambers et al., 1993; Orr et al., 1993). A number of studies examined the phosphorylation status of P-gp in cell lines selected for drug resistance and suggested that it played a key role in the resistant phenotype (Marsh and Center, 1987; Mellado and Horwitz, 1987; Meyers, 1989; Staats et al., 1990). Subsequently, a number of investigations focused on using chemical inhibitors of PKC isoforms to reduce the activity or expression of P-gp and restore drug sensitivity to the cells (Chambers et al., 1990, 1992; Bates et al., 1992). The inhibitors were highly successful and pointed to a regulatory role of phosphorylation for P-gp. However, two manuscripts in the mid-1990s using site-directed mutagenesis of the PKC motifs in the linker region deflated this strategy (Germann et al., 1996; Goodfellow et al., 1996). Disruption of the PKC-mediated phosphorylation status did not alter the ability of P-gp to confer drug resistance; this was despite clear evidence of an interaction between the two proteins (Yang et al., 1996). It is now recognized that the chemical inhibitors of PKC were substrates for transport by P-gp, and their effects were related to direct inhibition with anticancer drugs for transport rather than via altered expression (Sato et al., 1990; Germann et al., 1995; Smith and Zilfou, 1995).

Perhaps unsurprisingly, the strategy to alter P-gp phosphorylation status to circumvent drug resistance was discontinued. In the ensuing period a great deal of research has focused on defining the myriad signaling pathways that control cancer cell growth. Many of these pathways (e.g., Ras/MAPK, JNK, p38 MAPK, protein kinase A- and PKC-related proteins, and PI3K) involve cell proliferation status, mediate apoptotic signaling, or generate the stress response. These

TABLE 1
A selection of novel compounds found to reverse P-gp MDR by altering signal transduction

Compound	Probable Pathway or Target	Reference
SC-51089	Prostaglandin E2 receptor	Pekcec et al., 2009
PHIL-7 (idarubicin derivative)	JNK-phosphorylation	Peng et al., 2013
siRNA Bile extract	Wnt/ β -catenin	Shen et al., 2013
PEITC (phenethyl isothiocyanate)	Pi3k-Akt	Tang et al., 2013
Dioscin	NF- κ B	Wang et al., 2013
Parthenolide	NF- κ B and Hsp70	Xin et al., 2013
Procyanidin	NF- κ B and MAPK/ERK	Zhao et al., 2013

signaling pathways are known to modulate P-gp expression in cells, tumor spheroids, and animal models (Chen and Sikic, 2012; Sui et al., 2012; Breier et al., 2013). In addition, both chemical inhibitors and silencing strategies (e.g., siRNA) of enzymes within these pathways have been demonstrated to affect P-gp function. Table 1 provides a small and selected list (restricted to 2013) of signaling pathway inhibitors that alter P-gp function in drug-resistant cells.

Unfortunately, the vast majority of these studies have adopted a similar strategy to those involving PKC in the 1990s, namely that the reporter assay was based exclusively on measurement of overall cytotoxicity. On the basis of this previous experience, it may be argued that mechanistic information will be of paramount importance in the possibility of reviving this strategy to tackle MDR. Key parameters include whether 1) the phosphorylation (or other post-translational modification) status of P-gp, 2) the total cellular expression level of P-gp, or 3) the cellular localization of P-gp has been altered. It would also be informative to ascertain whether novel kinases or phosphatases directly interact with the transporter. Pharmacological investigations should address whether the chemical inhibitors of signaling pathways actually modulate P-gp function by competing with anticancer drugs for the transport process.

Differential Modulation of P-gp

At present, the common strategy of incorporating pharmacological inhibitors of P-gp in conventional treatment regimens is complicated with issues of specificity, toxicity, and differential modulation. It was shown previously that P-gp transport function, and subsequently MDR in cancer, is differentially modulated depending on the drug combination used and the site of interaction within the plasma membrane bilayer (Bebawy et al., 2001; Huang et al., 2007). Specifically, modulators appear to have a selective effect depending on the anticancer drug with which they are combined. For instance, it has been shown that verapamil reversed resistance of CEM/VLB100 cells to vinblastine and fluorescein-colchicine but not to colchicine (Bebawy et al., 2001; Huang et al., 2007). In contrast, chlorpromazine reversed resistance to vinblastine but not to fluorescein-colchicine and increased the degree of resistance to colchicine (Bebawy et al., 2001; Huang et al., 2007). Similarly, gypenoside extracts from *Gynnostemma pentaphyllum* effectively circumvent colchicine resistance by 15-fold in P-gp-overexpressed CEM/VLB100 cells, while having no effect on vinblastine and taxol resistance in the same cells (Huang et al., 2007). This concept of differential pharmacological modulation has since been proposed as a cellular mechanism potentially contributing to the emergence of pharmacoresistant schizophrenia (Bebawy and Chetty, 2008), supporting the significance and application of these early findings across different clinical conditions. This evidence provides us with a strategy for tailoring drug regimens to include MDR inhibitors according to the specific anticancer drug combinations used.

Perspectives and Summary

The vast majority of investigations aimed at overcoming drug resistance conferred by P-gp have employed the mantra of direct inhibition of the transporter. Unfortunately, this approach has not yet reached clinical success due to the complex array of drug toxicity, altered pharmacokinetics and adverse drug interactions. The current review has presented a number of alternative approaches to circumventing P-gp-mediated MDR. The novel strategies have been identified through the tireless efforts by researchers to gain a more complete understanding of this phenotype. These strategies will hopefully continue to develop and generate targeted, selective, and nontoxic strategies to eradicate the presence of MDR in cancer.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Callaghan, Luk, Bebawy.

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