

Strategies for overcoming ABC-transporters-mediated multidrug resistance (MDR) of tumor cells*

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The development of multidrug resistance (MDR) of tumors is a major cause of failure in anti-tumor chemotherapy. This type of crossresistance is due to the expression of ABC transporter glycoproteins actively effluxing the drug from the cells against the concentration gradient at the expense of metabolic energy, thus preventing the accumulation in cells of therapeutic concentration of active agents. In this review strategies for overcoming this adverse phenomenon are discussed. They comprise the control of expression of MDR glycoprotein transporters and control of the functioning of the expressed transporter proteins. The latter approach is discussed in more detail, comprising the following general strategies: (i) development of compounds that are not substrates of efflux pump(s), (ii) use of agents that inactivate (inhibit) MDR proteins, (iii) design of cytostatics characterized by fast cellular uptake, surpassing their mediated efflux, (iv) use of compounds competing with the drug for the MDR protein-mediated efflux. Positive and negative aspects of these strategies are analysed, with special attention put on strategy based on the use of MDR modulators in combination therapy, allowing the restoration of cytotoxic activity of clinical cytostatics towards resistant tumor cells.

Keywords: multidrug resistance, antitumor cytostatics, antimetabolites, modulators

Chemotherapy belongs to the most potent tools in contemporary medicine, enabling successful treatment of numerous microbial infections as well as neoplastic diseases. While the “chemotherapy era” has already brought solutions to many clinical problems, its potential is still not fully exploited and the development of new drugs, more effective and with better selectivity, is badly needed. Unfortunately, the brilliant achievements of chemotherapy have been endangered by the development of mutational or phenotypic resistance of pathogenic organisms towards applied chemotherapeutic agents, making them useless. This unfortunate phenomenon concerns all pathogens submitted to chemotherapeutic treatment, including neoplastic cells. In this review we will concentrate on the problems of resistance to anticancer drugs.

During the past five decades the development and use of anticancer agents has become one of the most important ways of controlling malignant diseases. Their action ends up with tumor cells' death by necrosis or apoptosis. However, the emergence of drug resistance has made many of the currently available chemotherapeutic agents ineffective. Consequently, the future of antitumor chemotherapy depends on the outcome of the extensive world effort aimed at the overcoming, on a rational basis, of the resistance phenomenon.

During the first decades of antitumor chemotherapy the development of resistance was, fortunately, of a specific character, addressed to particular antitumor drugs which induced a number of specific metabolic changes in tumor cells, making such drugs ineffective. These changes comprised

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Abbreviations and codes: ABC, ATP-binding cassette protein superfamily; BCRP, breast cancer resistance protein; BSO, buthionine sulfoxine; DX, doxorubicin; HL-60, human promyelocytic leukemia cell line; IDA, idarubicin; LRP, lung resistance related protein-dependent resistance; MDR, multidrug resistance; MIT, mitoxantrone; MRP, multidrug resistance-associated protein-dependent resistance; MRP1, member of MRP transporters family; ODNs, oligodeoxyribonucleotides; PDZ, patent application pending; Pgp (MDR1), P-glycoprotein; RI, resistance index (ratio of IC_{50} (resistant cells) to IC_{50} (sensitive cells) where IC_{50} is a drug concentration causing 50% of inhibition of cell growth); RNAi, RNA interference; VINC, vincristine; V_o , the initial velocity of cytostatic uptake.

mostly: membrane transport (failure of drug uptake), intracellular drug distribution (lowered drug concentration at target site), structural changes of targets (loss of drug-target affinity), enhancement of detoxification systems (stimulated drug metabolism), activation of DNA repair mechanisms, and inhibition of processes inducing apoptosis. Because of the specific character of this type of resistance, it was usually possible to circumvent it by switching from one antitumor drug to another or by appropriate chemical modification of the antitumor agent.

MULTIDRUG RESISTANCE OF TUMORS

The control of tumor drug resistance dramatically worsened due to the emergence of a novel type of resistance, discovered in 1976 by Juliano and Ling, the multidrug resistance (MDR), which became a major impediment to the success of cancer chemotherapy. MDR tumor cells are crossresistant to a broad variety of chemotherapeutic agents, even chemically and functionally unrelated ones. The MDR phenomenon occurs upon expression or overexpression of a specific group of broad substrate spectrum membrane phosphoglycoproteins, capable of actively transporting diverse chemotherapeutic agents out of the cells against the concentration gradient. These drug efflux pumps operate at the expense of metabolic energy, leading to reduced intracellular drug accumulation. Consequently, MDR tumor cells can evade the cytotoxic effect of the drugs.

The phosphoglycoprotein drug efflux pumps belong to the broader group of the ABC superfamily of membrane transporters. It should be noted that many of them are constitutively present in various normal tissues, like epithelial cells of the colon, kidney, adrenal, pancreas or liver, which substantially complicates the development of agents which would selectively counteract the MDR phenomenon. The physiological role of ABC proteins comprises active transport of endo- and xenobiotics, including detoxification processes. Consequently, tumors derived from these tissues exhibit intrinsic multidrug resistance to cytostatic agents even before chemotherapy is initiated. The MDR of tumors derived from other tissues appears phenotypically upon its induction by a cytostatic agent.

There is vast literature characterizing the chemical, biochemical and functional properties of MDR drug efflux pumps and describing their genetics. It is not the authors' aim to review this literature as we intend to concentrate on the strategies of overcoming the multidrug resistance. Therefore, in this matter, we refer the readers to selected original publications and recent review articles (Riordan & Ling, 1979; Chen *et al.*, 1986; Azzaria *et al.*, 1989; Ambudkar *et al.*, 1992; 1999; Cole *et al.*, 1992; Gottes-

man & Pastan, 1993; Muller *et al.*, 1994; Fardel *et al.*, 1996; Seelig, 1998; Dalton & Scheper, 1999; Konig *et al.*, 1999; Maliepaard *et al.*, 1999; Orłowski & Garrigos, 1999; Borst *et al.*, 2000; Litman *et al.*, 2000; 2001; Scheffer *et al.*, 2000; Dean *et al.*, 2001a; 2001b; Loo & Clarke, 2001; Gottesman, 2002; Gottesman *et al.*, 2002; Hagenbuch *et al.*, 2002; Schinkel & Jonker, 2003; Yang *et al.*, 2003; Haimour *et al.*, 2004). However, we think it useful to shortly summarize the current status of knowledge on the phosphoglycoprotein drug efflux pumps.

The most studied, best characterized, and of clinical importance is the first discovered multidrug transporter, named P-glycoprotein and abbreviated as Pgp or MDR1 protein, encoded by the *MDR1* gene (Chen *et al.*, 1986; Gottesman & Pastan, 1993; Ambudkar *et al.*, 1999). The 170-kDa P-glycoprotein has an overall molecular architecture similar to that of all ABC transporters. The Pgp molecule is composed of two halves, each consisting of transmembrane α -helices and the cytoplasmic ATP-binding domain. The two halves are joined by a single polypeptide chain. The protein molecule contains also the substrate(s) binding domain(s). The transmembrane regions form the drug translocating pathway (Loo & Clarke, 2001), while the ATP-binding sites, exhibiting ATPase activity, provide the metabolic energy upon ATP hydrolysis enabling the active drug efflux (Azzaria *et al.*, 1989; Ambudkar *et al.*, 1992). ATP hydrolysis induces, after binding of the substrate, conformation changes of the protein molecule, expelling out of the cell the substrate(s) (Gottesman & Pastan, 1993). Although the crystal structure has been elucidated only for an ABC transporter protein of bacterial origin, nevertheless it helped to construct a structural model for the open conformation of the MDR1 P-glycoprotein, contributing essentially to better understanding of the molecular mechanism of transporter functioning (Seigneuret & Garnier-Suillerot, 2003). The broad substrate spectrum of Pgp comprises preferably neutral or cationic amphiphilic organic compounds. The recognition of multiple substrates is based on their particular structural features (Seelig, 1998). Of importance are the number, strength and spacial location of electron-donating groups for hydrogen bonding with hydrogen donors from defined amino-acid sequence in P-glycoprotein. The structure-activity relationships were concerning the substrate-Pgp interaction also studied by QSAR analysis (Litman *et al.*, 1997a). The Pgp transporter is intrinsically present in the intestine, liver, kidney and blood-brain barrier.

Other ABC multidrug transporters comprise MRP and BCRP proteins (Schinkel & Jonker, 2003). The most studied of them and of growing clinical importance are the MRP transporters. They constitute a family of, up to now, nine isoformic members (Konig *et al.*, 1999; Borst *et al.*, 2000; Yabuuchi

et al., 2001; Gottesman, 2002; Haimeur *et al.*, 2004). Individual proteins are numbered successively. The most studied one and well characterized is MRP1, a 190-kDa protein. The presence in normal tissues of MRP transporters is quite widespread. Members of the MRP family are structurally and functionally similar to Pgp, however, they differ from the latter as concerns their direct substrates. The substrate spectrum of cytostatic agents is similar to that of Pgp but MRPs, being organic anion transporters, mediate the efflux of these drugs mainly after formation of anionic drug conjugates with glutathione, glucuronate or sulfate, upon the action of appropriate transferases. The most important and studied are the S-conjugates with glutathione (Cole *et al.*, 1992; Muller *et al.*, 1994; Jedlitschky *et al.*, 1996). MRP proteins can also mediate drug efflux by their cotransport with glutathione (Renes *et al.*, 1999).

The BCRP is 95-kDa phosphoglycoprotein drug transporter with a somewhat different structure. Unlike Pgp and MRP it contains only one transmembrane and one ATP binding domain. Therefore it is also called a "half transporter" (Doyle *et al.*, 1998). Its drug substrate spectrum and mechanism of drug transport are quite similar to those of Pgp.

In the recent years another type of drug transporter has been recognized which is not associated with the cytoplasmic membrane, but operates by controlling the drug transport from the nucleus to the cytoplasm *via* vaults. This is a 110-kDa vault protein, the lung resistance protein (LRP) (Izquierdo *et al.*, 1996; Dalton & Scheper, 1999; Scheffer *et al.*, 2000). Its drug substrate spectrum is similar to that of Pgp.

STRATEGIES TO OVERCOME MDR IN CANCER CELLS

The design of nontoxic agents that would overcome the MDR of tumors has been a challenging area for pharmaceutical development. This world-wide effort combines various strategies, based on diverse mechanisms and effects. These strategies use two general approaches: (i) rational design of agents that retain their cytostatic activity towards MDR tumor cells, and (ii) development of augmenting compounds able to restore the cytotoxicity of available antitumor drugs against resistant cells. The latter approach is aimed at interfering with either the expression of the transporter proteins or their functioning.

CONTROL OF EXPRESSION OF MDR PROTEINS

Most of the information available concerns the *MDR1* gene. Since its amplification is not a prerequi-

site for Pgp-related resistance in human tumor cells (Ohishi *et al.*, 2002), the potential targets of gene expression inhibition can be either RNA or protein.

The literature on the structure, function and expression of the *MDR1* gene was recently reviewed (Scotto & Johnson, 2001). Its promoter activity is up-regulated by various stimuli, including cytostatics. However, of interest for the MDR problem is the possibility of down-regulation of MDR gene expression.

Relevant studies have not yet reached the level of clinical importance; however, some interesting and promising observations have been made that point to some novel possibilities of interfering with the transporter protein expression.

An *MDR1* specific polypeptide transcriptional repressor has been screened out (Bartsevich & Juliano, 2000). Modification of *MDR1* promoter region was achieved by 5-azacytidine (Efferth *et al.*, 2001). In consequence, the transcriptional activity was inhibited, and resistant K562 cells were transformed to a non-MDR type. Interesting results were obtained with ecteinascidin 743, an isoquinoline derivative of marine origin (D'Incalci, 1998). This cytostatic agent of high activity selectively inhibits the activation of induced *MDR1* gene blocking its transcription, without affecting constitutive *MDR1* expression (Jin *et al.*, 2000; Scotto & Johnson, 2001). Another example of targeting an *MDR1*-specific transcription factor is HMN-176, an active metabolite of the antitumor agent HMN-214, a stilbazole derivative (Tanaka *et al.*, 2003). Its action restores the chemosensitivity of MDR cells. Attention should also be drawn to the interesting results obtained with a designed transcriptional repressor targeted to the *MDR1* promoter, whose action results in the inhibition of *MDR1* gene transcription (Xu *et al.*, 2002). The authors also reviewed the alternative therapeutic strategy based on selective inhibition of Pgp expression by targeting *MDR1*-specific transcription factors.

A lot of attention has been paid to the control of MDR protein expression at the level of interference with mRNA by the antisense strategy. The formation of duplexes with the target mRNA by complementary oligodeoxyribonucleotides (ODNs) causes interruption of translation and transformation of resistant tumor cells to the non-MDR type. The major problems concerning the application of antisense strategy comprise the specificity of the ODNs for the chosen RNA target, the susceptibility to blood serum nucleases, and cellular uptake with the application of appropriate vectors (reviewed in: Crooke, 1992; Walder & Walder, 1988; Garcia-Chaumont *et al.* 2000). Although the usefulness of the antisense strategy for the reduction of expression of MDR proteins has not been confirmed in animal experiments yet, the accumulated knowledge (Cucco & Calabretta, 1996; Stewart *et al.*, 1996; Alahari *et al.*,

1998; Gao *et al.*, 1998; Volm, 1998; Astriab-Fisher *et al.*, 2000; 2002; 2004; Pakunlu *et al.*, 2003; Kang *et al.*, 2004; Matsumoto *et al.*, 2004) and patent literature (Ojima *et al.*, 1998) on this matter point to the potential applicability of the antisense strategy approach in reversing MDR.

Another means of affecting a target mRNA is to use catalytic RNA, a ribozyme (Phylactou, 2001). Ribozymes are small RNA molecules (30–40 nucleotides) that hybridize to a complementary sequence of mRNA and catalyze site-specific cleavage of the substrate. Anti-MDR ribozymes, introduced into the cells, can reverse MDR by cleavage of MDR1 and MRP mRNA (Masuda *et al.*, 1998; Kobayashi *et al.*, 1999; Wang *et al.*, 1999; Nagata *et al.*, 2002; Wang *et al.*, 2003).

Effects similar to the action of ribozymes in reversing MDR were obtained by the RNA interference (RNAi) approach, named also post-transcriptional gene silencing, induced by siRNA. RNAi is a conserved biological response to double-stranded RNA, which results in sequence-specific gene silencing (Hannon, 2002). The introduction of an MDR1-targeted small (21 nucleotides) siRNA duplex into MDR cancer cells inhibits specifically the expression of MDR1 by causing degradation of the complementary MDR1 mRNA (Wu *et al.*, 2003). Gene silencing induced by RNA interference has been shown to be specific and potent. siRNAs target the expression of the genes from which the siRNA sequences are derived without detectable effects on the expression of unrelated genes (Fire *et al.*, 1998; Elbashir *et al.*, 2001). Thus the RNAi approach may offer an alternative strategy for overcoming MDR, especially so because of the high selectivity, although its use is limited as the silencing effect on Pgp expression is short-lived, not exceeding 24 h (Hannon, 2002). Because the half-life of Pgp is 14–17 h (Muller *et al.*, 1995), a greater decrease in Pgp expression may be attained through the use of a DNA vector-based siRNA expression system (Paddison *et al.*, 2002; Sui *et al.*, 2002).

Expression of MDR genes is governed by appropriate signals. Inhibition of signal transduction can thus be another potential approach to reverse the MDR phenotype. At the moment not much is known about this matter, however, some interesting information concerning signals for Pgp induction is available (Yang *et al.*, 2001).

The posttranslational modifications of ABC transporter proteins may provide an alternative group of potential targets for MDR reversal. The protein modifications comprise N-glycosylation and phosphorylation. Pgp is N-glycosylated at one position on the first extracellular loop of the C-terminal. Although the use of a glycosylation inhibitor (tunicamycin) and of mutant MDR cells has shown that glycosylation of Pgp is not a prerequisite for its multidrug transport function (Endicott & Ling, 1989;

Ichikawa *et al.*, 1991; Gottesman & Pastan, 1993; Schinkel *et al.*, 1993), it has been suggested that N-glycosylation may contribute to the correct folding, proper routing or stabilization of the Pgp molecule. Perhaps a better perspective for drug development may be provided by inhibitors of protein phosphorylation (Glazer, 1998). Pgp can be phosphorylated by a variety of serine/threonine kinases, such as protein kinase C (PKC), cAMP-dependent protein kinases, and calmodulin-dependent protein kinases (reviewed in: Ford, 1996; Volm, 1998; Idriss *et al.*, 2000; Szabo *et al.*, 2000; Wiese & Pajeva, 2001). Phosphorylation occurs at the cytoplasmic linker region joining the halves of the Pgp molecule. This last step in posttranslational modification is indispensable for the transporter activity of Pgp, thus making protein kinases potential targets for MDR reversal. However, there is a serious problem with the selectivity of kinase inhibitors (Gupta *et al.*, 1996; Wielinga *et al.*, 1997; Castro *et al.*, 1999; Conseil *et al.*, 2001). Also the present knowledge concerning the role of the different phosphorylation states for the functioning of Pgp is rather limited. Inhibitors of PKC, such as H-87, staurosporine and their derivatives are able to reverse MDR effectively (Sato *et al.*, 1990). Simultaneously many compounds that can modulate MDR have also been shown to inhibit PKC (Aftab *et al.*, 1991). Perhaps selective inhibition of Pgp phosphorylation will be more likely with the use of some peptides able to selectively inhibit PKC- α (Gupta *et al.*, 1996). These peptides contain a sequence corresponding to the pseudosubstrate region of PKC- α , and consequently are able to inhibit this enzyme. The phosphorylation step in the expression of active MDR transporters also concerns the MRP1 protein (Sato *et al.*, 1990). Protein kinase inhibitors reduce the phosphorylation of this transporter and inhibit its activity (Gekeler *et al.*, 1995; Ma *et al.*, 1995).

OVERCOMING THE FUNCTIONING OF MDR MEMBRANE TRANSPORTER PROTEINS

There are several general strategies to circumvent the drug efflux action of expressed MDR transporter proteins: (i) development of compounds that are not substrates of the efflux pump(s), (ii) use of agents that inactivate (inhibit) MDR proteins, (iii) design of cytostatics characterized by fast cellular uptake, surpassing their MDR-mediated efflux, (iv) use of compounds competing with a drug for the MDR protein mediated efflux. Some additional specific strategies have also been applied for overcoming MRP and LRP resistance. The reduction of MRP resistance can be also achieved by affecting the intracellular formation of anionic drug conjugates. The formation of such conjugates with glutathione can be indirectly inhibited by buthionine sulfoximine (BSO),

a potent inhibitor of glutathione synthesis (Griffith & Meister, 1979), or by the inhibition of glutathione reductase by *N,N*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) (Vanhoefer *et al.*, 1997). Inhibition of drug transport from the nucleus to the cytoplasm, mediated by LRP protein, was achieved with the PAK-104P inhibitor, a pyridine derivative (Kitazono *et al.*, 2001).

The final effects of the general strategies illustrated in Fig. 1 comprise: drug influx with no or reduced efflux (strategies (i) and (ii), Fig. 1A), drug influx surpassing its efflux (strategy (iii), Fig. 1B), and competitive inhibition of drug efflux (strategy (iv), Fig. 1C). All these effects lead to the retaining

in MDR tumor cells of a therapeutic concentration of the drug.

Strategies (ii) and (iv) are of the combination therapy type requiring the use of more than one active agent: the cytostatic drug and an augmenting agent. Regardless of the type of action of the latter ones (reversible, irreversible, competitive, noncompetitive), these compounds bear interchangeable names: MDR-modulators, inhibitors, chemosensitizers, reversing agents. This is quite misleading, as these names do not refer directly to the mechanism of MDR overcoming.

It is important to note that the use of MDR modulators, albeit beneficial, could also have undesirable side effects *in vivo* because the ABC proteins are expressed also in normal tissues, such as the adrenal, gravid uterus, kidney, liver, colon and capillary and the capillary endothelial cells in the brain (Thiebaut *et al.*, 1987; Ambudkar *et al.*, 1992; Tatsuta *et al.*, 1992). MDR modulators could block their normal physiological function. Additionally, such chemosensitizers could cause increased toxic effects by inhibiting the efflux of the cytotoxic agent mediated by the ABC proteins in normal tissues. Therefore in designing all types of MDR-reversing agents selectivity of their action is of paramount importance.

Regarding these obstacles the nonsubstrate strategy has an advantage of not disturbing the physiological functioning of the ABC proteins in normal tissues. However, this strategy has strong limitations, as MDR proteins exhibit a very broad substrate spectrum comprising many groups of chemicals and it is very difficult to identify nonsubstrate ones. Nevertheless some such compounds have been found in random testing. Although they represent diverse chemical types, attempts have been made to find a rationale for their structural and physicochemical characteristics (Litman *et al.*, 1997a; Seelig, 1998). Some examples of more important cytostatics which are not recognized as substrates by MDR pumps comprise: paclitaxel – not a substrate of MRP1 (Huang *et al.*, 1997), lamellarin D – not a Pgp substrate (Vanhuyse *et al.*, 2005), epothilone B – not recognized by either Pgp or MRP (Chou *et al.*, 1998), and camptothecin derivatives – poor substrates for BCRP (Perego *et al.*, 2001). A systematic search for “non-substrate” compounds yielded several other products (Gottesman *et al.*, 2002). Special consideration should be given to MRP non-substrates. Such compounds are very rarely found in comparison with non-substrate of other MDR transporters. Also the mechanism of overcoming MRP by this type of agents has not been unambiguously elucidated yet. Unlike with other transporters the substrate properties concerning MRP should be considered regarding two processes: transferase reactions generating the anionic drug conjugates and their binding to the MRP transporter.

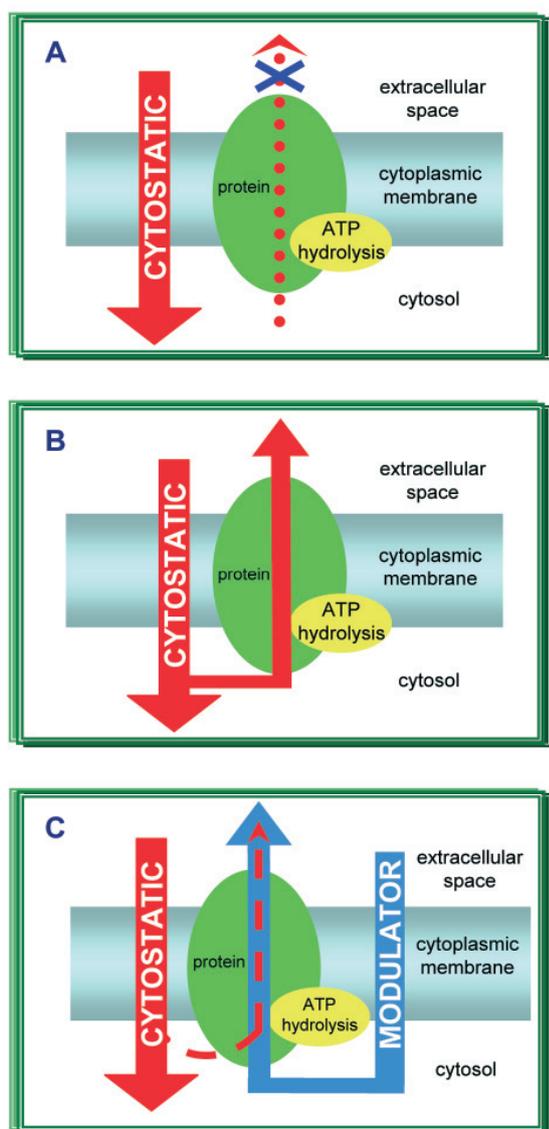


Figure 1. Three types of drug cellular migration events in overcoming MDR.

A, influx without mediated efflux; B, influx surpassing mediated efflux; C, competitive modulator action. Borowski E, Bontemps-Gracz MM, Piwowska A (2004) *Biotechnology International Review Conference*, November 14–18, 2004, Vienna, Austria. Abstracts, Abstract C-10.

The only broader group of MDR non-substrates comprises antimetabolites. Although these cytostatics are considered as MDR-overcoming drugs, the reasoning behind this classification has not been clearly defined. MDR transporters, albeit of a very broad substrate spectrum, recognize cellular components and metabolites indispensable for the cell function as endobiotics, the compounds which should not be effluxed. Thus antimetabolite, as close metabolite analogues mimicking the endobiotic cellular components, should not be recognized by the transporters as xenobiotics.

There is little literature dealing with the mechanism of overcoming MDR by antimetabolites. It has been evidenced that 5-fluorouracil (Zeng *et al.*, 2004; Jin *et al.*, 2005), 5-fluorouridine (Zeng *et al.*, 2004), gemcitabine (Bergman *et al.*, 2001), cytarabine (Hait *et al.*, 1993; Bergman *et al.*, 2001; Lofgren *et al.*, 2004); 6-thioguanine (Hait *et al.*, 1993), and methotrexate (Litman *et al.*, 2001), are not substrates of Pgp. Cytarabine is not a substrate of the BCRP transporter either (Stam *et al.*, 2004). Dideoxynucleosides are not recognized by Pgp in kidney transport (Lung & Bendayan, 1999). It is unfortunate that some antimetabolites, such as 6-methylguanidine, 3-deazauridine, tiazofurine, while retaining their cytotoxicity towards cells expressing ABC proteins, behave in part as xenobiotics so that they induce the overexpression of genes encoding these proteins, thus increasing resistance to other antitumor drugs (Lu *et al.*, 2002). Metabolic modification of antimetabolites can affect their substrate properties for MDR transporters. Methotrexate is a good substrate for the MRP protein in short treatment, but on prolonged exposure to the drug polyglutamylation occurs and the modified drug is no more effluxed (Jansen *et al.*, 1999; Kruh *et al.*, 2001). On the other hand, cytarabine is not cytotoxic for MRP expressing cells as it is effluxed as a conjugate formed with glutathione. Preincubation of cells with BSO, an inhibitor of glutathione synthesis, restores cytarabine cytotoxicity (Takemura *et al.*, 2001).

It is unfortunate that for this important group of MDR-overcoming antitumor drugs no systematic studies have been performed concerning the role of structural factors in their substrate properties for various MDR transporters. We have shown that antimetabolites which structurally differ considerably from their natural equivalents are, to a lesser or greater extent, recognizable by MDR transporters and are effluxed (see legend to Fig. 1: Borowski *et al.*, 2004). Some results obtained by other authors can be interpreted in the same way. The deeply modified folic acid analogues ZD1694 and GW1843 are good substrates for MRP proteins (Hooijberg *et al.*, 1999). The same effect was also observed with modified nucleoside analogues which were effluxed by MRP4 and MRP5 (Borst *et al.*, 2000).

Beside the "non substrates" concept and inhibition of formation of anionic conjugates with MRP substrates, either by the inhibition of glutathione synthesis or by affecting the activity of appropriate transferases, the final effect of drug influx without its efflux can be also achieved by inhibition or inactivation of the MDR transporter proteins. This approach comprises allosteric inhibition and irreversible (covalent) or reversible inactivation of transporter proteins. Most of the accumulated knowledge on this matter concerns the Pgp transporter.

The substrate-drug binding behavior of Pgp is complex, with several binding sites involved. They comprise transport sites, where translocation of the drug substrate across the lipid bilayer can occur, and regulatory sites which modulate Pgp function. Pgp modulators fall into two types: those that are transported and those that are not. Competitive and noncompetitive kinetics are observed for different modulators. Modulators which bind to Pgp but failing transport prevent the transport of other agents (Dey *et al.*, 1997; Litman *et al.*, 1997b; Scala *et al.*, 1997; Martin *et al.*, 2000).

Allosteric mechanism of inhibition of Pgp has been shown for several modulators (Maki *et al.*, 2003). One of them is *cis*-(Z)-flupentixol, a thioxanthene derivative. This compound does not interfere with substrate recognition by Pgp, but prevents substrate translocation and dissociation by allosteric modulation of human Pgp. Several anthranilic acid derivatives modulate Pgp-mediated transport by an allosteric effect on substrate recognition or ATP hydrolysis (Martin *et al.*, 1997; 1999). Similarly, indolizin sulfone SR33557 affects substrate binding to Pgp through interaction with a site other than the substrate recognition one (Martin *et al.*, 1997). Some other modulators like dexniguldipine and prenylamine inhibit interaction of other drugs with Pgp by a noncompetitive mechanism (Ferry *et al.*, 1995; Boer *et al.*, 1996). An interesting group of Pgp non-transportable inhibitors are flavonoids. These compounds interact with cytosolic domains of Pgp and its ATP binding sites, inhibiting the ATPase activity of this protein (Conseil *et al.*, 1998; Choi *et al.*, 2004).

Several covalent inhibitors of Pgp have been studied. Some ATP analogues, including photoaffinity labels, block the nucleotide binding domains and inhibit the ATPase activity of Pgp. The photoaffinity analogues react with a lysine residue in the nucleotide binding domains. There is some chance for selectivity in the inhibition of ATP hydrolysis because the catalytic sites in Pgp are conformationally flexible and of relatively low affinity and specificity compared to other transport ATPases (Al-Shawi *et al.*, 1994). Interesting results were obtained with a non-nucleotide type covalent inhibitor of Pgp ATPase activity, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). The inhibition occurs already after the inhibitor

binds to the ATP-binding catalytic site in a 1:1 molar ratio, indicating that the inactivation of only one of the two Pgp nucleotide binding sites is sufficient for complete inactivation. This is understandable, as both ATP-binding sites are indispensable for the transport activity due to the alternating cooperative supply of energy by both sites (Al-Shawi & Senior, 1993; Senior & Bhagat, 1998; Senior *et al.*, 1998).

The "non substrate strategy" in overcoming MDR is of interest also because it enables the advantageous monotherapy treatment, not requiring the use of any augmenting agents. The same desirable effect can be also obtained by the design of cytostatics with a cellular uptake faster than their mediated efflux (strategy (iii), Fig. 1B). Drug uptake can occur *via*: (1) diffusion, e.g., doxorubicin, (2) mediated transport, e.g., nucleoside analogues, (3) endocytosis, e.g., immunotoxins (Gottesman, 2002). The most desirable and most frequent is drug uptake by simple diffusion across the lipidic cytoplasmic membrane. The kinetics of diffusion is linearly concentration dependent. On the other hand, the MDR transporters mediated drug efflux is not linear and is of saturation kinetics type. Thus compounds characterised by very fast influx, faster than their mediated efflux at the transporters' saturation point, could be retained in cells in desirable concentration. It has been generally accepted that the lipophilicity of drugs is a factor favoring their uptake by diffusion. However, the increase of lipophilicity of cytostatics by appropriate chemical modification is not sufficient to achieve the goal. In our studies we have shown that in drug migration across the membrane not only the lipophilic-

ity, but to a great extent also structural factors as such are of essential importance. We have identified such factors in two important groups of cytostatics, namely anthraquinone and acridine compounds (Stefanska *et al.*, 1999). According to our concept, the inclusion into their molecules of one or two fused five- or six-membered heterocyclic ring(s) makes these molecules able to overcome MDR. This is an indispensable qualitative factor, while the modification of lipophilicity of such compounds by appropriate substituents can further quantitatively influence their activity towards MDR cells. This hypothesis has led to the development of several novel groups of active polycyclic anthraquinone and acridine analogues (Fig. 2 and references there). It has been evidenced that polycyclic compounds of such type overcome MDR due to the speed of their uptake surpassing the rate of MDR transporters-mediated efflux (Tkaczyk-Gobis *et al.*, 2001; Tarasiuk *et al.*, 2002; 2004; Fig 1: Borowski *et al.*, 2004). The cytotoxic activity of representative compounds (Fig 1: Borowski *et al.*, 2004) from several groups of polycyclic anthraquinone and acridine analogues towards Pgp and MRP cell lines is presented in Fig. 3. It should be noted that the compounds examined have very favorable resistance indexes in both types of resistant cells, as compared to those of the reference compounds doxorubicin and mitoxantrone. As exemplified in Fig. 4A the initial velocities of uptake and cellular accumulation of a tetracyclic anthraquinone analogue in sensitive cells are much better than in the case of the reference tricyclic idarubicin. An exemplary experiment with a pentacyclic acridine ana-

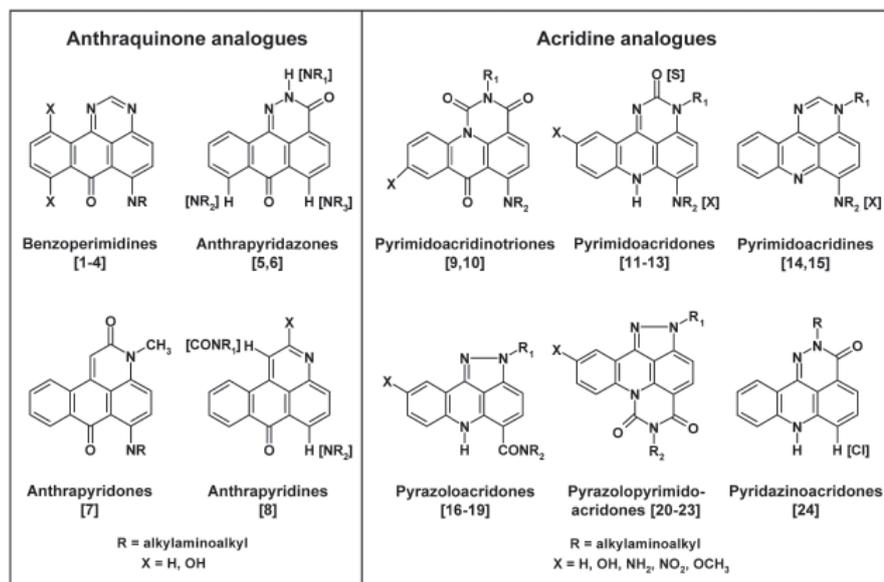


Figure 2. The structures of MDR-overcoming anthraquinone and acridine compounds.

[1-4] Stefanska *et al.*, 1993; 1999; Tkaczyk-Gobis *et al.*, 2001; Dzieduszycka *et al.*, 2002; [5, 6] Stefanska *et al.*, 2003; Fig 1: Borowski *et al.*, 2004; [7] Tarasiuk *et al.*, 2002; [8] Dzieduszycka *et al.*, 2005; [9,10] Antonini *et al.*, 1995; 1999a; [11-13] Antonini *et al.*, 1996; 1999b; Fig 1: Borowski *et al.*, 2004; [14, 15] Antonini *et al.*, 1999b; Fig 1: Borowski *et al.*, 2004; [16-19] Antonini *et al.*, 2001; Bontemps-Gracz *et al.*, 2002; Tarasiuk *et al.*, 2004; Fig 1: Borowski *et al.*, 2004; [20-23] Antonini *et al.*, 2002; Bontemps-Gracz *et al.*, 2002; Tarasiuk *et al.*, 2004; Fig 1: Borowski *et al.*, 2004; [24] Stefanska *et al.*, 2005.

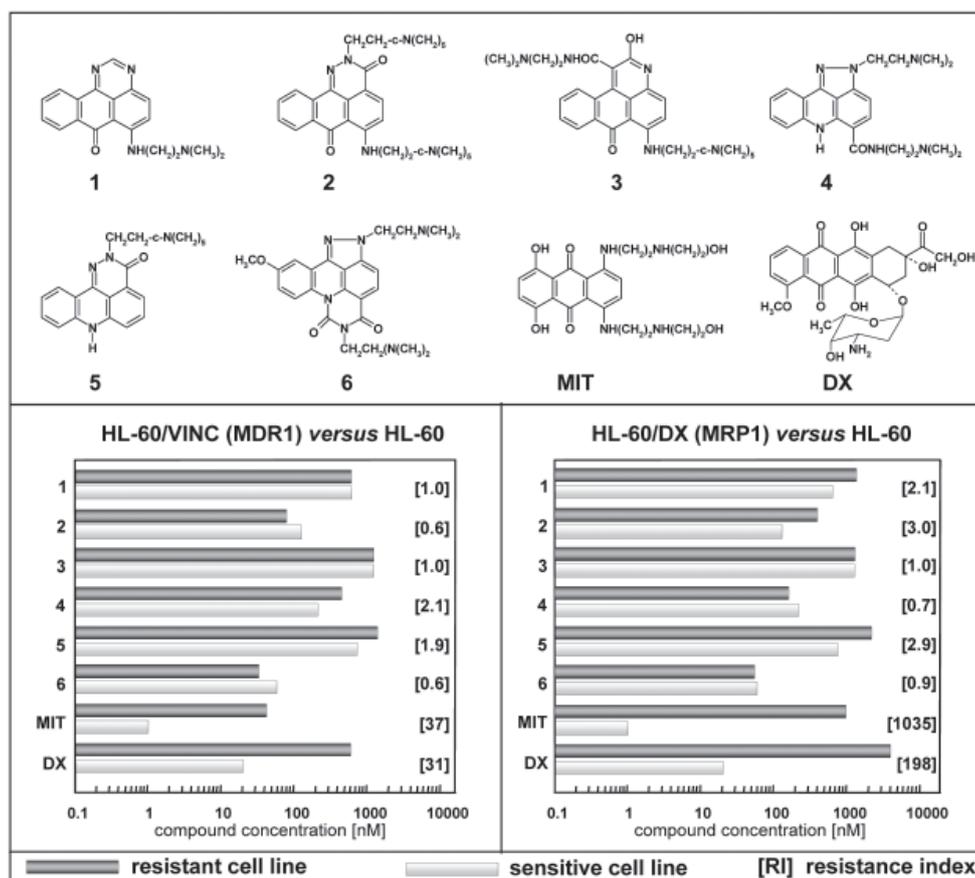


Figure 3. Resistance indexes for representative anthraquinone and acridine compounds (Fig. 1: Borowski *et al.*, 2004). The cytotoxic activity *in vitro* was determined by cell counting or by protein content of the cells (Bontemps-Gracz *et al.*, 1991) after 72 h of continuous exposure to the cytostatic.

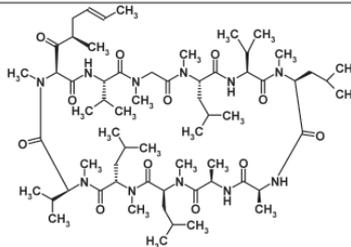
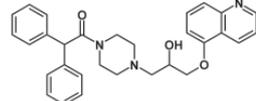
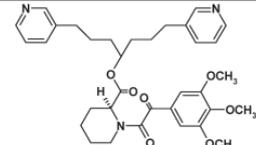
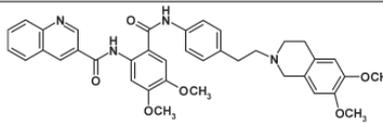
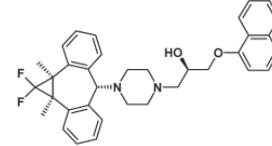
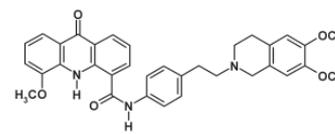
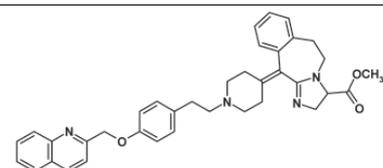
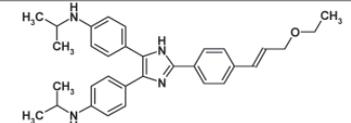
logue (Fig. 4B) shows that its uptake characteristics are practically identical in sensitive as well as in Pgp- and MRP1-expressing cells.

Competitive MDR modulators are the most studied and most advanced in the design of agents of potential importance, essentially contributing to overcoming multidrug resistance of tumors. Being MDR pumps' substrates these compounds compete with cytostatics for the MDR transporter proteins, thus inhibiting the efflux of the latter (Fig. 1C). This strategy was established following the pioneering studies of Tsuruo and coworkers in 1981 and the discovery of the activity of the first competitive MDR modulator verapamil. Since then, abundant literature has become available describing numerous competitive MDR modulators, to a lesser or greater extent restoring the cytotoxic activity of cytostatics against MDR tumor cells. Active agents belong to very diverse chemical groups and exhibit varied properties in respect to their selectivity for various target ABC transporters and undesirable side-effects. The abundance of this literature prompts us to refer the readers to selected more recent review (Volm, 1998; Krishna & Mayer, 2000; 2001; Lehne, 2000; Dantzig *et al.*, 2001; Avendano & Menendez, 2002; Liscovitch & Lavie, 2002; Teodori *et al.*, 2002; Kawase & Moto-

hashi, 2003; Robert & Jarry, 2003; Kellen, 2003; Lee, 2004). The development of these agents was aided by the application of the combinatorial chemistry approach, identification of physiochemical requirements, SAR analysis and QSAR methods (Zamora *et al.*, 1988; Ojima *et al.*, 1998; Kim, 2001; Wiese & Pajeva, 2001). The development of active chemical compounds as MDR modulators was accompanied by extensive biological, biomedical and pharmacological studies, as well as clinical trials (reviewed in Sandor *et al.*, 1998; Ambudkar *et al.*, 1999; Tan *et al.*, 2000; van Zuylen *et al.*, 2000; Toppmeyer *et al.*, 2002; Dantzig *et al.*, 2003; Goldman, 2003; Mizuno *et al.*, 2003; Thomas & Coley, 2003).

Regarding the positive features and the negative side-effects of the examined compounds, three generations of MDR modulators have been distinguished. Compounds discovered by random screening constitute first generation agents (e.g., cyclosporin A, verapamil). They exhibit poor modulatory activity and unacceptable toxicity when used in higher doses (Krisha & Meyer, 2000; Schinkel & Jonker, 2003). Many of them are also substrates for other ABC transporters and enzyme systems, resulting in unpredictable pharmacokinetic interactions in the presence of chemotherapeutic agents.

Table 1. MDR modulators of 2nd and 3rd generation in clinical trials

Modulator	Structure	Target	Trials phase	Reference
PSC833 Valspodar (cyclosporin D derivative)		Pgp	III	Liscovitch & Lavie, 2002; Robert & Jarry, 2003; Teodori <i>et al.</i> , 2002; Thomas & Coley, 2003, and reference cited therein; Minderman <i>et al.</i> , 2004; http://clinicaltrials.gov ; www.ontogen.com ; www.biospace.com
MS209 (quinoline derivative)		Pgp MRP	I / II	
VX710 Biricodar Incel (pipercolinate derivative)		Pgp MRP BCRP	II	
XR9576 Tariquidar (anthranilamide derivative)		Pgp MRP	I / II / III	
LY335979 RS-33295-198 Zosuquidar (dibenzosuberane derivative)		Pgp	II / III	
GF120918 GG918 Elacridar (acridone carboxamide derivative)		Pgp BCRP	I	
R-101933 Laniquidar (benzazepine derivative)		Pgp	II / III	
ONT093 OC144-093 (diarylimidazole derivative)		Pgp	I / II	

Second generation modulators exhibit much improved properties, to such an extent that some of them have reached the level of clinical trials. These compounds came not only from random screening but also from appropriate chemical modifications of 1st generation compounds. Also effective compounds, of novel structural types have been identified. Second generation modulators, like verapamil analogues, valspodar (nonimmunosuppressive analogue of cyclosporin), and biricodar, exhibited a higher modulatory activity and were less toxic, but still were unacceptable due to pharmacokinetic interactions with other transporter proteins. Modula-

tors of third generation exhibit essential improvement in the effectiveness and pharmacological profile. They include tariquidar, zosuquidar, laniquidar and ONT093. These compounds exhibit high MDR modulatory potency, selectivity for target MDR transporter(s), low toxicity, and lack of pharmacokinetic interactions. Selected optimal compounds of the 2nd and 3rd generations are undergoing intensive clinical trials (Table 1), however, none of them, as yet, has been registered for routine clinical use.

Perspectives for success in overcoming MDR of tumors by the application of MDR modulators are quite optimistic; however, this is not yet the

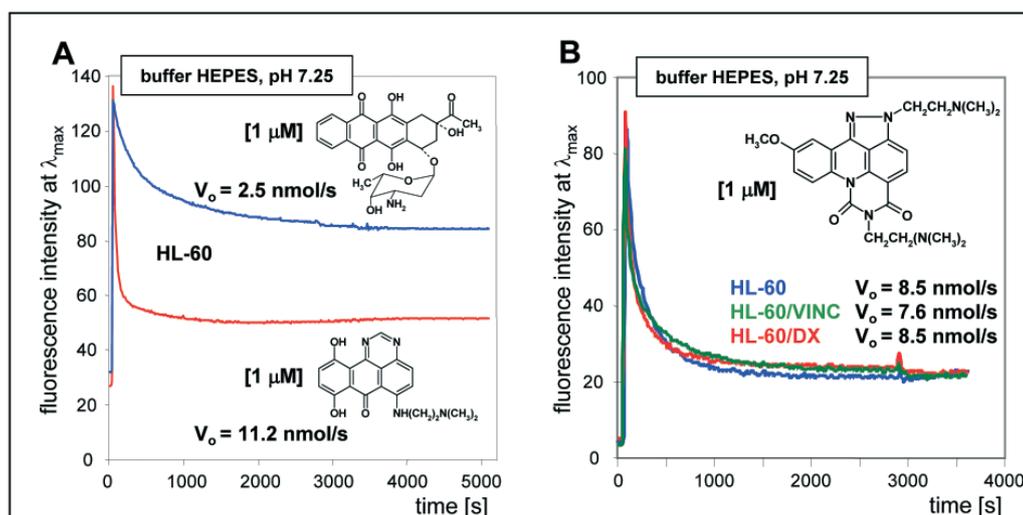


Figure 4. Uptake characteristics of cytostatics (as in Fig. 1: Borowski *et al.*, 2004).

The initial influx velocities and accumulation of A, benzoperimidine and reference idarubicin (sensitive cells); B, pyrazolopyrimidoacridone (sensitive, MDR1 and MRP1 resistant cells). Drug migration followed by fluorimetric method (Tarsiuk *et al.*, 1989).

end of this story. The search for still more effective compounds, with a better selectivity and pharmacological profile should be continued. Perhaps of importance in this endeavor should be broadening by medicinal chemists of the panel of structural types

of active agents, increasing the chance for the development of optimal MDR modulators.

In our studies we have identified novel structural types of competitive modulators of MDR. These are poly- (tetra- and penta-) cyclic anthraquinone and/or acridine analogues. This group of compounds was first developed as MDR-overcoming ones on the basis of a strategy discussed above and visualised in Fig. 1B. The compounds are substrates of MDR transporters. Their appropriate modifications aimed at retaining the transporters' substrate properties but diminishing their cytotoxicity gave a group of effective competitive MDR modulators. Perhaps the most interesting is the tetracyclic anthraquinone analogue PDZ (patent application pending). The compound, used in a noncytotoxic concentration, restores the cytotoxic activity of a number of clinical antitumor agents towards resistant MDR1 and MRP1 leukemic cell lines (Table 2). This effect results from the restoration of drug accumulation in the resistant cells (Fig. 5).

Table 2. Restoration of *in vitro* cytotoxic activity of clinical cytostatics by modulator PDZ used in nontoxic concentration 13.5 nM (as in Fig. 1: Borowski *et al.*, 2004)

Cytostatic		Resistance index RI	
		HL-60/VINC MDR1 resistance	HL-60/DX MRP1 resistance
Actinomycin D	alone	39.73	27.00
	with PDZ	1.25	4.25
Doxorubicin	alone	32.26	216.10
	with PDZ	1.29	1.45
Etoposide	alone	125.62	250.66
	with PDZ	1.30	1.26
Mitoxantrone	alone	33.30	927.40
	with PDZ	1.44	1.22
Taxol	alone	1454.28	1.10
	with PDZ	1.13	–
Vinblastine	alone	294.80	3.90
	with PDZ	1.33	1.33

The cytotoxic activity *in vitro* was determined by cell counting or by protein content of the cells (Bontemps-Gracz *et al.*, 1991) after 72 h of continuous exposure to the cytostatic.

ALTERNATIVE WAYS OF APPROACHING THE MDR PROBLEM

In addition to the reversal of multidrug resistance by chemicals interfering either with the expression of transporter proteins or with their function, there are some other interesting strategies.

Various substances modify the fluidity of membranes, affect membrane-bound structures, and change intracellular pH and/or electric membrane potential. The interaction sites of transporter proteins with the lipid bilayer are mostly in lipophilic amino-acid sequence located in the transmembrane

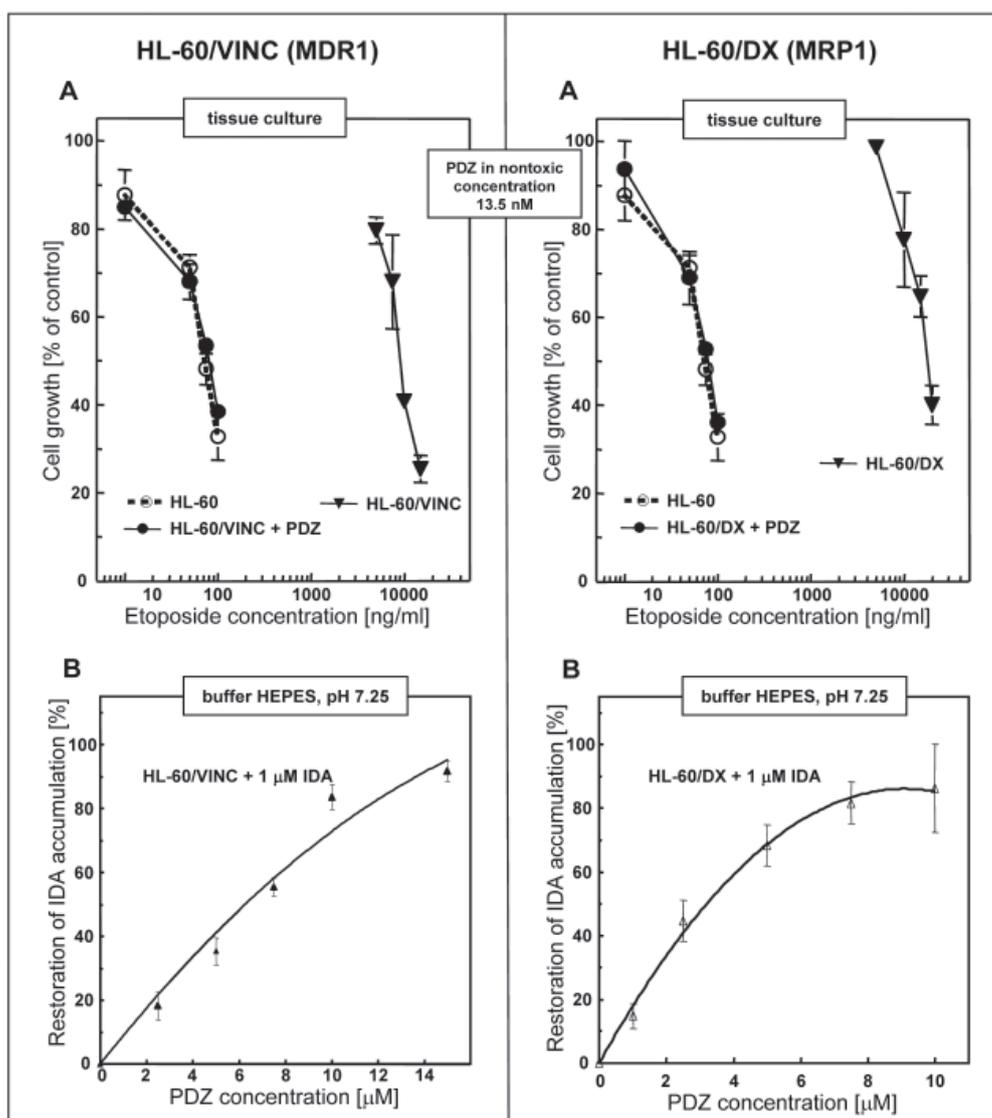


Figure 5. Restoration of cytotoxic activity and of cellular accumulation of clinical cytostatics by the modulator PDZ (as in Fig. 1: Borowski *et al.*, 2004).

A. The cytotoxic activity *in vitro* was determined by cell counting or by protein content of the cells (Bontemps-Gracq *et al.*, 1991) after 72 h of continuous exposure to the cytostatic. B. Idarubicin accumulation followed by fluorimetric method (Marbeuf-Gueye *et al.*, 2000).

regions, and can be affected by chemicals modifying the membrane physicochemical properties. Membrane modifications on their own can chemosensitize MDR cells even without the use of transporter inhibitors or modulators. The action of detergents or membrane fluidization by ether, other anaesthetics, or some other agents abolishes Pgp ATPase activity and modulates efflux from multidrug-resistant cells, due to the suggested flippase activity of Pgp (Regev *et al.*, 1999; Ferte, 2000).

Cytostatic agents cause necrotic or apoptotic tumor cell death, but it is apoptosis which plays a critical role in chemotherapy-induced tumor cells killing. The susceptibility of tumor cells to apoptosis upon treatment with cytostatics appears to depend on the balance between the proapoptotic and surviv-

al (anti-apoptotic) signals (reviewed in: Tsuruo *et al.*, 2003; Yang *et al.*, 2003; Pommier *et al.*, 2004; Secchiero *et al.*, 2004). This balance is shifted in MDR cells towards the survival as a consequence of the diminished intracellular drug concentration. The advantageous balance can be restored by introducing target-specific proapoptotic therapies. Once the apoptotic process is started it leads to the activation of the caspase cascade that in turn causes proteolytic degradation of a variety of important proteins and leads to the destruction of DNA (Fulda *et al.*, 1998). Thus, several target sites for proapoptotic therapies can be identified (Yang *et al.*, 2003) along the signal transduction pathway (Secchiero *et al.*, 2004). Perhaps the most interesting as targets are proteasomes, the major site of degradation of abnormal or short-lived

regulatory proteins, such as transcription factors or cyclins. Modulation of proteasome activity by inhibitors can affect the signal transduction pathway(s) associated with apoptosis and in consequence enhance it (Adams, 2002), thus compensating for the negative effect of the lowered concentration of a drug in MDR cells. A very promising proteasome inhibition was achieved by bortezomib (PS-341), a dipeptide boronate, which strongly stimulates apoptosis (reviewed in Yang *et al.*, 2003). However, it should be remembered that proteasomes are expressed also in normal tissues and play essential roles in various cellular functions. Therefore, nonselective inhibition of proteasomes might have adverse effects. Moreover, the inhibitor should be able to evade the action of the MDR pump, or else an appropriate pump modulator should be used.

Another alternative approach to deal with MDR cells is based on immunological methods. Monoclonal antibodies were first used to identify the expression of Pgp (Hamada & Tsuruo, 1986), however, they can also be applied to inhibit Pgp specifically because of their high selectivity against well defined epitopes, and the resulting ability to abolish the MDR phenotype of tumor cells (Hamada *et al.*, 1990; Mechetner & Roninson, 1992). The combination of anti-Pgp monoclonal antibodies with Pgp modulators may also be useful in enhancing the reversal of Pgp-mediated MDR (Watanabe *et al.*, 1997). Monoclonal antibodies themselves also affect the proliferation of Pgp-expressing tumor cells (Tsuruo *et al.*, 1989; Heike *et al.*, 1990; Efferth *et al.*, 1991; Mechetner & Roninson, 1992). The best results were obtained in a combination therapy using a monoclonal antibody, chemosensitizers, and an antitumor drug (Naito *et al.*, 1993a; 1993b). Also immunotoxins could be potentially valuable for the treatment of multidrug resistant cells. Their molecules consist of a monoclonal antibody coupled to a cytotoxic agent *via* a linker. The hybrid toxin binds to its specific target antigen and exerts its cytotoxic effect after internalization. Several such compounds have been obtained which exhibit cytotoxic activity towards Pgp-expressing cells with only marginal inhibitory effect in sensitive cells. The extent of cell killing correlated with the level of Pgp expression (FitzGerald *et al.*, 1987; Efferth & Volm, 1993). Of interest are also bispecific antibodies, representing chimeric proteins with each of the two antigen binding sites recognizing different antigens (Van Dijk *et al.*, 1989). The bispecific antibodies concept may allow an improvement of selectivity towards particular tissues. The antibody-directed approaches might be promising alternatives to conventional chemotherapy of MDR tumors. However, successful drug development can only be achieved if the following three major problems are overcome: (i) the immunogenic activity of animal antibodies in humans, (ii)

inadequate antibody concentration in solid tumors, (iii) lack of selectivity regarding Pgp-expressing tumors and normal cells (Hamada *et al.*, 1990; Efferth & Volm, 1992).

It has been shown that some protein-drug conjugates exhibit increased tumor uptake (Sinn *et al.*, 1990; Stehle *et al.*, 1997). Consequently, an MDR-reversing effect of bovine serum albumin-conjugated doxorubicin was shown in doxorubicin-resistant tumors (Pommerenke *et al.*, 1995).

A rather unusual and exciting concept has been put forward recently concerning the exploitation of the MDR effect for selectively killing resistant cancer cells, while sparing sensitive normal cells (Blagosklonny, 2003). The majority of current anticancer drugs attack cancer-nonspecific targets, such as DNA and microtubules, and hence do not discriminate between cancer and normal cells, resulting in harmful side effects. Many cytostatics induce apoptosis in cancer as well as in normal cells, executed by caspases (Reed, 2002). The use of a caspase inhibitor selected to be a substrate for the MDR pumps, protects normal cells from apoptosis upon the action of a cytostatic agent which, in contrast, is chosen such as not be a substrate of the drug efflux pumps. In consequence, only the MDR cells are not protected from the cell cycle inhibitor. In a model experiment (Blagosklonny, 2003), the cytostatic agent flavopiridol and the caspase inhibitor Z-DEVD-fink were used. Flavopiridol is an inducer of apoptosis (Kitada *et al.*, 2000) and is not a good substrate of Pgp or MRP (Boerner *et al.*, 2001; Robey *et al.*, 2001). On the other hand, the caspase inhibitor used (Blagosklonny, 2001) is a substrate of drug transporters. In consequence, good selective toxicity was recorded using the sensitive cell line HL-60 and its Pgp and MRP1 resistant sublines. Unfortunately, normal cells with intrinsic expression of transporter proteins are expected to behave as MDR tumor ones.

Another original concept of the same author (Blagosklonny, 2003) is based on the idea of "cytostatic protectors", which would reversibly arrest the cell cycle and inhibit proliferation, thus protecting normal cells from anticancer drugs that preferentially kill cells in a particular phase of the cell cycle (Shah & Schwartz, 2001; Gardner, 2002). The protector should be a substrate of MDR pumps, while the cytostatic agent should not (Blagosklonny, 1999).

The phenomenon of multidrug resistance enabled the development of an interesting concept for cancer gene therapy (reviewed in: Volm, 1998; Gottesman, 2002; Tsuruo *et al.*, 2003). The *MDR1* gene is expressed in several tissues, but is not widely expressed in bone marrow cells. This may be one of the reasons for severe side effects of some cytostatic agents being especially visible in bone marrow cells, which is the dose-limiting factor during the treatment of cancer patients. It may be possible to

protect haematopoietic cells from toxic side effects of cancer chemotherapy by transducing the *MDR1* gene, thus allowing the elevation of doses of cytostatics for cancer patients. For this purpose retrovirus-mediated gene transfer can be used (Pastan *et al.*, 1988; Gottesman *et al.*, 1994). This therapeutic approach is interesting because it could be applied to a wide range of currently resistant solid tumors.

CONCLUSIONS AND PERSPECTIVES

Multidrug resistance (MDR) of tumors is a major cause of failures in antitumor chemotherapy. Many diverse strategies for overcoming this adverse phenomenon have been proposed. The strategy based on development of MDR-modulating compounds used as augmenting agents in combination antitumor therapy is among the most promising ones. This strategy should allow the restoration of therapeutic effectiveness of already available clinical cytostatics, thus evading the threat of an apocalypse which otherwise awaits us when these cytostatics are no more effective due to the development of MDR of tumors.

Although positive effects can be expected in cancer chemotherapy by the application of MDR modulators as augmenting agents for overcoming multidrug resistance of neoplastic cells, we should be aware of various dangers accompanying their use. The major problem concerns the adverse side-effects which might occur if the modulator is not selective enough. In this respect two most important aspects should be taken into the consideration in the rational design of optimal MDR modulators.

Cytotoxic agents inducing ABC transporters often induce also cytochrome P450 enzymes (Lum & Gosland, 1995). These agents, being substrates for MDR transporters, also often are metabolised

by cytochrome P450 isoenzyme 3A4 in a detoxification process. Some MDR modulators can also be substrates for this enzyme. The competition between cytostatic agents and MDR transporter modulators for cytochrome P450 3A4 can result in unpredictable pharmacokinetic interactions that might lead to increased serum concentration of the cytotoxic agent and potentially put patients at risk of cytotoxic drug overexposure (Fischer *et al.*, 1998; Rowinsky *et al.*, 1998; Bates *et al.*, 2001; Gottesman *et al.*, 2002). Consequently, it may be difficult to establish safe but effective doses of the coadministered drug and the modulator.

Another problem concerning the adverse side-effects of MDR modulators is their selectivity for the target MDR transporters. Such agents may also function as substrates for other ABC transporter proteins, inhibition of which could affect detoxification and other transport activities of normal cells and tissues. Many of these transporters have a well defined physiological role, often involving the elimination of xenobiotics, in specialized tissues and organs like liver, kidney, and gastrointestinal tract (Krishna & Mayer, 2000).

Further fundamental studies should be done to better understand the mechanisms of the discussed adverse effects at the molecular level and to build a sound theoretical basis for the rational design and development of optimal agents for effective combination antitumor chemotherapy.

Acknowledgements

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