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Reversal of multidrug resistance in tumor cells *in vitro*

Ph.D. Thesis

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CONTENT

INTRODUCTION

1.1. General introduction

Cancer is the second most frequent disease group on a global basis. The hope for cure of most cancers can be probably fulfilled by systemic treatments such as chemotherapy and immunotherapy. Chemotherapy proven to be effective has led to the cure of many cancers such as leukemias, lymphomas, and sarcomas. The common clinical problem in the successful treatment of cancer is the resistance of tumor cells to chemotherapeutic drugs.

Resistance to chemotherapeutics has recently been observed to be emerging among viruses, bacteria, fungi, protozoa and cancer cells. The basic reason for the development of this resistance is a Darwinian selection, which ensures the accommodation of living organisms to an altered environment (1).

1.2. Different mechanisms of resistance

Drug resistance can arise as a consequence of various biochemical mechanisms (Figure 1.). These include: reduced drug delivery, decreased drug uptake, increased drug efflux, reduced metabolic activation of the drug, increased deactivation of the drug, sequestration of the drug to prevent interaction with target site, increase in intracellular concentration of target sites, structural alterations in the target site, duplication of the functions of the target site, and increased repair of damaged target site.

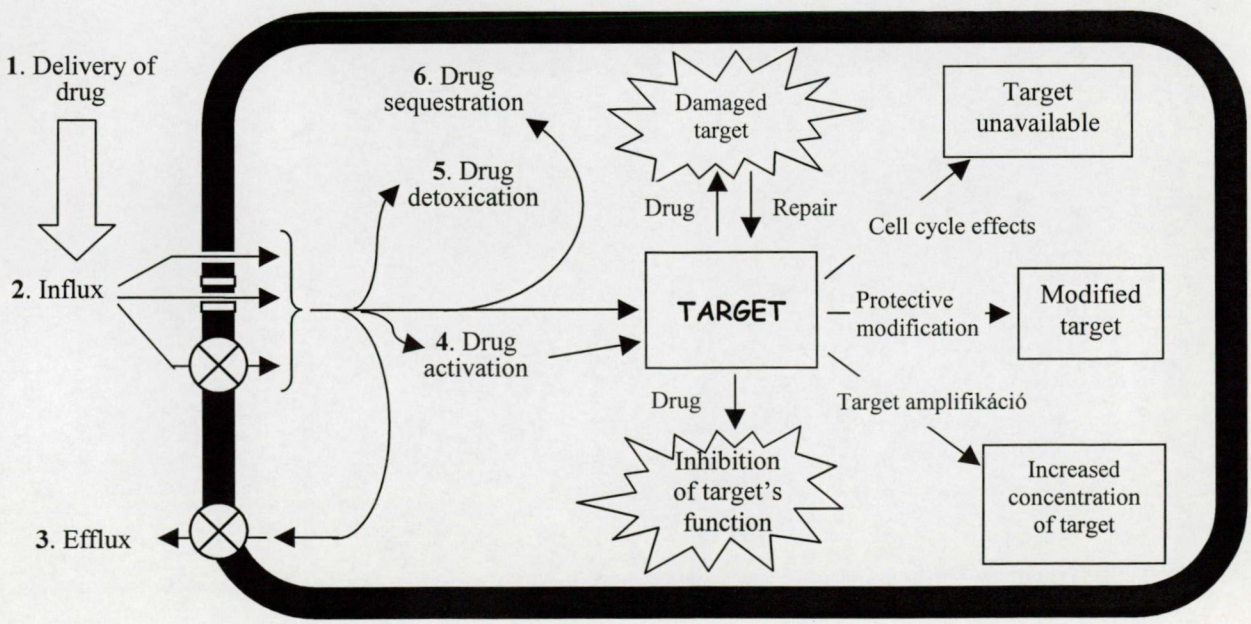

Figure 1. Biochemical mechanisms of drug resistance

It is important to realise that resistance to a particular drug can be achieved by more than one mechanism. On occasions, particularly in the case of changes in either drug transportation or drug detoxication, protection against more than one chemical is invariably observed. This can be manifested as cross-resistance to structurally-related and unrelated chemicals. This latter phenomenon has been referred to as multidrug resistance (mdr)(2).

Apart from multidrug resistance (MDR), other resistance mechanisms exist, e.g., the mdr-associated protein (MRP) (3), which is mediated by a 190 kDa membrane glycoprotein (4), and the lung resistance protein (LRP) which a 110 kDa protein involved in nuclear cytoplasmic drug transport (5). Enhanced gluthione-S-transferase (6) vesicular drug binding and compartmentalization of drugs, e.g. mitoxantrone (7) and DNA topoisomerase mutation-related resistance have been also observed in tumor cells. The widespread use of anticancer drugs underlines the importance of elucidating the various mechanisms of resistance and is based on the characterization of different mdr phenotypes.

1.3. The ABC superfamily of membrane transporter proteins

P-glycoprotein belongs to a large group of functional proteins that share common structural and functional properties. This superfamily of proteins has been named the ABC (ATP Binding Cassette) family of membrane traffic ATPases. There are at least 40 members of this ABC superfamily of proteins in bacteria, including nutrient, peptide, polysaccharide, toxin and drug transporters. There are many examples of these ABC proteins in eukaryotic cells as well, including a pigment transporter in *Drosophila melanogaster* (8), a pump that appears to mediate chloroquine resistance in *Plasmodium falciparum*, *pfmdr* (9,10), a transporter for the peptide mating factor of yeast called *STE6* (11,12), CFTR, the product of the cystic fibrosis gene (13,14), a peroxisomal membrane pump in the liver (15) which, when mutation results in a fatal cerebro-hepato-renal dysfunction (16), and two linked genes associated with transport of peptides into the endoplasmic reticulum for class I antigen presentation recently named Tap-1 and Tap-2 (17), and others.

The general structure of these transporters includes a set of six transmembrane domains, which are generally not homologous to others sharing amino acids with hydrophobic properties, having by an ATP binding cassette. In the bacterial systems, the subunit with the transmembrane domains and the ABC proteins may be separate or fused. In eukaryotic systems, a set of six transmembrane domains may be fused in to a single ABC protein, as for the peroxisomal membrane protein, endoplasmic reticulum peptide transporters, *Drosophila*

pigment transporter, and the antigen peptide transporters, or two of these may be fused together to give 12 transmembrane domains and two ATP sites, such as the CFTR, STE6 and MDR proteins. Although this family has not yet been fully described, it appears that the ABC transporters localised to intracellular membranes contain only one set of six transmembrane domains and one ATP site, whereas the transporters localised to the plasma membrane have 12 transmembrane domains and two ATP sites. Several secondary transporters such as the glucose carrier, anion exchanger, and Na/H exchanger, which do not have ATP sites, when localised to the plasma membrane also contain 12 transmembrane domains, but homologous intracellular organelle transporters contain six transmembrane domains, and function as dimers (18,19). Based on this analogy, it is tempting to suggest that the minimal functional unit of the ABC transporters may also require 12 transmembrane domains and two ATP sites.

Broad-spectrum resistance to chemotherapy in human cancer has been called multidrug resistance. This resistance is due to decreased accumulation of drugs in cells where the efflux of cytostatics is mediated by an energy-dependent drug transporter protein. One of the biochemical aspects by which tumor cells manifest multidrug resistance is the overexpression of an integral plasma membrane P-glycoprotein or P-170, the product of an SOS gene. This is the multidrug resistance 1 (*MDR1*) gene. P-glycoprotein (P-gp) acts as a drug efflux pump that actively extrudes drugs from tumor cells, thereby decreasing the concentration of chemotherapeutic agents in resistant cancer cells or HIV-1 protease inhibitors (20).

The overexpression of the MDR1, MRP and LRP genes explains only a subset of multidrug resistance. More studies identify resistance-related abnormalities of the enzyme topoisomerase II (21,22) with respect to overexpression of the genes involved in the glutathione S-transferase system (23,24), and the expression of other genes (25,26), has provided additional mechanisms by which multidrug resistance can be conferred. Even though these phenomena are associated with multidrug resistance, the breadth of the resistance associated with each, is not as broad as the multidrug resistance often encountered in the clinic.

1.4. Genes responsible for multidrug resistance

Different P-gp isoforms have been identified, and these are encoded by a family of closely related genes. They are referred to as *pgp* genes in hamsters and rats, *mdr* genes in mice, and *MDR* genes in man (27). Based on their 3'-untranslated regions the mammalian multidrug resistance genes are divided into three classes of genes, termed class I, class II and

class III as in Table 1. (28).

The rodent class I and class II genes appear to be more closely related to each other in structure and function than either gene is to the class III gene. Overexpression of class I or class II P-gp renders cells multidrug resistant, while class III P-gp are not capable of conveying multidrug resistance. The structural similarities between two halves of the transmembrane P-gp molecule led to the hypothesis that the P-gp gene family arose from the duplication of a primordial gene (29), however, detailed analysis of the *MDR1* exon/intron structure indicates that this is probably not the case (30).

Table 1. Mammalian multidrug resistance genes

Species	Class I	Class II	Class III
Human	*MDR1*		*MDR3 (MDR2)*
Mouse	*mdr3 (mdr1 a)*	*mdr1 (mdr1 b)*	*mdr2*
Hamster	*pgp1*	*pgp2*	*pgp3*
Rat	*pgp1*	*pgp2 (mdr1 b)*	*pgp3 (mdr2)*

The *MDR1* gene has been localized in the human chromosome 7 band p21-21.1 (31) on a 600-kb *NruI* fragment, and the entire *MDR1* coding region is contained on a 120-kb *XhoI* fragment (32). This gene extends for more than 100 kb and encompasses 28 introns, 26 of which interrupt the protein-coding sequence. Its messenger RNA has a size of 4.7 kb, and its coding region therefore represents less than 5 % of its total length. The human *MDR2* gene has the same number of 28 exons, 27 coding, but is shorter and only extends for 74 kb. The function of the closely related *MDR2* gene is not known, and its substrates are not yet identified (33).

One of the most intriguing aspects of P-gp biology is the broad substrate specificity of the molecule, which can be altered by point mutations. Spontaneous base pair substitutions in the human *MDR1* gene, resulting in a glycine to valine change at amino acid 185, located at the cytoplasmic border of the third transmembrane domain, change the pattern of resistance by the increased ability to pump colchicine and etoposide, with a decrease in the ability to pump vinblastine and actinomycin D (34). Both intact halves of the P-gp molecule, as well as the two nucleotide binding sites seem to be essential for drug transport (35). Evidence is accumulating that transmembrane domains of P-gp are important for the interaction of chemotherapeutic drugs with P-gp (36). For example, predicted transmembrane domain 3, 6 and 11 are likely to play a role in substrate recognition, binding or release. Point mutations,

either spontaneously occurring or genetically engineered, within or in close proximity to these putative transmembrane domains alter cross-resistance pattern. An important role for the transmembrane domains of the P-gp molecule for drug transport fits with the hydrophobic vacuum cleaner model of the function of P-gp (37). As a consequence of its broad substrate specificity, the function of the P-gp molecule can be inhibited by non-cytotoxic compounds that also have a high affinity for the drug binding site on surface of the P-gp.

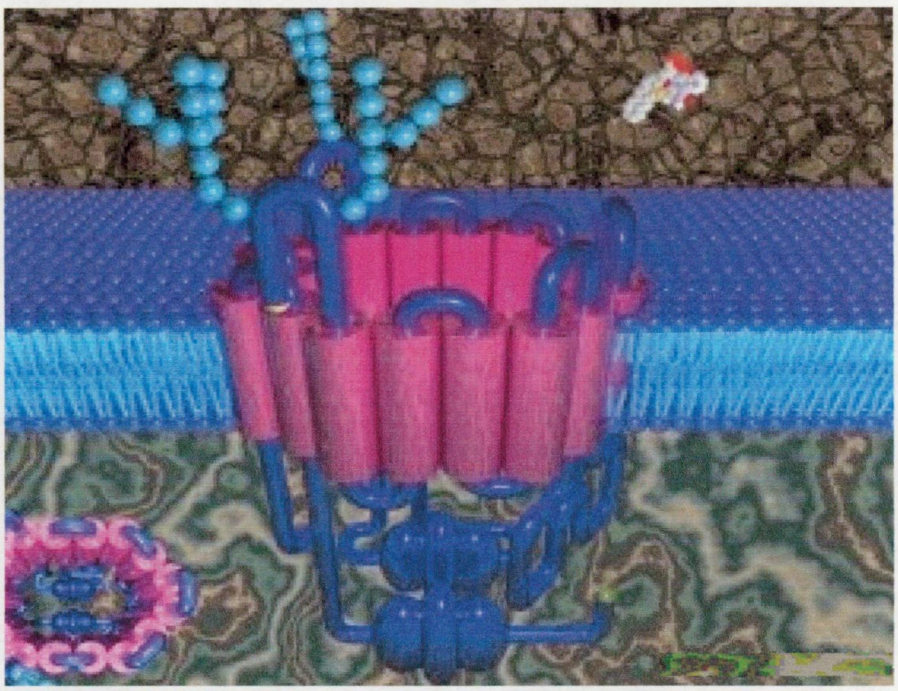

Figure 2. Structure of the multidrug resistant efflux pump

1.5. Sequence, structure, expression and function of P-glycoprotein

P-gp is 1280 amino acids in lenght. The primary amino acid sequence predicts a protein with 12 transmembrane domains in two homologous halves, each containing six transmembrane regions and two large intracytoplasmic loops encoding an ATP-binding site. P-gp consists of two homologous halves, with 43 % amino acid-sequence identity between the amino- and carboxy-terminal halves (38,39). The degree of the homology varies throughout the sequence, and it is much stronger near the C-terminus than in the rest of the protein. Each half of P-gp consists of short, highly hydrophilic N-terminal region, a long hydrophobic region, and a long - relatively hydrophilic - cytoplasmic C-terminal region. The mass predicted from the deduced amino acid composition is 140 kDa (40), but size fractionation by polyacrylamide gel electrophoresis indicates a size of ~170-180 kDa. This discrepancy seems

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due to glycosylation and phosphorylation of P-gp (41). This general structure appears to be indicative of membrane channels and transporters (e.g., in human voltage-sensitive Na^+ channel, cystic fibrosis transmembrane conductance regulator) and is a consistent motif throughout evolution (42).

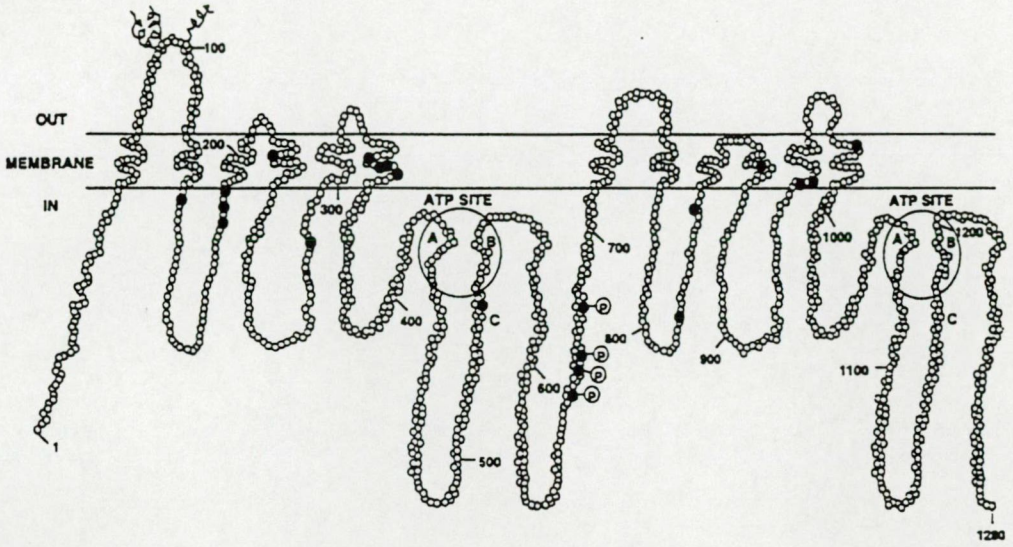

Figure 3. Schematic model of the structural organization of human multidrug transporter, based on amino acid sequence.

P-gp uses energy in the form of ATP to transport drugs through a channel, formed by the transmembrane segments (43,44).

Table 2. Potential physiological functions of P-gps in mammals.

1.	**Protection against exogenous toxins ingested with food:** Expression in small intestine, colon, blood-tissue barrier sites
2.	**Excretion of metabolites or toxins:** Expression in liver canalicular membrane, kidney
3.	**Transport of steroid hormones:** Expression in adrenal gland, demonstrated transport of cortisol, corticosterone, aldosterone
4.	**Extrusion of (poly-)peptides (cytokines) not exported from the cell via the classical signal/cleavage pathway:** Compare yeast *St6*, *E.coli HlyB*, mammalian endoplasmic reticulum peptide transporters
5.	**Ion transport and cell volume regulation:** Activation of an endogenous Cl^- channel activity
6.	**Lymphocyte cytotoxicity:** Possible involvement in NK-cell-mediated cytotoxicity
7.	**Transport of prenylcysteine methyl esters**
8.	**Intracellular vesicular transport of cholesterol**

P-gp is found generally in polarized epithelial cell layers, where it localizes at the apical (or luminal) surface of the cell. This localisation indicates that a major function of P-gp is the protection of organisms against many of the toxic xenobiotics to which they can be exposed in nature (45).

Lowered extracellular pH (46), heat shock and arsenite (47), cytotoxic agents (48), transfection with oncogens (49) and with human immundeficiency virus-1 (50), UV irradiation (51) increase the expression of the *MDR1* gene in both human and rodent cell lines.

1.6. Expression of multidrug transporter in normal tissues, the normal function of „mdr"

The highest level of human *MDR1* protein and mRNA expression are found in the adrenal gland, kidney, jejunum, colon and endothelial cells of the blood-brain barrier, whereas human *MDR2* is strongly expressed in the liver.

Immunohistochemical analysis with specific anti-P-gp antibodies reveal that P-gps are expressed in a polarized manner on the apical membrane of secretory epithelial cells lining luminal spaces such as the glandular epithelial cells of the endometrium in the pregnant uterus, the biliary canaliculi of hepatocytes, the brush border of renal proximal tubules, pancreatic ductules, columnar epithelium of the intestine and in endothelial cells of the blood-brain barrier and in the testes (19). Expression of P-gp at the surface of epithelial cells lining the luminal spaces of the intestine, kidney and liver suggest that P-gp plays a protective role at these sites. Additional experimental evidence suggests that: 1) endothelial cells from the blood-brain barrier can carry out unidirectional drug transport *in vitro* (52) to protect the brain from toxic natural products (18); 2) pluripotent stem cells of the hemapoietic system can transport the P-gp substrate fluorescent dye Rhodamine 123 (R123) (53); 3) the cardiac glycoside, digoxin, which is eliminated by glomerular filtration and tubular excretion as a P-gp substrate (54). On the other hand, expression of P-gp at other sites such as the pancreatic ductules, the adrenal cortex and the endometrial glands of the pregnant uterus, suggests that P-gp may play a role in hormone transport. P-gp can transport steroid hormones such as aldosterone, dexamethasone, cortisol (55) and corticosterone.

The tissue-specific expression of the *MDR1*gene indicates that levels of *MDR1* RNA may be regulated in mammals. Studies have demonstrated increases in RNA levels for P-gp after partial hepatectomy (56,57), treatment with chemotherapeutic drugs and cytotoxic stress such

as heat shock (47). These studies demonstrate increased P-gp mRNA levels in response to cell injury which supports the role for P-gp in protecting cells against damage by toxic products.

1.7. Glycosylation of P-glycoprotein

P-gps are post-translationally modified by glycosylation and phosphorylation. P-gp is N-glycosylated at one position on the first extracellular loop of the C-terminal. Experiments in which multidrug resistant cells were grown in the glycosylation inhibitor of tunicamycin (58,59) and mutant multidrug resistant cells greatly reduced glycosylation of P-gp demonstrating that glycosylation of P-gp is not required for its multidrug transport function (60,61). However, it has been suggested that N-glycosylation may contribute to correct folding, proper routing and stabilization of the molecule.

1.8. Phosphorylation of P-glycoprotein

Early studies suggested that the phosphorylation state of P-gp affects its function. P-gp is phosphorylated on serine and threonine residues, and is a substrate for a number of cellular kinases (62,63). Studies in which protein kinase C has been specifically overexpressed, activated by phorbol esters (64,65) or inhibited by staurosporine (66,67,68) have demonstrated that P-gp is a substrate for protein kinase C phosphorylation (69,70). In human P-gp, protein kinase C phosphorylation sites are Ser-661, Ser-671 (71), cAMP dependent protein kinase phosphorylation sites are Ser-667, Ser-671 and Ser-683 (72). These phosphorylation sites are confined to a central cytosolic segment that connects the two homologous halves of P-gp.

It is interesting that some of the multidrug resistance reversing agents such as verapamil and trifluoroperazine, which bind and inhibit P-gp, also increase P-gp phosphorylation (73,74). These studies suggest that different kinases are involved in P-gp phosphorylation, namely cellular drug resistance is increased, which is correlated with decreased intracellular drug accumulation.

1.9. Interaction of P-glycoprotein with drug molecules

The hypothesis that P-gp protects multidrug resistant cells by pumping cytotoxic drugs through the plasma membrane out of the cell, suggest that P-gp contains specific binding sites for these drugs, and sites would be labeled by photoaffinity drug analogs. Many different photoaffinity derivatives of multidrug resistant drugs have been synthesized and shown to

bind to P-gp. The most frequently used of such binding agents are the drug analog ^{3}H-vinblastine (75), the P-gp modulator calcium channel blocker ^{3}H-azidopine (76), verapamil (77), an α1-adrenergic receptor ligand ^{125}I-iodoarylazidoprazosin (78), and others like colchicin (79), iodomycin (80), cyclosporin A (81), and forskolin (82).

More studies established that at least two regions of the membrane-associated portion of P-gp were involved in binding photoactivable ligands (83,84):

- one minor site located within the amino terminal half and
- a major site mapping to the carboxy terminal half.

Further studies on a series of antibodies directed against discrete P-gp peptides demonstrated that prazozine and azidopine bound to the same site on P-gp, limited mostly to two 4 and 5 kDa cryptic peptides mapping immediately downstream of the last membrane domains of each half of P-gp (85). This study suggested that symmetrical regions in each half of P-gp are involved in drug binding and transport.

Cornwell et al., (86) prepared two different photoaffinity vinblastine analogs and showed compounds specifically labeled P-gp. The specificity of labeling was defined by three criteria:

- the photoaffinity vinblastine analogs labeled a 170 kDa protein in the plasma membrane of multidrug resistant cells, but did not label any such protein in the plasma membrane vesicles prepared from the drug sensitive parent cells,
- it was possible to inhibit the action by adding excess vinblastine to the reaction,
- the 170 kDa labeled protein was immunoprecipitated by antibodies raised against P-gp (87).

Excess vinblastine competed succesfully to prevent ^{3}H-vinblastine from binding to plasma membrane vesicles prepared from multidrug resistant cells. These result show that vinblastine binding and labeling is a saturable process, and from this one may infer that vinblastine binds to a specific site on P-gp. Other drugs, which are also presumed to be substrates for P-gp transport, did not inhibit vinblastine binding and labeling. For instance, daunomycin inhibited vinblastine binding, or tomato lectin inhibited promethazine binding to P-gp, but colchicine and actinomycin D did not, even though the multidrug resistant cells are cross-resistant to all of these drugs (44).

Many different drugs are able to interfere with the activity of the multidrug transporter and also reverse the multidrug resistance phenotype of cultured cells. These include the calcium channel blocker verapamil, the antiarrhythmic quinidine, the antihypertensive reserpine and the immune suppressant cyclosporin A (88). Since verapamil is also a substrate

for P-gp (89) and inhibits ATP-dependent transport of vinblastine into vesicles containing P-gp (44), it is very likely that verapamil works as a competitive inhibitor of the multidrug transporter.

Photoaffinity analogs of verapamil (90), colchicine (91) and daunomycin (80) have since been prepared and used to specifically label P-gp. These results demonstrate that these drugs have binding sites on P-gp and support the hypothesis that P-gp is responsible for pumping these drugs out of the multidrug resistant cells.

Probably all the drugs share a single site for the transport by P-gp, but there is more than one site on the transporter for binding.

1.10. Expression of multidrug transporter in human cancers

A preliminary survey of more than 400 different human cancers demonstrated the widespread expression of the *MDR1* gene in human cancers with both intrinsic and acquired multidrug resistance (92). Cancer of the liver, colon, kidney, pancreas and adrenal, which are generally drug-resistant, express high levels of *MDR1* RNA equivalent to levels found in four-fold to six-fold multidrug resistant tissue culture cells (92). Similar levels of expression are found in a minority of human leukemias and non-Hodgkin's lymphoma, chronic myelogenous leukemia in blast crisis, astrocytoma before chemotherapy, and in higher percentages of treated leukemias, lymphomas, neuroblastomas, sarcomas, cancer of the breast and ovary from patients who have received chemotherapy.

The process of malignant transformation in cancer derived from tissues that do not normally express P-gp can activate expression of the *MDR1* gene (32). Increased expression of the *MDR1* gene is commonly seen in tumors treated with chemotherapy that have relapsed during the course of, or after chemotherapy; for example breast cancer, ovarian cancer, lymphoma, leukemia, neuroblastoma, pheochromocytoma, rhabdomyosarcoma and multiple myeloma. In these cases, it is presumed that small numbers of *MDR1*-expressing cells were present when therapy was initiated, and this population survived chemotherapy and caused the relapse, but a direct effect of chemotherapy to induce *MDR1* gene expression is also possible (93).

1.11. Mechanism of action

The early studies of P-gp-positive multidrug resistant cells clearly demonstrated that the emergence of multidrug resistance in these cells was linked to a marked decrease in the

intracellular accumulation of the various drugs to which the cells expressed resistance to. This reduced cellular drug accumulation was strictly energy (ATP) dependent and was concomitant with an ATP-dependent increase in drug release from these cells. Two major hypotheses for the mechanism of P-gp action to explain such reduced drug accumulation have been put forward (37,60,94).

The first hypothesis is that P-gp functions as a drug transporter (efflux pump), which can act on a broad range of structurally unrelated molecules and uses the energy of ATP hydrolysis to mediate drug efflux. Experimental data supporting the notion that P-gp functions as a drug transporter include the observations that:

- P-gp binds the ATP analog, has ATPase activity, and mutations in either of its predicted ATP-binding domains abrogate its function,
- P-gp binds drug analogs, and mutations in its predicted transmembrane domains modulate substrate specificity by altering drug binding to the protein,
- P-gp shares homology with a number of prokaryotic and eukaryotic membrane proteins implicated in the ATP-dependent transport of various types of substrates across the membrane,
- transport studies in intact cells and plasma membrane vesicles from P-gp expressing cells indeed suggest that P-gp mediates increased ATP-dependent drug binding and transport into these vesicles (44,86,95).

The second opposing hypothesis proposes that P-gp itself is not a drug transporter, but has an indirect role in modifying the intracellular environment to create either an electrochemical - or a pH - gradient. In turn, these gradients would act in an indirect fashion to drive the movement of charged drugs across the membrane. The major appeal of this hypothesis is its explanation of the unusual ability of P-gp to act on a vast number of structurally unrelated substrates, which yet share a high degree of hydrophobicity and are positively charged at neutral pH. In this model, P-gp was proposed to function as an outwardly directed ATP channel, creating an electrochemical ATP-gradient and possibly providing a driving force for drug efflux. Another model for a P-gp mechanism is based on the observation that P-gp-positive cells demonstrate an altered intracellular pH (96). In this model P-gp would function either directly or indirectly to increase intracellular pH and lower the electrical membrane potential, in consequence of which:

- charged hydrophobic compounds such as anticancer cytostatics or multidrug resistance reversing drugs (lipophylic cations) might be differently retained in P-gp-

positive and negative cells,

- the pH-dependent binding of drug molecules to their respective, but structurally unrelated targets might also be altered by P-gp overexpression.

These hypotheses suggest that the P-gp action may have a dual function.

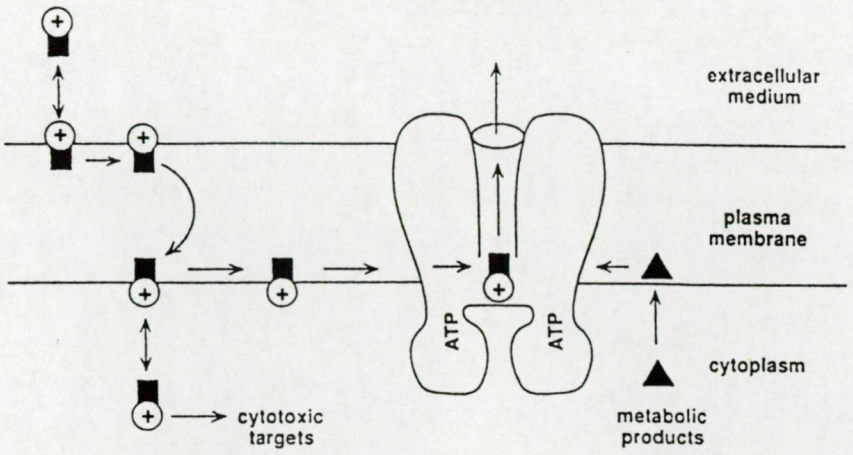

Figure 4. Mechanism of action the mdr efflux pump

A model for the action of P-gp, which incorporates these ideas, is shown in Figure 4 (38). The first feature of this mechanism is that drugs can be detected and expelled as they enter the plasma membrane in the manner of a hydrophobic vacuum cleaner (97). Anticancer drugs with a hydrophobic domain and a positively charged domain have been demonstrated, to diffuse into and across the plasma membrane, where they encounter the multidrug transporter by lateral diffusion. The transporter uses energy transduced from two essential ATP binding sites to pump the drug out of the membrane (35,98,99). The hydrophobic metabolic product, produced in the cytoplasm or in the membrane, is shown as a potential substrate for the transporter. One pathway of drug or metabolic efflux within the transporter is shown in Figure 4 for simplicity, but there may be more than one (100).

1.12. Methods of MDR1 detection

The detection of multidrug resistance has both theoretical and clinical significance. These approaches include assessing *MDR1* gene amplification, measuring *MDR1* mRNA levels and detecting P-gp. General methods detecting *MDR1* gene in tissues are shown in Table 3.

A number of monoclonal antibodies are available for the detection of P-gp. The monoclonal antibodies C219 is used for both immunoblot analysis and immunohistochemical detection of P-gp, but it is nonspecific. Another antibody C494 is specific for P-gp. This

antibody also recognizes a cytoplasmic epitope and thus also requires fixation prior to immunohistochemical staining.

Table 3. Methods of *MDR1* detection

Analysis of RNA	*Analysis of protein*
Northern blot, slot blot	Western blot
RNAse protection	Immunohistochemistry
In situ hybridization	Flow cytometry
Polymerase chain reaction (PCR)	

Antibody MRK-16 has high affinity for P-gp. MRK-16 recognizes an external epitope on the outer surface of the plasma membrane of living cells and it does not require prior fixation.

The primary advantages of immunohistochemistry in the clinical setting include the ability to discriminate P-gp expression in normal cells from tumor cells. Immunohistochemistry is sensitive enough to detect low-level expression of P-gp.

Polymerase chain reaction is probably the most sensitive, rapid and easy means of detecting *MDR1* mRNA. It is possible to analyze the most minute of clinical specimens for diagnostic purpose.

1.13. Clinical relevance to reverse multidrug resistance

After the discovery that the P-gp molecule can be expressed in human tumors, clinical trials have been initiated with the aim to block the P-gp drug efflux pump, and in that way make anticancer drugs more effective (101). A large number of compounds has been noted to overcome P-gp mediated multidrug resistance (Table 4).

Chemosensitizers in general are lipid-soluble at physiological pH and possess a basic nitrogen atom and at least two planar aromatic rings.

The calcium channel blocker verapamil is able to overcome P-gp mediated multidrug resistance to vincristine and doxorubicin, both *in vitro* and *in vivo*. Verapamil is capable of overcoming clinical drug resistance, but cardiotoxicity has been close-limiting. Cardiotoxicity induced by verapamil may be reduced by the use of specific stereoisomers. Both the R and S optical isomers of verapamil are equally effective in reversing multidrug resistance, but the R isomer is 10 times less cardiotoxic than the S isomer. Clinical studies are currently underway with R-verapamil as a chemosensitizer, likewise the immunosuppressive drug cyclosporin and its non-immunosuppressive analogue SDZ PSC 833.

Table 4. Chemosensitizers: agents capable of inhibiting P-gp *in vitro*

Agents that reverse drug resistance	**Anticancer drugs**
Calcium channel blockers (verapamil, nifedipine, azidopine, dihydroperidines)	Vinca alkaloids (vinblastine, vincristine)
Anti-arrhytmics (quinidine, amiodarone)	Anthracyclines (doxorubicine)
Antihypertensives (reserpine)	Epipodophyllotoxins (etoposide)
Antibiotics (hydrophobic cephalosporins)	Antibiotics (actinomycin D)
Antihistamines (terfenadine)	Others (mitomycin C, taxol, mithramycin, topotecan)
Immunosuppressants (cyclosporin A, FK506, rapamycin)	**Other cytotoxic agents**
Steroid hormones (progesterone)	Antimicrotubule drugs (colchicine, podophyllotoxin)
Modified steroids (tamoxifen, tirilazad)	Protein synthesis inhibitors (puromycin, emetine)
Lipophilic cations (tetraphenylphosphonium)	DNA intercalators (ethidium bromide)
Diterpenes (forskolin)	Toxic peptides (valinomycin, gramicidin, D,N-aceyl-leucyl-leucyl-norleucinal (ALLN))
Detergents (Tween-80)	
Antidepressants (thioperidone)	
Antipsychotics (phenothiazines)	
Many other hydrophobic, amphipathic drugs and their analogs	

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AIMS OF THE THESIS

Multidrug resistance is a very important problem and the treatment failures of cancer patients are related to multidrug resistance to a great extent.

Therefore, we planned to investigate the opportunity of reversal of multidrug resistance by synthetic and natural compounds in a well-defined system with the use of human *MDR1* transfected mouse lymphoma cell lines and leukocytes from patient for *ex vivo*.

- Firstly, the antiproliferative effect of various resistance modifiers will be tested by using MTT method. Secondly, the multidrug resistance reversal effect will be studied in subtoxic or non-toxic concentrations.
- The complex formation of the resistance modifiers, phenothiazines and related compounds, on nucleic acids such as DNA, tRNA will be studied.
- The effect of one representative compound will be tested on the *MDR* gene activity by the application of RTase PCR.
- For the multidrug resistance reversal effect, the drug accumulation will be tested by the widely used Rhodamine 123 extrusion assay.
- For inhibition of the drug efflux pump localised in mouse lymphoma cells (transfected by human *MDR1* gene) substituted phenothiazines, benzothiazines, 3-benzazepines will be used as well as important plant-derived natural compounds.
- For studing the specificity of efflux pump inhibition three stereoisomer pairs will be selected.
- The mechanism of action of resistance modifiers will be analysed to a limited extent (gene regulation, activity of P-gp, importance of the first loop of P-gp).
- The effect of some resistance modifiers will be studied as well on the drug accumulation of leukocytes isolated from leukemic patients *ex vivo*.
- The combination of resistance modifiers and some cytostatics will be tested on the multidrug resistant cell line to obtain evidence for additive or synergistic interactions.

According to our working hypothesis some of the non-toxic resistance modifiers can be combined with anticancer chemotherapeutics. Therefore our results may contribute to the improvement of rational drug design to reverse multidrug resistance of cancer cells.

MATERIALS

3.1. Chemicals

Promethazine (PZ) and verapamil were obtained from EGIS Works, Hungarian Pharmaceutical Company, Budapest and trifluoroperazine (TFP) from Richter-Gedeon, Hungarian Pharmaceutical Company, Budapest.

H3001 H31OH H36ON RZ541 ellipticines synthetized by Prof. György Hajós (KKKI, Budapest), mepacrine, (-)butaclamol, (+)butaclamol (Research Biochemicals Inc. P.O.Box 181 Wayland, M.A 01778), trans(e)-clopenthixol, cis(z)-clopenthixol (Lundbeck CO.A/S. 2500 Kobenhavn-Valby, Denmark) were obtained.

Platidiam, bleomycin, methotrexate (Lachema, Brno), novantron, daunorubicin, fluorouracil (La Roche Ltd, Basel), thaliblastine, vinblastin, vincristine (Richter-Gedeon, Budapest), reserpine and yohimbine were obtained from Meiji Pharmaceutical University, Tokyo.

The 7,8-dioxo-CPZ were synthetized by Prof. Sándor Földeák (102). Chlorpromazine (CPZ), 6,9-dihydroxi-CPZ, 6,9-dioxo-CPZ, (Psychopharmacological Res, Branch NIMH, Rockwille, Md, USA), 5-oxo-5-H-benzo(a)phenothiazines, 6-hydroxy-5-oxo-5H-benzo(a)phenothiazines, 6-methyl-5-oxo-benzo(a)phenothiazines were synthetized by Prof. Noboru Motohashi (103). Cyclosporin A (CsA), cyclosporin D (PSC-833) were obtained from the Food and Drug Administration, Washington, USA.

Four phenothiazine derivatives, i.e., 10-[3-(phthalimido)propyl]-2-chloro-10*H*-phenothiazine [**1**], 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [**2**], 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**], 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [**4**] were prepared as described previously (104).

The six 2,3,4,5,-tetrahydro-3-benzazepinones [**1-6**] (7,8-dimethoxy-2-methyl-3-methanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [**1**], 7,8-dimethoxy-2-methyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [**2**], 3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [**3**], 7,8-dimethoxy-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [**4**], 7,8-dimethoxy-2-isopropyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [**5**], 7,8-dimethoxy-2-phenyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [**6**]) and three 2,3,4,5,-tetrahydro-1H-3-benzazepines [**7,8,10**] (7,8-dihydroxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-

benzazepine [7], 7,8-dihydroxy-3-methyl-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [8], 7,8-dimethoxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [10]) were synthesized as recently published (105). Compound 7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine [9] was synthesized, according to the literature (106). Tomato lectin, dopamine-hydrochloride (DA) was purchased from Sigma (St. Louis, MO, USA), norepinephrine (NE) was purchased from Aldrich (Milwoukee, WI, USA).

6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [7], 2-methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [8], 1,3-dimethyl-6,12-dihydro-1-benzopyrano [3,4-b][1,4]benzothiazin-6-one [9], .1,4-dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4] benzothiazin-6-one [10], 3,4-dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [11], 2-chloro-3-methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [12] were synthesized by a method recently published (107). 6-methylcoumarin [13], 7-methylcoumarin [14], ethyl 3-coumarincarboxylate [15] were purchased from Aldrich Chemical Co. (St. Louis, MO, USA).

D-methotrimeprazine, L-methotrimeprazine were obtained from EGYT Pharmacochemical Works Budapest, Hungary.

Crocin, triglucosyl crocetin, diglucosyl crocetin, picrocrocin were prepared by Prof. Y. Shoyama, Kyushu University, Fukuoka, Japan.

Ginsenosides, Rb1, Rc, Rd, Re and Rg1 were isolated from white ginseng by repeated column chromatography on silica-gel as reported elsewhere (108).

Cannabis: cannabinol, cannabidiol, cannabidiolic acid, delta8-tetrahydrocannabinol (Δ8-THC), delta9-tetrahydrocannabinol (Δ9-THC) were kindly supplied by Dr. Keizo Watanabe, Narcotics Laboratory Section, Division of Narcotic Drugs, United Nations, Vienna International Center, Vienna, Austria.

Amitriptyline, fluphenazine, maprotiline, trimipramine, desipramine, imipramine, haloperidol, doxepin, Rhodamine 123 (R123), daunorubicin (DR) were obtained from Sigma (St. Louis, MO, USA).

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT), sodium chloride, sodium dodecylsulphate (SDS), phosphate-buffered saline (PBS), dimethylsulphoxide (DMSO), colchicine, L-glutamine, streptomycin, nystatin were obtained from Sigma (St. Louis, MO, USA).

3.2. Cells and cell cultures

The L5178Y parent (par) mouse T cell lymphoma and its, multidrug resistant (mdr), transformed subline with *MDR1/A* gene were grown in McCoy's 5A medium supplemented with 10 % heat-inactivated horse serum in the presence of colchicine (109). The sensitive leukemia cell lines, L1210 and its *MDR1* gene transfected resistant pair were obtained from Prof. Gottesman of NIH, and were maintained as described earlier (110). The sensitive KB-3-1 and the resistant KB-V-1 human adenocarcinoma cell lines were cultured as described previously (111).

Brain capillary endothelial cells of a BB19 cell line were cultured in monolayers, in RPMI medium supplemented with 10 % FCS and 10 % human AB serum, and 50 μg/mL endothelial growth factor (Sigma).

3.3. Patients

Patients SzL, PL and NV were consenting blood donors at the University Medical School of Debrecen.

Peripheral human blood (PBL) samples were obtained from volunteer cancer patients and PBL were prepared by Ficoll-Hypaque density gradient centrifugation as described elsewhere (112).

METHODS

4.1. Indirect immunfluorescence analysis of P-glycoprotein

Endothelial cells ($2x10^6$ cells in 50 μL) were incubated for 45 min on ice with 50 μL of a 1:50 dilution of monoclonal antibody C219 (Signet Lab Inc., Massachusetts, USA), specific for P-gp. Cells were washed three times with ice-cold PBS containing 1 % FCS and stained for 45 min on ice with a 1:200 dilution of fluoresceinated $F(ab')_2$ fragments of goat anti-mouse Ig (DAKO, Carpinteria, USA). Cells were washed three times with FASCtar plus (Becton-Dickinson) and analyzed with fluorescence microscopy and a flow cytometer at 488 nm excitation. Background fluorescence was measured by incubation of the cells with buffer only instead of C219 mAb.

4.2. The inhibition of MDR1 gene expression

The par and mdr cell lines were incubated in the presence of 0.5-2.0 μg/mL TFP and PZ for 48 hours at 37 °C in 5 % CO_2 as usual. For isolation of the total cellular RNA the cells were washed in ice cold sodium chloride, centrifuged. RNA was prepared from drug-treated and control cells with the miniprep-RNA protocol. cDNA was prepared from the 1.0 μg isolated RNA by reverse transcriptase (RT) (113). The RT assay was run at 42 °C for 60 min followed by a denaturation at 95 °C for 5 min and a cooling step at 5 °C for 5 min. The amplification was performed with a polymerase chain reaction (PCR) at 94 °C for 5 min and continued for 37 cycles of melting 94 °C for 30 seconds and annealing extending with Taq polymerase 62 °C 50 seconds followed by a final step at 72 °C 60 seconds. The PCR products were separated in 2 % agarose in the presence of ethidium and semiquantitated by densitometry (Wayne-Rasband, NIMH, Bethesda, USA). The TFP and PZ apparently inhibited the synthesis of *MDR1* RNA by the inhibition of gene expression. Primers for PCR were *MDR1* and β-actin.

4.3. The binding of resistance modifiers to nucleic acids

4.3.1. Spectrophotometric studies

The spectra of a series of solutions of compounds were investigated in the presence of increasing amounts of CT DNA or *E.coli* tRNA in the Institute of Pharmacy, University of Manchester. The spectra were recorded on a Cary Varian IE spectrophotometer in 0.03 M tris

buffer containing 0.018 M NaCl at pH 7.00 for 2.50 x 10^{-5} M solution of compound and are annotated with the polynucleotid:ligand ratios for up to 10:1 polynucleotid:ligand based on polynucleotid phosphate:ligand.

4.3.2. Thermal denaturation studies

All experiments with polynucleotides, DNA and tRNA were conducted in 0.03 M tris(hydroxymethyl) aminomethane buffer containing 0.018 M NaCl, adjusted to pH 7.40 by addition of dilute hydrochloric acid; and with or without the addition of 0.01 $MgCl_2$ in the case of t-RNA.

Thermal denaturation profiles of drug:polynucleotide complexes were recorded on a Cary Varian Model IE spectrophotometer using the Cary temperature controller connected to a Cary 1/3 multicell block. The six-cuvette cell holder was heated by a circulating solution of ethylene glycol, electronically controlled and programmed. The cuvette temperature was recorded by means of a probe inserted into one of the cuvettes. Six sample and six reference cuvettes were used each time. Two of the samples were for polynucleotide alone, and in general all runs were duplicated. All cells were allowed to equilibrate to the starting temperature for at least 5 minutes. All cuvettes, including that containing the temperature probe, were stoppered with teflon caps after debubbling, and space was allowed for expansion of the solutions.

4.4. Assay for measuring antiproliferative activity

Greiner 96-well flat-bottom microtrays were used throughout the study. For diluting and distributing cells and reagents, an 8-channel pipette was used. L5178Y par and mdr tumor cells were resuspended in the growth medium and seeded into the microtrays at appropriate concentrations (4000/0.2 mL/well). The growth medium was McCoy's 5A medium containing 10 % horse serum, L-glutamine (1 mL/100mL), streptomycin (1mL/100 mL), and nystatin (0.1mL/100 mL). Then, from 0 to 50 μg/mL of the compounds were added to the wells (5 μL/well). Cell controls and medium controls were set up in each tray. Cell control wells received 0.2 mL/well of growth medium, and medium control wells had no cell suspension but 0.2 mL of growth medium. The total volume of medium per well at this point was 0.2 mL. The trays were incubated further at 37 °C for 3 days after cell seeding in a CO_2 incubator.

At the end of the incubation period, the cells in the wells were stained with 20 μL MTT of 5 mg/mL stock solution for 4 hours, then 100 μL 10 % SDS dissolved in 1N HCl was given

and the samples were incubated for 24 hours. Inhibition of growth as well as the cytotoxic effects of the test samples were determined by measuring the optical density at 545 nm with a Awareness Stat Fax-2100 Technology Inc. vertical beam reader (reference 630 nm).

The average optical density of 2 wells of each dilution, as well as the controls, was calculated and the percentage inhibition was determined according to the formula:

$$100 - \frac{\text{(OD sample - OD medium control)}}{\text{(OD cell control - OD medium control)}} \times 100$$

4.5. Assay for measuring combined antiproliferative effect of cytostatics and resistance modifiers

For the checkerboard titration the anticancer compounds were diluted in the Grainer 96-well flat-bottom microplate. The stock solution of anticancer compounds and various modifiers were prepared and diluted in McCoy's 5A medium. The anticancer compounds were tested by using two-fold dilutions at concentrations ranging from 0,09 to 10 μg/mL. The modifiers were tested by using also two-fold dilutions at concentrations ranging from 0,05 to 25 μg/mL. The modifiers were added to the well with cytostatics in various concentration individually, then the volume was completed to 100 mL. Then the L5178Y par and mdr tumor cells were distributed in the wells in the growth medium at concentrations of 5000/0.2 mL/well. The plates were incubated at 37 °C for 3 days in a CO_2 incubator.

At the end of the incubation period the cells in the wells were stained with 20 μL MTT of 5 mg/mL stock solution for 4 hour, then 100 μL 10 % SDS was added with 1N HCl treatment for 24 hour. Inhibition of growth of the test samples were determined and quantified by measuring the optical density at 545 nm with a Awareness Stat Fax-2100 Technology Inc. vertical beam reader (reference 630 nm).

The inhibition of growth was calculated and the percentage inhibition was determined according to the formula:

$$100 - \frac{\text{(OD sample - OD medium control)}}{\text{(OD cell control - OD medium control)}} \times 100$$

In order to evaluate the activity of combinations of cytostatics and resistance modifier more accurately the fractional inhibitory concentrations (FIC) indices were calculated as $FIC_A + FIC_B$, where FIC_A and FIC_B represent the minimum concentrations that inhibited the growth of tumor cells for resistance modifier A and cytostatics B, respectively.

$$FIC_A = \frac{MIC_A \text{ combination}}{MIC_A \text{ alone}} \qquad FIC_B = \frac{MIC_B \text{ combination}}{MIC_B \text{ alone}}$$

A mean FIC index was calculated based on the following equation: $FIC_{index} = FIC_A + FIC_B$ and the interpretation made as follows: synergistic (< 0.5), additive (0.5 – 1.0), indifferent (>1), or antagonistic (> 4.0).

4.6. Multidrug resistance reversal effect, by decreasing Rhodamine 123 efflux

The L5178 mouse T cell lymhoma cell line was infected with the *MDR1/A* retrovirus as previously described (114). *MDR1* expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the mdr phenotype. The L5178 MDR cell line, and the L5178Y par cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine (1 mL/100mL), streptomycin (1mL/100 mL), and nystatin (0.1mL/100 mL). The cells were adjusted to a concentration of $2x10^6$/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquots to Eppendorf centrifuge tubes. Then the tested compounds were added in various concentrations (0.2-100.0 μL) of the 1.0 mg/mL stock solutions, and the samples were incubated for 10 min at room temperature. Then 10 μL (5.2 μM final concentration) indicator R123 was added to the samples, and the cells were incubated for a further 20 min at 37 °C, washed twice and resuspended in 0.5 mL PBS for analysis. The fluorescence of cell population was mesaured by flow cytometry with a Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the R123 exclusion experiments (101). The percentage of the control mean fluorescence intensity was calculated for par and mdr cell lines as compared to untreated cells. An activity ratio was calculated by the following equation on the basis of measured fluorescence values:

$$R = \frac{\text{mdr treated / mdr control}}{\text{par treated / par control}}$$

4.7. Quantum-chemical calculations for quantitative structure activity relationships studies (QSAR)

The molecular orbital calculations by a parametric method 3 (PM3) were performed with a MOPAC program (Version 6.01) at the Meiji Pharmaceutical University, Tokyo (117). The geometries of the neutral species (10-[3-(phthalimido)propyl]-2-chloro-10*H*-phenothiazine [**1**], 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [**2**], 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**] 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [**4**]) were optimized with respect to all geometrical parameters with the Broyden-Fletcher-Goldfarb-Shanno algorithm incorporated in the program. For these calculations, the FACOM M770 computer at the Josai University Information Sciences Center (Saitama) was used. Some parameters of the molecules were selected for structure-activity relationship studies such as HOMO-, LUMO-energies, dipole moment values, 1st excited singlet state dipole moment and logP values etc.

4.8. Treatment of patients

Treatment of patients was performed at the University Clinic of Debrecen, and leukocytes were seperated and studied for drug accumulation *ex vivo*.

Patient SzL was diagnosed as having acute myeloid leukemia (AML). The first regimen of treatment was with daunorubic (DR) (45 mg/m^2 body surface for 3 day duration) and Alexan (100 mg/m^2 body surface for 7 day duration). After relapse, the second regimen of treatment was with TAD/COAP administration (Tioguanin, Alexan, Daunorubicin / Cyclophosphamid, Vincristine, Alexan, Prednisolone). At the time of blood sampling the WBC was 28.5 x 10^9 cells/L and the blast count was 38 % as measured by Giemsa staining. This patient was in complete relapse at the time of the flow cytometric analysis and later deceased. Patient PL was diagnosed with AML. The first regimen of treatment was with adriamycin (AM), (45 mg/m^2 body surface for 3 day duration) and Alexan (10 mg/m^2 body surface for 7 day duration). After relapse, the second regimen involved TAD/COAP. At the time of blood sampling the WBC was 2.2 x 10^9 cells/L and blasts were not detected. At the time of writing the manucript the patient is asymptomatic. Patient NV was diagnosed with AML. The first regimen of treatment was with DR (45 mg/m^2 body surface for 3 day duration) and Alexan (100 mg/m^2 body surface for 7 day duration). This treatment was repeated after objective improvement. At the time of the blood sample WBC was 7.1 x 10^9 cells/L and the blast count was 9.8 %. This patient later died.

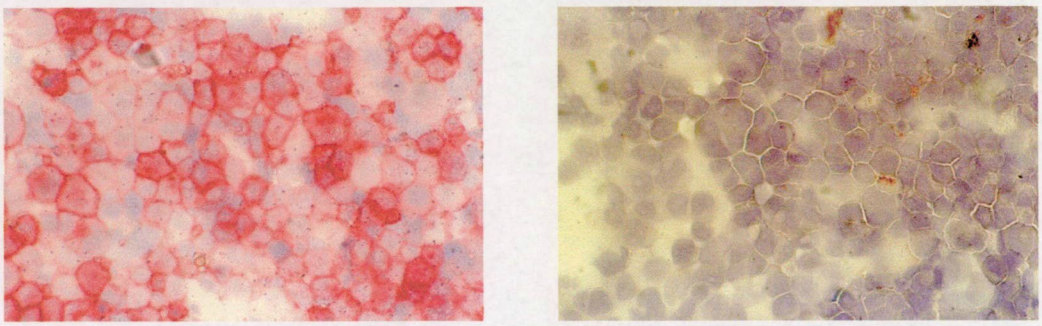
RESULTS AND DISCUSSION

5.1. Regulation of multidrug resistance gene expression by reduction of the MDR1 gene activity

The large number of cancer patients and the emerging mdr of cancer cells often leads to the ineffectiveness of chemotherapy. The majority of anticancer medicines do not inhibit multidrug resistance but can induce the expression of the *MDR1* gene. Therefore, we supposed that physicians have to change the strategy of chemotherapy. One way is to prevent the induction of *MDR1* gene.

The *MDR1* gene can be considered as a member of the family of stress-induced genes. Due to some similarities, the *MDR1* gene promoter can also be activated by various environmental stresses, carcinogens and anticancer drugs. Therefore it was suggested that the *MDR1* gene promoter could be a target for stress-induced gene regulation.

In our experiments the presence of P-gp in drug treated lymphoma cells and functionally similar brain capillary endothelial cells were examined in trypsinized and acetone fixed cells with the use of two monoclonal antibodies. One of these, mAb 4E 3.16 requires the epitope of *MDR1* to be on the exterior surface of the cell membrane. The second one, C219 recognizes an internal highly conserved sequence found in both *MDR3* and *MDR1* isoforms of P-gp, and was studied by us with a fluorescence microscope. In this way the external and internal fragments of P-gp were identified in both cell lines. Trypsin treatment (0.25 % trypsin for 5 min) markedly decreased the expression of binding of mAb specific for the external fragment of P-gp on the surface of lymphoma or brain capillary endothelial cells. The binding of mAb to the internal compartments of P-gp was not changed after trypsinization.



A **B**

Figure 5. Identification of P-gp with 4E 3.16 monoclonal antibody on KB-3-1 multidrug resistant (**A**) and sensitive cells, as a control (**B**) in fluorescence microscopy. (KB-3-1cell lines are present of Prof. Gottesman, National Institutes of Health, Bethesda, Maryland, USA).

In our experiments we investigated how to prevent the induction of the *MDR1* gene by the PZ and TFP. On the Figure 6. we can see, that PZ and TFP are able to downregulate the *MDR1* gene expression. If P-gp is not overexpressed then anticancer drugs possibly accumulate in effective concentrations in the tumor cells. At any rate the PZ and TFP reduced the gene expression in the tumor cells, when the cells were cultured in the presence of low concentration of the drugs for 48 hours (Figure 6.).

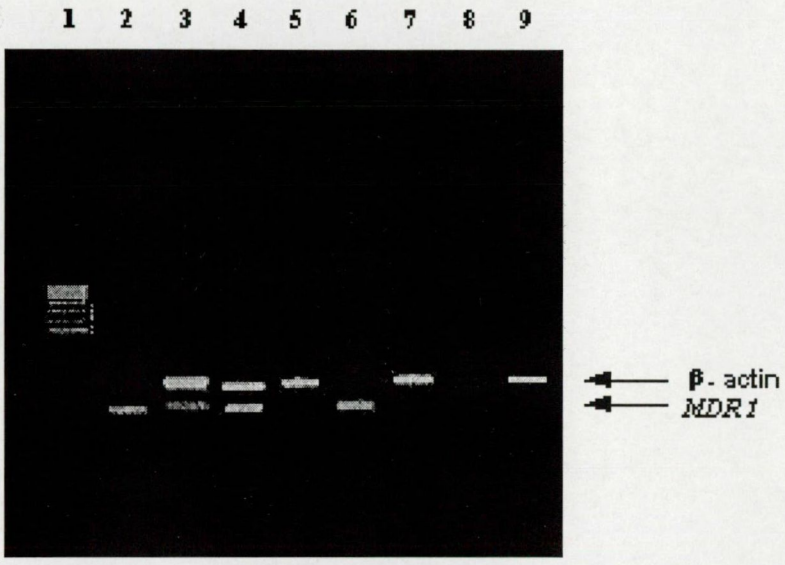


Figure 6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *MDR1* mRNA expression in the presence of TFP and PZ.

Lane 1. DNA molecular weight markers (Boehringer, Mannheim, Germany). *Lane 2. MDR1* transfected cells grown in the presence of 0.5 μg TFP (*MDR1* without β-actin band). *Lane 3. MDR1* transfected cells grown in the presence of 0.5 μg TFP (*MDR1* and β-actin bands). *Lane 4. MDR1* transfected cells untreated (*MDR1* and β-actin bands). *Lane 5. MDR1* transfected cells grown in the presence of 2.0 μg PZ (β-actin primer without *MDR1* primer). *Lane 6.* mdr control primer (without β-actin primer). *Lane 7.* control par cells (with β-actin primer). *Lane 8.* negative control, par cells only (mdr primer). *Lane 9. MDR1* transfected cells grown in the presence of 2.0 μg PZ (β-actin and *MDR1* bands).

The results show that at least two independent biological processes are responsible for the reversal of multidrug resistance of tumor cells, such as the reduced activity of the efflux pump and the down regulation of *MDR1* gene.

5.2. Possible binding of resistance modifiers to DNA or RNA

UV visible spectrophotometry

When a ligand possessing a chromophore binds to a polynucleotide its UV spectrum will be altered as consequence of the change in its environment. It is well known that the long

wavelength absorption band of ligands which intercalate between the base-pairs of a polynucleotide undergoes a shift to longer wavelengths with a corresponding decrease in absorbance, i.e. a bathochromic and hypochromic change, respectively (Table 5.). This has been used extensively in the investigation of the intercalation of acridines into DNA (118).

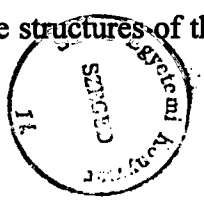
Interaction with DNA

The chains of the polynucleotide duplex melt apart when the hydrogen bonds between their base-pairs break on increasing the temperature or by titration with acid or alkali. Melting can be monitored conveniently by an increase in absorbance (hyperchromic effect) that results from the disruption of base stacking. The mid-point melting temperature (Tm) is a characteristic of polynucleotide stability and is affected by several conditions including the number of triple hydrogen bonded GC pairs it contains: the larger the mole function of GC pairs, the higher the temperature or pH of the melting transition.

Measurement of the increase in the thermal denaturation (ΔTm) of DNA or a homopolymer as a result of ligand : polynucleotide complex formation, compared to that of polynucleotide alone measured in the same experiment, provides a good starting point for studying the binding of a polynucleotide to different compounds. The value of ΔTm is not directly convertible to a binding constant as it represents an averaging of the binding constants over the multiple sequences of the heterogenous sites of the polynucleotide. However, the ΔTm value reflects the affinity of the resistance modifier compound for the polynucleotide or DNA and can give an indication of sequence specificity (e.g. G-C pairs) for polynucleotide-binding molecules but, because of the differences in the inherent Tm values of various biopolymers, the magnitudes of ΔTm values for different polymers cannot be compared directly.

1. The tricyclic antidepressants: butaclamol and clopenthixol and crocin produced no increase in Tm in DNA indicating that no intercalation into DNA was occurring with these compounds. The negative value for ΔTm may indicate some weakening of the hydrogen bonding of the DNA helix as caused by these compounds. An essential prerequisite for intercalation is a linear or near linear aromatic ring system. This is not present in butaclamol, clopenthixol or crocin (see structure of butaclamol ring system Fig. 13.), so intercalation does not occur (Table 5.).

2. The ellipticine derivatives, H3001 and RZ541 compounds, closely resemble the structure of ellipticine, as may be seen from the structures of the two compounds overlaid by



ellipticine. However, H3001 shows a more obvious intercalation than does RZ541 (Table 5.), in that the shift in ΔTm is greater with H3001, and there is a pronounced bathochromic (shift to longer wavelenght) and hypochromic (decrease in absorption) shift with H3001. Both compounds have a planar aromatic structure. However, there is a second essential prerequisite for intercalation. The compounds must possess a protonatable nitrogen group. Both compounds possess a 1,2,4 triazine ring system which should have a pKa in the region 7-8 which would mean weak protonation at pH 7.0. However, in addition, both compounds possess a fourth nitrogen. In H3001 this is exposed as part of a pyrollidine ring and would be more basic than the indoline ring nitrogen of RZ541. Consequently one would expect that H3001 would be more protonated at pH 7.0 than RZ541 leading to a more pronounced intercalation.

Table 5. Interaction of some tricyclic and other compounds with DNA and tRNA

Compounds	ΔTm (DNA) °C	λmax Cpd (nm)	λmax Cpd + DNA (nm)	Bathochromic shift (nm)	ΔTm (tRNA) °C	ΔTm (tRNA + Mg ions) °C
H3001	1.0	313.9	315.5	1.6	2.5	3.4
H31OH	2.5	389.0	407.7	18.7	-9.0	3.4
H36ON	2.1	415.6	417.2	1.6	-10.0	-9.7
RZ541	0.6	321.0	321.0	-	14.5	-10.0
7,8 dioxoCPZ	2.3	502.2	503.9	1.7	-	-
Mepacrine	21.0				20.0	17.7
(-)butaclamol	-5.0				0.0	-16.7
(+)butaclamol	-0.5				5.5	-
Cis-clopenthixol	-4.0				-	-11.7
Trans-clopenthixol	-4.0				-	-
Crocin	-2.4	474.4	474.4	-	-3.0	5.3

Tm DNA = 71.4 °C, Tm tRNA = 50.0 °C, Tm tRNA + Mg ions = 67.7 °C

H31OH and H36ON compounds more closely resemble 7,8 dioxoCPZ in structure and both (like 7,8 dioxoCPZ) show a much more pronounced intercalation into DNA than either H3001 or RZ541 (compare the ΔTm and bathochromic/hypochromic shift values give Table 5.). Both compounds possess near planar aromatic ring systems, but H31OH (and 7,8 dioxCPZ) have a tertiary amine side chain nitrogen (pKa about 10.0) which would be fully protonated at pH 7.0 whilst H36ON is a quaternary salt and is permanently charged. This could explain the increased intercalation seen for these compounds.

Interaction with RNA

It is known that magnesium ions can stabilise the structure of tRNA and that in the absence of stabilising anions tRNA has a considerably more flexible structure (119). Totsuka et al. claim that tighter binding to tRNA in the presence of stabilising magnesium ions indicates that the ligand binds somewhere near the variable loop region.

The results indicate that H3001 and H31OH are binding to tRNA and in this variable loop region, as opposed to mepacrine which is unaffected by the presence of magnesium ions and can be presumed to be binding at a different site on the tRNA molecule.

RZ541 appears to bind to the unstabilised structure of tRNA, presumably by a non-specific hydrophobic binding, but binding is abolished in the stabilised structure and it causes a large decrease in the thermal stability of the tRNA. It is possible that RZ541 cleaves the t-RNA molecule in the presence of magnesium ions as does (-)butaclamol and cis-clopenthixol in a manner similar to the enediyne C-1027 (120) whilst the quaternary ammonium compound H36ON causes a large decrease in thermal stability of tRNA in both the presence and absence of magnesium ions possibly by disrupting hydrogen bonding.

5.3. Mdr reversal effect of some representative compounds

Firstly some well-known anticancer drugs were tested for mdr reversing activity. Platidiam, novantron, fluorouracil, bleomycin and methotrexate did not affect the efflux pump activity (Table 6.) or even decreased the drug accumulation.

Table 6. The effect of some anticancer drugs and resistance modifiers on R123 accumulation by mdr mouse lymphoma cells.

Compounds	Concentration (μg/mL)	Fluorescence activity ratio
Verapamil	8.0	2.29
Platidiam	2.0	0.53
	20.0	0.60
Novantron	2.0	0.67
	20.0	0.70
Fluorouracil	2.0	0.47
	20.0	0.73
Bleomycin	0.5	0.88
	5.0	0.80
Methotrexate	5.0	1.00

However, some vinca alkaloids, e.g., vinblastin, vincristin and thaliblastin enchanced the R123 accumulation in the mdr cells. At the same time the structurally similar reserpine had a

very slight effect, and that yohimbin was ineffective (Table 7.).

Table 7. The effect of some vinca alkaloids and structurally related compounds on R123 accumulation by mdr mouse lymphoma cells.

Compounds	Concentration (μg/mL)	Fluorescence activity ratio
Thaliblastine	2.0	20.4
	20.0	13.5
Vinblastine	2.0	22.3
	20.0	23.9
Vincristine	2.0	16.7
	20.0	34.5
Reserpine	2.0	0.85
	20.0	1.43
Yohimbine	2.0	0.49
	20.0	0.68

Secondly, some phenothiazines which are known to reverse antibiotic resistance of bacteria, were investigated.

Some ring-substituted chlorpromazine derivatives had a moderate effect or were ineffective. However, benzo(a)phenothiazines were effective in elevating the R123 accumulation of mouse lymphoma cells in relatively high concentration from 2.0 to 20 μg/mL and in this study the 5-oxo-benzo(a)phenothiazine was the most active compound (Table 8.).

Table 8. The effect of some phenothiazines and benzo[a]phenothiazines on R123 accumulation by mdr mouse lymphoma cells.

Compounds	Concentration (μg/mL)	Fluorescence activity ratio
Verapamil	8.0	3.81
7,8-dioxochlorpromazine	2.0	0.89
	20.0	0.70
6,9-dihydroxichlorpromazine	2.0	0.82
	20.0	0.79
6,9-dioxochlorpromazine	2.0	0.79
	20.0	2.38
5-oxo-5H-benzo[a]phenothiazine	2.0	0.85
	20.0	1.40
6-hydroxy-5-oxo-5H- benzo[a]phenothiazine	2.0	1.20
	20.0	1.00
6-methyl-5-oxo- benzo[a]phenothiazine	2.0	3.44
	20.0	2.92

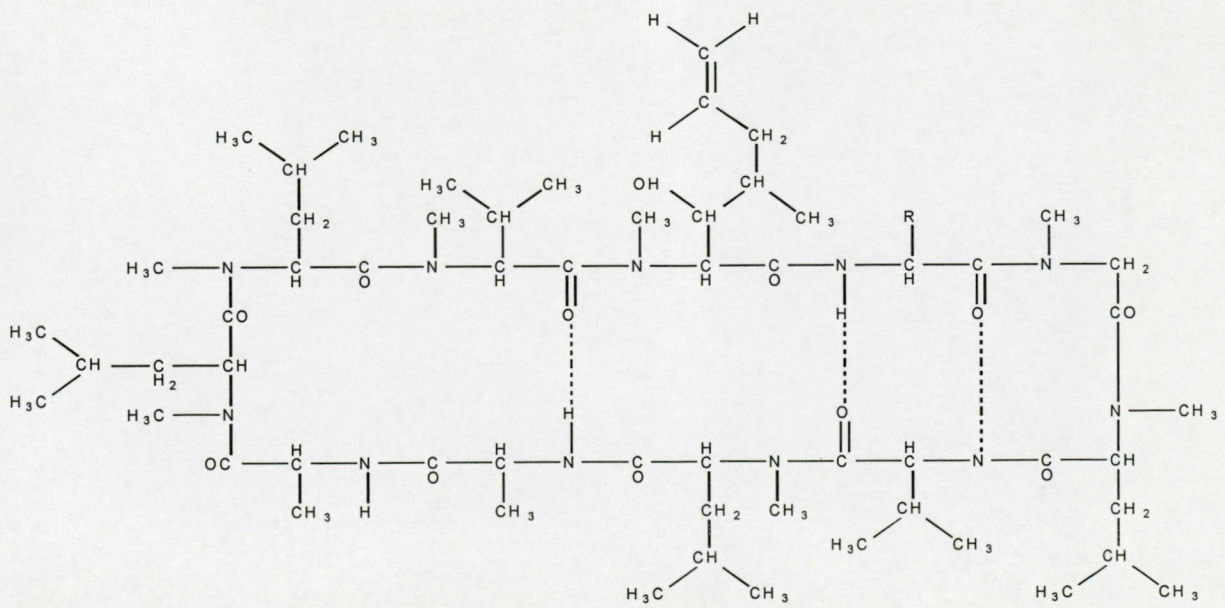
The same phenothiazine compounds were less effective on the drug efflux of human brain

capillary endothelial cells than on mdr cells (Table 9.). The inhibition was low, but called our attention to the role of chemical structure of the phenothiazines in the mdr reversal effect.

Table 9. The effect of substituted phenothiazines on R123 accumulation by human brain capillary endothelial cells.

Compounds	Concentration (μg/mL)	Fluorescence activity ratio
Verapamil	8.0	0.81
7,8-dioxochlorpromazine	2.0	1.11
	20.0	0.85
6,9-dihydroxychlorpromazine	2.0	1.06
	20.0	1.03
6,9-dioxochlorpromazine	2.0	1.05
	20.0	0.80
5-oxo-5H-benzo[a]phenothiazine	2.0	1.10
	20.0	0.69

To clarify the effect of chemical structure in the biological active two cyclosporin, were tested. The cyclosporin A and PSC 833 were much more inhibitory to the P-gp function of mouse lymphoma cells than the previously tested phenothiazines; the cyclosporin D had a higher activity than cyclosporin A (Table 10.). This may give an opportunity to improve the mdr reversal effect by further changes in the chemical structure of the polypeptide (Figure 3.). The conclusion of the experiments is that mdr can be reversed, and the effect is dependent on the chemical structure.



Cyclosporin A: R= $-CH_2CH_3$ Cyclosporin D: R= $-CH(CH_3)_2$

Figure 7. Chemical structure of cyclosporin A and D.

Table 10. The effect of cyclosporins on R123 accumulation by mdr mouse lymphoma cells.

Compounds	Concentration (μg/mL)	Fluorescence activity ratio
Cyclosporin A	2.0	12.72
(Sandimmune)	4.0	24.87
	8.0	50.04
	10.0	51.00
Cyclosporin D	2.0	99.29
(PSC-833)	4.0	100.47
	8.0	102.78
	10.0	112.57

The different activity of various substituted phenothiazines or cyclosporins could be related to the binding site of the compounds on the mdr P-gp. Therefore, the importance of the structure of the binding site was analysed indirectly by exploiting the differences in chemical structure of transmembrane loops of P-gp.

It is known that, the first loop is glycosilated by polylactosamine. Since the tomato lectine (TL) is specific for the polylactose amine this lectin was tested in our system. The mdr reversal effect of PZ was determined by TL treatment (Table 11.). The binding of PZ (or TFP) to the P-gp of the tumor cells was decreased by the interaction of TL with the polylactosamine moiety of the first loop of P-gp. That means that the first loop has a key role in the biological effect of P-gp. The TL itself was not able to affect the efflux pump activity in the tumor cells, but the inhibition by PZ (or TFP) was reduced. The TL reduced the inhibitory effect of PZ (or TFP) (Table 11.), showing that the localisation (or accessibility) of the P-gp is not the same in the two cell lines.

Table 11. Effect of TL on the mdr of mouse lymphoma and human brain capillary endothelial cells in the presence of PZ.

Compounds	Concentration (μg/mL)	Fluorescence activity ratio
Mouse lymphoma		
Promethazine	5	3.80
Tomato lectin	50	0.90
Tomato lectin + promethazine	50 + 5	2.56
Human brain capillary endothel		
Promethazine	5	0.71
Tomato lectin	50	1.17
Tomato lectin + promethazine	50 + 5	0.82

The results show that there are some differences in the lectin sensitivities of the two cell

lines. Based on the results we suppose that the difference can be the consequence of different localization of the two efflux proteins in the membrane lattice. It is possible that more than one mechanism is involved in the action of PZ and TL on the efflux pump of tumor cells. The effectiviness of PZ called our attention to the role of calcium channels and calmodulin. Therefore the effect of new phenothiazines were further studied (Table 12.).

5.4. Mdr reversal effect of phthalimido- and chloroethyl-phenothiazines

The fluorescence activity ratio of the group 1, i.e., 10-[3-(phthalimido)propyl]-2-chloro-10*H*-phenothiazine [**1**] (n=3) and 1-(2-chloroethyl)-3-(2-trifluoromethyl-10*H*-phenothiazin-10-yl)propyl-1-urea [**4**] (n=3) at concentrations of $1\text{-}2\text{x}10^{-5}$ M, was shown to have a stronger activity 10.65 and 5.34, respectively. The fluorescence activity ratio of the group 2, i.e., 10-[4-(phthalimido)butyl]-2-chloro-10*H*-phenothiazine [**2**] (n=4) and 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazin-10-yl)butyl-1-urea [**3**] (n=4) was weaker with values of 8.91 and 4.52, respectively. The relationship between multidrug resistance reversal effects of phenothiazines at a concentration of $2\text{x}10^{-5}$ M also showed a similar correlation with respect to 1-(2-chloroethyl)-3-(2-chloro-10*H*-phenothiazine-10-yl)butyl-1-urea [**3**] (n=4) (Table 12.).

Table 12. Effect of phenothiazines [**1, 2, 3, 4**] on multidrug resistance L5178 cells.

Compounds	Conc. (M)	FSC	SSC	FL-1	Fluorescence activity ratio
Par control		504.86	184.75	724.50	
Mdr control		552.95	211.52	23.80	
Verapamil	4 μg/ml	545.75	216.62	57.84	2.43
10-[N-(phthalimido)alkyl]-2-substituted-10H-phenothiazines					
[1]	$1\text{x}10^{-5}$	541.63	215.98	253.59	10.65
	$2\text{x}10^{-5}$	538.05	219.75	564.72	23.72
[2]	$1\text{x}10^{-5}$	535.16	220.48	212.14	8.91
	$2\text{x}10^{-5}$	537.95	22.88	369.03	15.50
1-(2-chloroethyl)-3-(2-substituted-10H-phenothiazin-10-yl)alkyl-1-ureas					
[3]	$1\text{x}10^{-5}$	535.93	210.71	107.66	4.52
	$2\text{x}10^{-5}$	534.05	211.90	298.51	12.54
[4]	$1\text{x}10^{-5}$	535.31	216.00	127.30	5.34
	$2\text{x}10^{-5}$	532.75	226.20	21.26	9.21

From the measurement of fluorescence activity ratio by R123, among four phenothiazines, i.e., [**1**], [**2**], [**3**] and [**4**], a correlation was found between multidrug resistance reversal effect and chemical structure (Table 12.).

Moreover, the phenothiazines [**1, 2, 3, 4**] did not show any mutagenic effect, but were antimutagenic according to the Ames test (121). Therefore, we suspect that the direct antitumor activity of phenothiazines can be exploited for experimental drug design.

Some correlation between biological effect and chemical structure was attempted. The dipole moments (in Debye unit, D) π-LUMO, or π–HOMO energies (eV unit), and the distance (Å unit) between nitrogen of phenothiazine ring system and the terminal nitrogen of the aliphatic chain group were calculated for estimating the relationships between electronic structure and antitumor activity. A significant relationship between dipole moment, π-LUMO, or π–HOMO energies and antitumor activity could not be established (Table 13.).

Table 13. Calculated dipole moments, π-LUMO, π–HOMO energies and distance between N10 and nitrogen atom on four componds [**1, 2, 3, 4**] by PM3 method

Compounds	**$(CH_2)n$**	**Dipole moment (Debye unit)**	**π-LUMO (eV unit)**	**π–HOMO (eV unit)**	**D[1)]**
[**1**]	3	3.31	-1.188	-7.997	5.04
[**2**]	4	4.31	-1.195	-7.982	6.33
[**3**]	4	2.92	-0.549	-8.162	6.30
[**4**]	3	4.34	-0.972	-8.433	5.00

[1)] Distance between N10 and nitrogen atom (in Å units)

In all cases, the PM3 optimized geometries were slightly nonplanar, and calculated structures are shown in Figure 8.

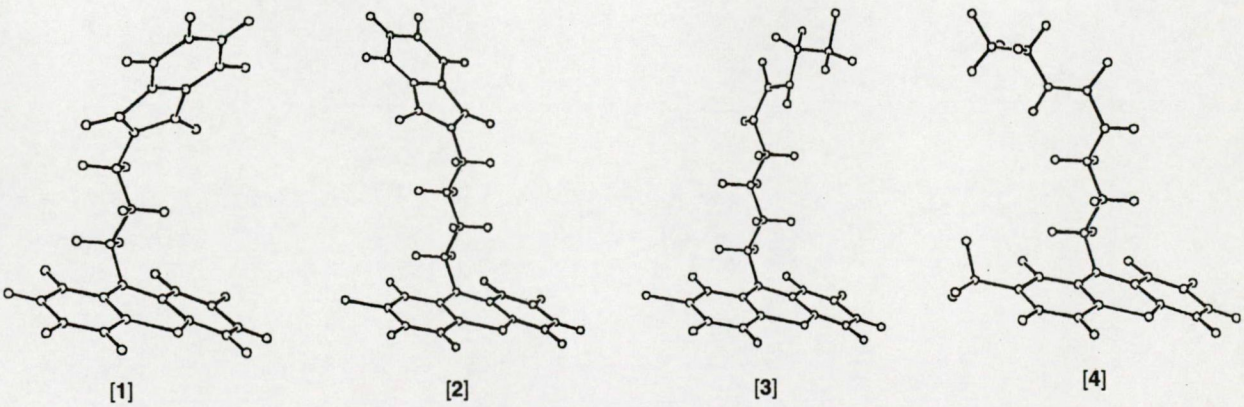


Figure 8. The optimized structures of 4 compounds [**1, 2, 3, 4**] by PM3 method.

We suppose that accessibility of a lone electron pair on the ring nitrogen of the phenothiazine and the distance between this nitrogen and the terminal nitrogen of methylene group might have a role on the activity of the compounds. The 3-(phthalimido)-propyl group

of [**1**] and 1-(2-chloroethyl-1-urea)-3-propyl group of [**4**] have the tendency of orientating inside of the phenothiazine skeleton. On the other hand, (phthalimido)-butyl group of [**2**] or a 1-(2-chloroethyl-1-urea)-3-butyl group of [**3**] tends to orientate away from the phenothiazine skeleton (Figure 8.). This is an apparent difference between group 1 compounds and group 2 compounds. This active conformation of group 1 compounds might be convenient for attack on the electrostatic P-gp membrane sites (122). Additionally, the proposed binding can be supported by the C-C bonds of phenothiazine ring 1.390-1.405 Å. The double bonds were 1.386-1.398 Å except for the central bonds such as C4a-C10a with 1.402-1.408 Å, the C9a-N10 and N10-C10a were 1.437-1.449 Å and 1.437-1.450 Å, respectively, and the C4a-S5 and S5-C5a were 1.755-1.760 Å, respectively. This may be taken to mean that the antitumor activity of four phenothiazines [**1, 2, 3, 4**] depends on the number of a methylene groups in the sidechains (n=3, 4, 5).

We could conclude from these results that electron-distribution contributions are strongly related to a lone electron pair on the ring nitrogen of the phenothiazine and on the terminal nitrogen (3N or 4N) of alkyl group.

In the investigation of the antitumor activity of „half-mustard type" phenothiazines against a total of 54 tumor cell lines such as leukemia, melanoma, lung, colon, ovarian, breast, renal and prostate cancer, and brain tumor cell lines we found that phenothiazines with a butylene linkage showed higher antitumor activity than those with a propylene linkage. It is possible that the anticancer effect of phenothiazines might depend on a particular membrane structure in the cancer cells or on the presence of a sensitive part of the cell cycle. The phenothiazines showed „cancer type-specific" antiproliferative action. It is obvious that the colon cancer, leukemia and melanoma are more sensitive than prostate or ovarian cancer. The compounds might act on cancer cells via alkylurea-induced alkylation of proteins or on DNA by an intercalation mechanism.

5.5. Mdr reversal effect of some new 3-benzazepines

The mdr reversing effects of 2,3,4,5-tetrahydro-3-benzazepinones [**1-6**] and 2,3,4,5-tetrahydro-1H-3-benzazepines [**7, 8, 9, 10**], and two neurotransmitters such as dopamine (DA) and norepinephrine (NE) were compared to that of verapamil in terms of chemical structures, with the use of a L5178 mouse lymphoma cell line. The effect was measured in terms of the magnitude of the fluorescence ratio between treated and untreated groups of cells. Compound [**8**] has the highest activity on mdr reversal, among 12 compounds used in this research. This

compound was two-fold more potent than verapamil. Then, compound [**8**] might be an anti-mdr inducing agent of great interest, because the affinity of [**8**] to dopamine D1 and D2 receptors was reduced by introduction of a trifluoromethyl (CF_3) group at position 2 of the benzazepine ring (105).

We have reported that benzazepines [**7-9**] induced apoptotic cell death in human promyelocytic leukemia HL-60 cells and the cytotoxic activities of [**7-9**] were 1.3, 1.3 and 2.0 times higher than that of DA, respectively. Additionally, it was found that apoptosis induced by these benzazepines [**7-9**] is coupled with their radical generation (105). These results are show in Table 14.

Table 14. Mdr reversal effect of some new 3-benzazepines.

Compounds	Conc. μg/mL	FSC	SSC	FL-1	Fluorescence activity ratio
Par control		504.86	184.75	1060.98	49.98
Mdr control		552.95	211.52	21.23	1.00
Verapamil	5	545.74	216.62	88.72	4.18
Benzazepines:					
[**1**]	5	473.30	188.91	9.85	0.46
[**2**]	5	425.16	205.05	18.69	0.88
[**3**]	5	494.57	193.54	12.97	0.61
[**4**]	5	467.29	188.79	16.39	0.77
[**5**]	5	491.72	190.79	44.77	2.11
[**6**]	5	507.50	197.30	13.89	0.65
[**7**]	5	469.78	187.09	17.01	0.80
[**8**]	5	447.77	191.53	177.91	8.38
[**9**]	5	477.28	189.43	16.12	0.76
[**10**]	5	507.71	198.41	28.68	1.35
Dopamines:					
Dopamine	2	579.02	251.38	21.77	0.84
	20	571.20	251.12	23.47	0.93
Norepinephrine	2	569.10	256.09	21.32	0.82
	20	564.14	254.11	19.53	0.80

The effectiveness of phenothiazines called our attention to the role of some drug binding receptors, and calcium channel (and possibly calmodulin) in the mdr reversing effect. To examine this benzazepine compounds were tested and were found effective inhibitors.

5.6. Mdr reversal in tumor cells by 6,12-dihydro-1-benzopyrano [3,4-b][1,4] benzothiazin-6-ones compounds

Benzo[*a*]phenothiazines have antitumor activity against some tumor cells (103). They induce significant apoptosis of human myelogenous leukemic cell lines such as ML-1, U-937

and THP-1 (123), and affect the tobacco tissue culture and hormone requirement (124). Based on our estimated structure-activity relationships, it was expected that 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones may have some antitumor activity and may cause mdr reversal in resistant tumor cells due to their electron donating properties.

The effect of 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones **[7-12]** and structurally „similar" coumarins **[13-15]** was tested on the mdr reversal in tumor cells. The coumarins used in this study were ineffective (Table 15.).

Table 15. The effect of 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones **[7-12]** and related coumarins **[13-15]** on the mdr reversal on L5178 tumor cells with multidrug resistance

Compounds	Conc. (μg/mL)	FSC	SSC	FL-1	Fluorescence activity ratio
Par control		501.31	335.65	1130.80	
Mdr control		552.47	385.44	18.78	
Verapamil	8	540.50	381.20	159.37	8.49
[7]	2	551.31	375.07	9.22	0.49
	20	53343	400.03	10.70	0.57
[8]	2	55739	375.09	10.71	0.57
	20	54154	388.52	29.27	1.56
[9]	2	55348	379.05	14.64	0.78
	20	54472	375.61	26.90	1.43
[10]	2	55191	385.77	13.82	0.74
	20	54369	380.65	20.86	1.11
[11]	2	54058	368.60	8.33	0.44
	20	55307	374.34	11.25	0.60
[12]	2	55050	386.64	9.61	0.51
	20	54476	382.16	11.91	0.63
[13]	2	55861	396.64	9.90	0.53
	20	55992	389.56	9.72	0.52
[14]	2	55913	393.86	10.94	0.58
[15]	2	52256	397.48	10.76	0.57
	20	51396	371.64	11.95	0.64

Three 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones of **[8]** (fluorescence activity ratio 1.56), **[9]** (fluorescence activity ratio 1.43) and **[10]** (fluorescence activity ratio 1.11) at 20 μg concentration had a moderate activity on mdr reversal (fluorescence activity ratio >1). However, three 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones of **[7, 11, 12]** (fluorescence activity ratios 0.57, 0.60 and 0.63, respectively) without methyl or benzo group at position 1 or 2 together reduced the R123 accumulation in tumor cells, probably by inducing the efflux pump mechanism or by causing a direct membrane injury (Table 15.).

5.7. The significance of multidrug resistance reversal

Based on the *in vitro* experiments we concluded, that the pretreatment of cancer cells with resistance modifiers like fluphenazine makes cells more sensitive to anticancer drugs such as 5-FU, cisplatin and daunorubicin.

These experiments were performed with the checkerboard method. In the experiments fluphenazine was used as the representative resistance modifiers, and the antiproliferative effect of three different cytostatics were tested in various concentrations in the presence of different concentrations of fluphenazine. The most effective synergistic effect was found between fluphenazine and daunorubicin, FIX = 0.05 (Figure 9.). The 5-FU and fluphenazine combination was less effective, FIX = 0,25 (Figure 10.). The cisplatin and fluphenazine combination was less effective FIX = 0.4 (Figure 11.), although the synergistic effect of fluphenazine on the antiproliferative effect of daunorubicin was still apparent.

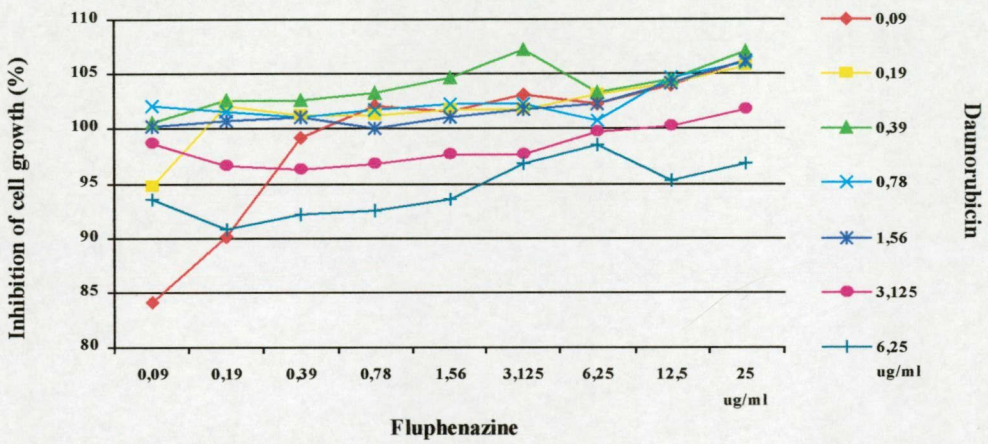


Figure 9. The inhibition effect of daunorubicin and fluphenazine on the mdr mouse lymphoma cells.

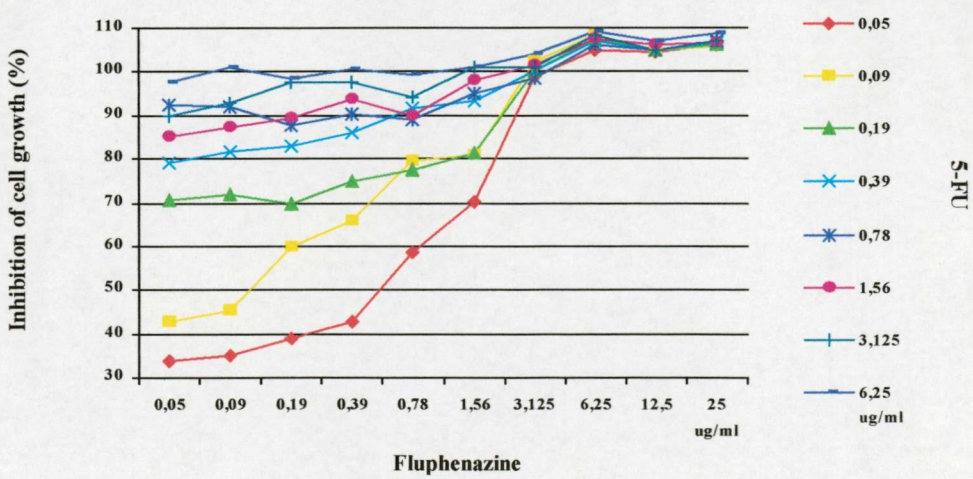


Figure 10. The inhibition effect of 5-FU and fluphenazine on the mdr cells of mouse lymphoma cells.

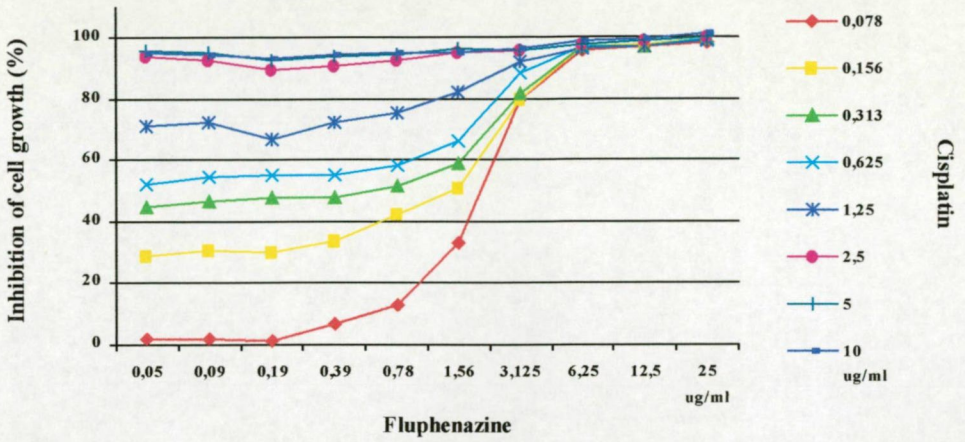


Figure 11. The inhibition effect of cisplatin and fluphenazine on the mdr mouse lymphoma cells.

5.8. The question of specificity ?

The side-effects of resistance modifiers on normal cells expressing the P-gp could be drug efflux because the organ-specific toxicity depends on the amount of P-gp in the various tissues. Mdr P-gp is also present in various normal cells (like capillary endothelium in the brain, colon, kidney and liver cells) and is associated with normal functions of cells. The P-gp, expressed in these tissues is responsible for the transport of toxic compounds into the CSF, bile or urine, etc. Consequently the reduced blood-biliary, blood-urine or blood-brain barrier can increase the organotoxicity of the antitumor drug. Therefore cancer or organ-specific efflux inhibitors could be used to increase cytostatic drug concentrations in malignant cells to avoid side-effects on other essential organs. For example, if drugs existed, which affect the blood-brain barrier selectively, they could be used for different purposes modifieing the effects of various drugs. Such drugs are able to increase the concentration of cytostatics in the tumor cells localized in the brain.

Substances entering or leaving the brain must pass through endothelial cells. The multidrug resistance P-gp in brain capillary endothelium may be one of the functional components of the blood-brain barrier, functioning as an active efflux pump (125).

On the other hand, cytokines can penetrate the blood-brain barrier at specific sites. One of the most important actions of the tumor necrosis factor (TNF), a pluripotent, proinflammatory cytokine, is that it can increase the permittivity of the barrier (126). It is interesting that endothelial cells both initiate and respond to a cascade of events triggered by cytokines. Enhanced formation of nitric oxide (NO), especially by the inducible nitric oxid synthase (iNOS), is largely stimulated by TNF. Nitric oxides are reactive intermediate molecules

functioning in vasodilatation, or even in neural transmission (127). TNF effect resulted in enhanced NO production. Western blot analysis showed enhanced expression of iNOS, which could be inhibited by pentoxifylline, an inhibitor of TNF action and NO synthesis. Flow cytometric analysis revealed that the brain capillary endothelial cells exerted P-gp expression (staining with the monoclonal antibody mAb C219), which was not influenced by TNF. However, the mdr function itself in these cells was decreased by TNF.

We assume that cultured endothelial cells are excellent tools for the investigation of the possible connection between NO production and mdr function, and for the estimation of the effect of different agents influencing these activities, which might be important in blood-brain barrier function, or even in cancer therapy of tumors originating from the central nervous system.

Table 16. Theoretical applications for general efflux pump inhibitors and organ-specific efflux pump inhibitors in combination with chemotherapeutics.

Type		**Specificity of**	
	Mdr-reversing compound's effect	*Anticancer drug accumulation*	*Expected results of combination*
A	GENERAL efflux Pump inhibitors: Quinidine Cyclosporin	SPECIFIC for: tissue or organ GENERAL for: uniform distribution in the body	Synergy Toxic or general immuno-suppression
B	SPECIFIC for tissue organ: Verapamil (cardiac) Trifluoperazine (CNS) Thiazinamium (lung)	SPECIFIC for: affinity to CNS, kidney, liver GENERAL for: Cytotoxic uniform distribution in the body	Strong synergy Synergy or organ-specific toxicity

Since the P-gp responsible for the multidrug resistance of the tumor cells also has a physiological function, (e.g., maintains the blood-brain barrier), it was reasonable to search for a specific mdr reversing compound and a selective antitumor drug (Table 16.).

5.9. The role of stereoselectivity

Chiral anticancer drugs exist since pharmacological differences have been found between stereoisomers. The single isomers of leucovorin, isophosphamide buthionine sulfaximine and verapamil are used in medical practice with better results than the racemic forms. However the role of chirality was not properly analysed (128, 129) in the reversal effect of drugs on the mdr. Despite similar drug targets in the cancer cells, the 50 % inhibitory concentration of

various anthracyclins, vinca alkaloids, podophyllotoxins, topoisomerase inhibitors and antibiotics varied to a great extent in multidrug resistant and sensitive cancer cells (130).

In the mdr cancer cells the efflux pump mechanism is responsible for treatment failures, therefore the inhibition of the efflux mechanism may result in an effective anticancer chemotherapy. However, we have to consider that normal cells also contain ABC transporters e.g., to function as detoxifiers. To avoid toxic side effects of novel resistance reversal compounds we need drugs with a selective inhibition of the mdr efflux without any effects on ABC transporters having physiological function in cancer cells. To achieve this effect three classes of known neuroleptic drugs with active and inactive stereoisomers were tested for mdr efflux inhibition and antiproliferative effects on sensitive and multidrug resistant cancer cell cultures, and the results compared with verapamil as a classic resistance modifier.

Before the reversal of multidrug resistance, the ID_{50} values of the compounds were determined and it was found that the parent cell line having 30 % higher ID_{50} was less sensitive to the stereoisomer pairs than the multidrug resistant cell line.

However, we have to consider that for determination of antiproliferative effect much smaller number of cells are treated for a much longer time than for the determination of reversal of mdr efflux pump (Table 17.).

Table 17. Determination of 50 % growth inhibitory dose of various stereoisomers on mdr and parent cells of mouse lymphoma.

Cell	Compounds	Cytotoxicity ID_{50} (μg/mL)
Parental	(-)Butaclamol	0.375
	(+)Butaclamol	0.400
	Cis-clopenthixol	0.697
	Trans-clopenthixol	0.325
	L-methotrimeprazine	0.450
	D-methotrimeprazine	0.375
MDR	(-)Butaclamol	0.285
	(+)Butaclamol	0.290
	Cis-clopenthixol	0.250
	Trans-clopenthixol	0.252
	L-methotrimeprazine	0.250
	D-methotrimeprazine	0.325

The effect of three different pairs of stereoisomers was studied on the activity of P-gp of the mouse lymphoma cells (Figure 12.). The levo- and dextro-methotrimeprazine enantiomer pairs had nearly the same effect by inhibiting the efflux pump activity of mdr P-gp in mouse

lymphoma cells. The results show that the two enantiomers do not differ significantly in the reversal of multidrug resistance.

The second pair used was the butaclamols. An interesting relationship was found between the mdr reversing effect and molecular configuration of the butaclamol stereoisomers. In these experiments the concentration dependent inhibition of stereoisomers on the efflux pump were compared. (-)Butaclamol was more effective than the biologically important (+)butaclamol enantiomer. The data indicated that there are no general rules for structure-activity relationships in the inhibition of mdr efflux pump activity by tricyclic compounds.

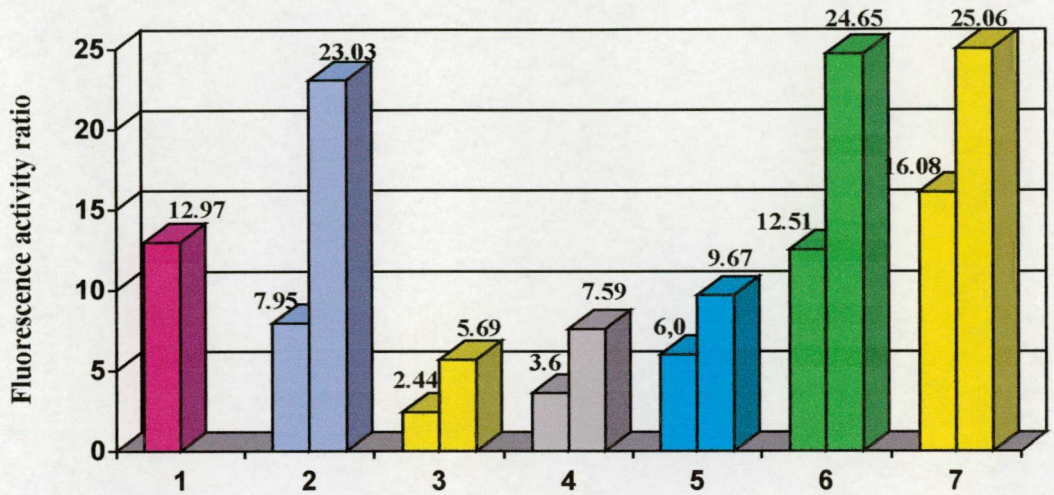


Figure 12. The effect of butaclamol, methotrimeprazine and clopenthixol isomers on the function of mdr efflux pump in mouse lymphoma cells.

1: Verapamil 5 μg/mL; **2:** (-)Butaclamol 0.5; 5 μg/mL; **3:** (+)Butaclamol 0,5; 5 μg/mL; **4:** L-methotrimeprazine 0.5; 5 μg/mL; **5:** D-methotrimeprazine 0.5; 5 μg/mL; **6:** Trans-clopenthixol 0.5; 5 μg/mL; **7:** Cis-clopenthixol 0.5; 5 μg/mL.

The third stereoisomer pair is the clopenthixols. The cis- and trans-clopenthixol had similar inhibitory effects on the mdr efflux pump of mouse lymphoma cells if results were compared to verapamil as control.

The (-) isomer of verapamil is 10-fold more potent as a calcium antagonist than the (+) isomer, however both enanthiomers were reported as equally effective in increasing cellular accumulation of anticancer drugs (131). The enantiomers of phenylalkylamines are equally potent in inhibiting drug transport by P-gp (132). However the potency of vinca stereoisomers in both wild type and mdr cells was dependent on the substituents of stereoisomeric form due to modulation of cytotoxicity of vinblastine (133). When the stereoisomers of verapamil and cyclosporins were compared on P-gp mediated efflux and NK cell mediated cytolysis

verapamils were more potent inhibitors of cytolysis than cyclosporins. On the contrary cyclosporins were more effective in inhibiting of the P-gp mediated R123 efflux than verapamil isomers (134).

The CNS - active and inactive - members of butaclamol enantiomers differed very much in mdr-reversal. That means that the drug binding has enantioselectivity on the P-gp. On the contrary, the similar effects of levo- and dextro-methotrimeprazine provided evidence against the enantioselective inhibition of the drug efflux pump.

How can the two different examples of enatiomer effects be exploited ? The CNS inactive enantiomers were inhibitory as in the case of methotrimeprazine and butaclamol. Therefore the mdr reversing effects of CNS inactive members of enantiomers could be exploited in the neoadjuvant chemotherapy of cancer to increase the effectiveness of several cytostatics in multidrug resistant cancer. The remarkable inhibitory effect of (-)butaclamol can be due to its higher log-P value, i.e., increased lipophilicity, or distribution coefficient with respect to a water-lipid system (logP).

Surprisingly, the stable stereoisomers of clopenthixol had similar concentration-dependent inhibition on the mdr efflux mechanism; again evidence for the lack of stereospecificity in drug binding of cancer cells (135). On the contrary, for normal cells in the CNS (136), in the heart (131), in bacterial plasmid replication inhibition of ATPase or cholinesterase enzymes (137), stereoselectivity and configuration of tricyclic compounds is essential (136).

Cis-clopenthixol inhibited the dopamine stimulated adenyl cyclase 10-fold compared to the trans isomer. In the case of methotrimeprazine enantiomers a significant stereoselective effect was observed on dopamine and 5-hydroxytryptamine (5-HT) receptors, whereas the opiate receptors did not discriminate between the levo- and dextrorotatory isomers (138).

The basis of selectivity of some stereoisomers may be due to a rigid configuration of the receptor sites. Another explanatory concept may be that the individual members of stereoisomer pairs have different energy levels for binding. Indeed, the cis-stereoisomer has a more stable configuration with a lower energy level than the trans-form of clopenthixol. The differences in the energy levels in the excited state can be even higher (139).

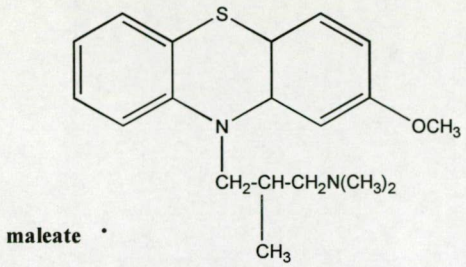
Based on the relatively high concentration of drugs used in our experiments, potential non-specific effects were suggested via interactions at various drug receptors. As an example sigma receptors have high affinity binding sites for several psychotropic drugs. Similar sites are located not only in the CNS but also occur in various peripheral tissues. The overexpression of these receptors is found in human tumors (140). Different neuroleptic

compounds affecting sigma receptors produced changes in cell morphology. Some neuroleptics lacking sigma affinity such as (+)butaclamol and clozapine had no effect on cellular morphology (141). However (-)butaclamol exhibited morphological changes resembling apoptosis. The binding sites are distinguishable by their affinity for stereospecific ligands (142).

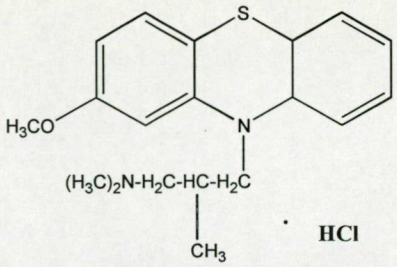
The (+)butaclamol has a high affinity binding site on the dopamine D_2 receptor (143) while the (-)butaclamol was 30 times less active. Interestingly, the (-) enantiomer was more active in the reversal of mdr efflux pump than the (+)butaclamol, which means that mdr reversal effect was not mediated by D_2-like structures, but is probably mediated by sigma receptors. 5-HT receptors are suggested to play a role in certain neuroleptic disorders, the therapeutic effect of (+)butaclamol and clozapine is localized on 5-HT receptors by inhibiting some effects of 5-HT. However the (-)butaclamol and (-)propranolol were less effective (144).

Considering the role of D_4 receptor or D_4-like structures on the P-gp, we can exclude D_4 specific binding on the P-gp because dopamine receptor antagonists which showed high affinity to the receptor had a rank order of haloperidol > chlorpromazine > (+)butaclamol > (-)butaclamol. The (+)butaclamol bound to D_4 receptors in a stereoselective manner, showed a higher affinity than its respective (-) enantiomer. The (+) enantiomer was found to be nearly 20-fold more effective than (-)butaclamol (145). The lack of dopamine receptor specific stereospecificity of mdr reversal excludes the involvement of D_2 and D_4-like binding sites on the P-gp. Rather our results refer to sigma receptor involvement for drug binding responsible for inhibition of drug efflux in the tumor cells.

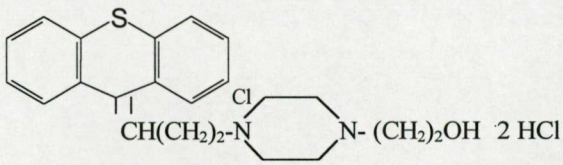
When mdr cells were exposed to tricyclic stereoisomers with lipophilic characteristic, the drug resistance was reversed due to the inhibition of efflux pump system. However, downregulation of the *MDR1* gene was also found in some cases (146, 147). The experiments involving the effect of verapamil stereoisomers showed no substantial difference in the potencies of (+) and (-) enantiomers in reversing the mdr efflux pump mechanism (132) and in agerelated clearance (148). The enantioselective mdr reversal effect of the pharmacologically inactive (-)butaclamol may be exploited in combination chemotherapy based on the different lipophilicity of the two stereoisomers.



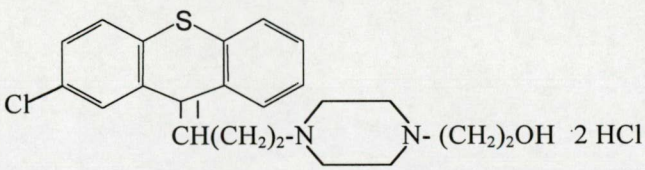
L-methotrimeprazine
L-10[3-(dimethylamino)-2-methylpropyl]-
2-methoxyphenothiazine



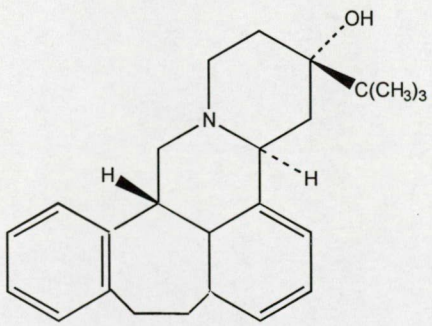
D-methotrimeprazine
D-10[3-(dimethylamino)-2-methylpropyl]-
2-methoxyphenothiazine



cis - Clopenthixol
4-[3-(2-chlorothioxanthene-9-ylidene)propyl]-1-piperazineethanol



trans - Clopenthixol
4-[3-(2-chlorothioxanthene-9-ylidene)propyl]-1-piperazineethanol



Butaclamol
3-(1,1-dimethylethyl)-2,3,4,4a,8,9,13b,14-octahydro-
1H-benzo[6,7]cyclohepta[1,2,3-de]pyrido-[2,1-a]isoquinolin-3-ol

Figure 13. The chemical structure of methotrimeprazine, clopenthixol and butaclamol enantiomers and stereoisomers.

5.10. Anti-mdr effect of natural compounds

Antitumor effects of crocine-, ginsenoside- and cannabis derivates

Various components isolated from medicinal plants have different antitumor effects: e.g., cytotoxic-, chemopreventive-, apoptosis- and differentiation induction. We selected three traditional medicines, saffron, ginseng and marihuana components for this study. The antitumor activity of saffron in mice transplanted with sarcoma-180, Ehrlich ascites carcinoma and Dalton's lymphoma ascites tumors was studied (149). Inhibitory effects of saffron on chemical carcinogenesis in mice using two-stage assay system (150) and the effect of crocetin on skin papillomas and Rous sarcoma (151) have also been described. Escribano et al. (152) reported that crocin inhibits the growth of HeLa cells and suggested apoptosis induction. More recently we found that crocin and crocetin derivatives inhibit skin tumor promotion in mice (153). It is well known that the ginseng extracts prevent cancer disease in the Chinese traditional medicine. Konoshima and his co-workers investigated the inhibitory effects of ginseng water extract and ginsenoside Rg1 for anti-tumor-promoting activities using an *in vitro* two-stage carcinogenesis assay system (154). The effects of marihuana components was described on the plasmid mediated bacterial resistance and pathogenicity in model experiments (155) and on *E. coli* adhesion to tissue culture cells (156).

Based on the similarity of drug resistance mechanisms in bacteria and cancer cells, it was worthwhile to study the activity of some cannabinoids, ginsenoside and saffron derivatives on the P-gp of cancer cells and tumor antigen expression of virus infected cells.

The primary screening of crocin was carried out with a short-term *in vitro* assay on EBV-EA activation. The inhibitory effects of crocetin glycosides on activation induced by TPA and viability of Raji cells were shown by a study of Molnár et al. (157).

These results showed the inhibitory effect of compounds isolated from active carotenoid fraction against EBV-EA induction. The components of ethanol extract of *Crocus sativus* (ECS) (crocetin gentibiose glucose ester and crocetin di-glucosyl ester) were less potent than crocin in inhibiting the EBV-EA induction effect. The comparative study among three analogues clearly suggests that gentibiose attached to the diterpenoid chain are important for crocin to exert its biological activity. The exposure of EBV genome carrying lymphoblastoid Raji cells derived from Burkitt lymphoma and exposure of adenovirus infected cells by some compounds resulted in altered T-antigen expression in Hep-2 cells (157). The crocin and diglucosyl-crocetin inhibited early tumor antigen expression in Hep-2 cells. In the presence of triglucosyl-crocetin the effect was less significant. The crocin had no antiviral effect on

Human herpes simplex virus-2 (157).

These findings led us to believe that crocetin glucoside may affect the activity of membrane proteins such as mdr P-gp (Table 18.). The resistance reversal effect of crocetin glycosides was tested on mouse lymphoma cells carrying the human *MDR1* gene. The data obtained with crocetin glycosides is summarized in Table 18. The triglucosyl-crocetin and diglucosyl-crocetin activity was marginal for 0.1 to 10.0 μg/mL concentrations, however, picrocrocin and crocin were ineffective in this aspect. Even drug accumulation was decreased in the treated cells, thus one cannot exclude enhanced membrane permeation or apoptosis.

Table 18. The reversal of multidrug resistance of mouse lymphoma cells by crocetin glycosides and picrocrocin

Compounds	Conc. μg/mL	FSC	SSC	FL-1	Fluorescence activity ratio
Par control		548.98	170.71	743.22	
Mdr control		649.28	259.87	36.03	
Verapamil	8	651.43	255.02	185.33	***5.14***
Crocin	10	695.35	235.23	23.25	***0.65***
	20	684.18	230.62	17.14	***0.48***
	100	687.09	231.37	13.54	***0.38***
Diglucosyl crocetin	10	697.47	230.71	22.45	***0.62***
	20	698.58	225.24	44.74	***1.24***
	100	695.02	239.36	32.68	***0.91***
Triglucosyl crocetin	10	708.07	220.63	40.79	***1.13***
	20	678.15	219.71	43.18	***1.20***
	100	682.96	219.59	37.77	***1.05***
Picrocrocin	10	693.62	224.73	27.28	***0.76***
	20	689.09	225.03	25.78	***0.72***
	100	665.07	219.06	25.12	***0.70***

The interpretation of multidrug resistance reversal effect of the compounds also gives some information about direct cytotoxic potential. The morphological changes were analysed by measuring the FSC and SSC values with the flow cytometer. The cell size increased, and at the same time, cellular granulation decreased. The viability of cells did not change during the experiment. As determined by the trypan blue test the cell viability was between 95 and 98 %.

Crocus sativus grows in South Asia and South Europe. Its medicinal value was recorded in traditional Chinese literature and was used to improve blood circulation (158). The compounds of saffron are able to inhibit chemical carcinogenesis and had direct antitumor action in animal experiments (149,150). *Crocus sativus* extracts were effective in the

treatment of central nervous system diseases including depression, anxiety, and led to improved memory functions (159).

It was shown that the saffron extracts contained characteristic compounds such as carotenoids; crocin and monoterpene aldehydes; picrocrocin and safranal (160). The majority of crocetin glycosides were able to inhibit the growth of human cancer cells *in vitro* (152).

In addition some plant phenolics were able to enhance the survival time of mice inoculated with NK/LY ascites tumor cells (161,162), others affected the genetic base of bacterial resistance, including plasmid transfer, which was also inhibited by psychoactive and inactive derivatives (155).

Crocine had an inhibitory effect on two-stage carcinogenesis with DMBA as an initiator and TPA as a promoter. When crocin was applied before the TPA treatment only 10 % of animals had papillomas and the formation of papillomas was delayed (153). Konoshima assumed that there are two possibilities by which crocin inhibits tumor formation: prevention of DNA damage, and work as an activator for DNA excision repair enzyme.

There were no data about the effects of these plant compounds on drug resistant cancer, although the molecular mechanism of the membrane effect was analysed (163).

It is possible that gene expression is blocked by the reduced promoter activity in the presence of crocin, or that a T-antigen shift is the consequence of physiochemical changes in the membrane lattice induced by charge transfer complex formation between particular membrane components and crocin derivatives.

Ginsenoside derivatives had a moderate inhibition on mdr efflux, and the effect also showed a dose dependence for 2.0 to 20.0 μg/mL. The ginsenoside Rc and Rd had the highest effect by reducing the activity of drug efflux pump. These two ginsenosides had nearly the same effect without any apparent cytotoxicity (Table 19.).

The effect of ginsenoside derivatives was dependent on their chemical structure. The compounds in general had moderate effect on the inhibition of multidrug resistance efflux pump of the human *MDR1* transfected mouse lymphoma cells.

The ginsenoside Rb1 was an exception, having the lowest effect possibly due to the bulky substitution at the R_3 site. This hypothesis was supported by the highest activity of the Rd derivative. In the latter compound the space-filling of the R_3 substituent is much smaller than in Rb1.

Table 19. The reversal of mdr of mouse lymphoma cells by naturally occurring glycosides

Compounds	Conc. μg/mL	FSC	SSC	FL-1	Fluorescence activity ratio
Par control		522.20	260.98	1010.84	
Mdr control		581.72	311.94	47.57	
Verapamil	8	578.25	312.83	374.40	***7.87***
Ginsenoside Rb1	2	585.29	304.65	75.78	***1.59***
	20	585.02	298.95	87.95	***1.85***
Ginsenoside Rc	2	589.62	308.93	245.17	***5.15***
	20	584.74	299.28	182.13	***3.83***
Ginsenoside Rd	2	582.43	300.19	278.08	***5.85***
	20	585.17	307.34	363.94	***7.65***
Ginsenoside Re	2	586.11	300.44	142.80	***3.00***
	20	588.32	301.59	145.84	***3.07***
Ginsenoside Rg1	2	589.12	315.78	99.32	***2.09***
	20	585.34	307.60	113.27	***2.38***

Some of the cannabis compounds showed inhibition on the R123 accumulation of tumor cells at 0.5-5.0 μg/mL concentrations (Table 20.). We could not detect any inhibitory effect in case of the hallucinogenic Δ8-Δ9 THC.

Table 20. The reversal of multidrug resistance of mouse lymphoma cells by some cannabis derivatives

Compounds	Conc. μg/mL	FSC	SSC	FL-1	Fluorescence activity ratio
Verapamil	5	528.86	253.87	62.89	***3.15***
Cannabinol	2	502.23	262.80	221.29	***11.08***
Cannabidiol	2	502.93	235.87	57.79	***2.89***
Cannabidiolic acid	2	490.11	248.36	10.91	***0.55***
Δ8 THC	0.5	497.26	246.43	13.58	***0.68***
Δ9 THC	0.5	491.21	244.91	14.45	***0.72***

The Δ8-THC had a marginal effect at cytotoxic concentrations while Δ9 had a remarkable effect at the same (2.0 μg/mL) concentration, which lead to a 10 percent reduction of cell size without any apparent changes in SSC values or cell viability.

The two effective compounds enhanced the NK cell activity (157), i.e., ginsenoside Rd and Rg1 may have a beneficial effect in tumor bearing animals. However, the ADCC activity of leukocytes was enhanced by ginsenoside Rc, Rg1 and Rb1 (157).

In the case of cannabinol, the electrophilic nature of the molecule may be responsible for the reversal of the multidrug resistance efflux pump. However, the extreme hydrophobicity and altered π-conjugation in the molecules of the two hallucinogenic compounds such as Δ8

and Δ9 THC might be responsible for their ineffectiveness.

These compounds can change the membrane structure and only a few of them are able to block the mdr efflux pump.

In addition, cannabinol can enhance the phagocytic index and TPA-induced chemiluminescent reaction of human polymorphonuclear leucocytes by enhancing oxidative bursts (156). The relevant immuno-modulating effect of the most active ginsenoside was studied in two functions of the human leucocytes such as NK cell activity and ADCC. The ginsenosides were able to enhance the natural killer cell activity, however the ADCC activity changed to a lesser extent.

In conclusion, we suggest that the reduced T-antigen expression, increased NK cell activity and inhibition membrane efflux pump responsible for the multidrug resistance, could be exploited in antitumor chemotherapy.

5.11. Anti-psychotic drugs reverse multidrug resistance of tumor cell lines and human AML cells ex vivo

Several compounds are in clinical trials for modulation of P-gp related resistance in cancer (33). It is now not uncommon to use anti-psychotic drugs in cancer patients (164, 165, 166). The concomitantly used cancer chemotherapeutic and anti-psychotic drugs may alter each others pharmacokinetics in cancer patients (167).

Tricyclic anti-psychotic drugs were shown to exert a variety of biological effects on the subcellular and cellular levels. For example, CPZ, TFP and clozapine inhibit certain proteases, acetylcholine esterase, and affect Ca^{2+} metabolism (168). Fluphenazine, a phenothiazine type anti-psychotic drug, some of its analogues (169), butaclamol stereoisomers (135) and the chemically related thioxanthene type compounds (170) were shown to inhibit P-gp function in some *MDR1* gene expressing cell lines. Most of these tricyclic- and other antidepressive drug molecules are lipophylic and possess a positive charge due to their protonated terminal chain nitrogen atom. These chemical characteristics were shown to be important for agents which affect the function of P-gp (37,171). Prochlorperazine, the tricyclic antiemetic drug, was shown to be effective as a resistance modifier in phase I clinical studies (172).

For the above reasons we have studied the effect of some anti-psychotic drugs on the function of P-gp. In our study, we used the transfected leukemia cell lines L1210 MDR, L5178 MDR and the human adenocarcinoma cell line KB-V-1 and peripheral blood

lymphocytes obtained from leukemic patients.

Treatment of L1210 MDR cells with CsA (5 μg/mL), a known P-gp blocker, increased the relative fluorescence intensity about 7-fold in the L1210 MDR cells. All tested anti-psychotic drugs increased the relative fluorescence intensity, in L1210 MDR cells, haloperidol and fluphenazine being the most active. In the L5178 MDR cell line fluphenazine and maprotiline were the most effective compounds. None of the tested drugs changed significantly R123 uptake into the parental cells. Similar results were obtained with KB cells using DR as fluorescent substrate (Table 21.).

Table 21. Influence of anti-psychotic drugs on the uptake of R123, the P-gp substrate, into L1210, L5178 cells and the MDR derivatives, KB-3-1 and KB-V-1 (MDR) cells.

Anti-psychotic drugs	Conc. μg/mL	Relative fluorescence intensity Cells:					
		L1210	L1210 MDR	L5178	L5178 MDR	KB-3-1	KB-V-1
Amitriptyline	0.5	1.19	3.02	0.62	3.27	-	-
	1	-	-	0.67	3.50	-	1.96
	5	1.19	5.87	0.54	4.81	0.86	5.98
Fluphenazine	0.5	1.49	4.02	0.75	4.45	-	-
	1	-	-	0.76	7.44	-	1.37
	5	1.16	8.45	0.68	13.30	0.73	2.89
Maproptiline	0.5	1.13	2.54	0.68	3.22	-	-
	1	-	-	0.60	3.08	-	-
	5	1.02	4.63	0.50	8.05	0.71	1.68
Trimipramine	1	1.22	2.91	-	-	-	1.37
	5	1.15	6.30	-	-	0.81	2.05
Desipramine	1	-	1.73	-	-	-	1.37
	5	-	2.80	-	-	0.78	1.55
Imipramine	0.5	-	1.28	0.82	3.04	-	-
	1	-	-	0.74	3.45	-	-
	5	-	2.82	0.70	4.80	-	1.98
Haloperidol	0.5	1.03	5.39	0.71	2.28	-	-
	1	-	-	0.64	2.04	-	1.99
	5	1.15	13.20	0.51	2.11	0.89	3.37
Doxepin	0.5	-	1.19	0.96	2.40	-	-
	1	-	-	0.81	2.67	-	-
	5	-	2.95	0.57	4.48	-	-
Cyclosporin A	5	1.21	7.05	0.85	12.08	0.90	4.51

In these experiments DR was used as fluorescent substrate of P-gp, since it was also used as a chemotherapeutic agent in patients. The antipsychotic drugs increased the relative fluorescence of KB-V-1 but not of the KB-3-1 parental cells. The increase was close to that of

CsA, the known P-gp blocker, for amitriptyline, fluphenazine and haloperidol. However, efflux of DR from KB-V-1 cells was not blocked to the same extent as in the L1210 MDR and L5178 MDR cells, relative to CsA (Table 21.). We attribute this lack of correlation between the three cell lines to the fact that we used different substrates, R123 and DR, with the L5178, L1210 and the KB cells, respectively, which fact may contribute to differential sensitization with CsA. It was shown previously that some P-gp blockers may exert their effects indirectly through the plasma membrane without being a substrate (110).

Because of the demonstrated blocking effect of the tested anti-psychotic drugs on P-gp, these drugs were tested in peripheral blood lymphocytes (PBL) of drug treatment resistant patients, with the use of DR as fluorescent substrate.

Relative to L1210 MDR, L5178MDR and to KB-V-1 cells CsA had a small effect on the DR efflux in PBL of the patients SzL and PL. However, most of the tested anti-psychotic drugs had comparable effects to CsA in PBL in both of the patients (Table 22.).

Table 22. Influence of anti-psychotic drugs on the uptake of DR, as P-gp substrate, into PBL of patients.

Anti-psychotic drugs	**Conc. μg/mL**	**Relative fluorescence intensity Patients:**		
		SzL	**PL**	**NV**
Amitriptiline	1	-	-	-
	5	1.48	1.39	1.10
Fluphenazine	1	-	-	-
	5	1.24	1.53	0.90
Maproptiline	1	-	-	-
	5	1.86	1.32	1.03
Trimipramine	1	-	-	-
	5	1.53	-	-
Desipramine	1	-	-	0.90
	5	1.65	-	-
Imipramine	1	-	-	-
	5	1.42	1.00	1.00
Haloperidol	1	-	-	-
	5	1.44	1.37	0.98
Cyclosporin A	5	1.39	1.32	0.90

Cells from AML patient NV, although resistant to treatment in the clinic, could not be sensitized by CsA or with the anti-psychotic drugs for uptake of DR (Table 22.). The reason for the difference among the three PBL for sensitization could not be established, however some basic information can be obtained for example by mAb staining. Future investigations are planned to include more detailed characterisation of clinically resistant AML cells, which

do not respond to P-gp blockers.

Anti-psychotic drugs are used at a blood level of 10 to 500 ng/mL, depending on the drug used and on the sensitivity of the patient (170,173). A double concentration of the highest clinical blood level, 1 μg/mL, showed small but significant blocking of P-gp in L1210 MDR, L5178 MDR and KB-V-1 cells (Table 21.). PBL of the patients could not be tested at a 1 μg/mL dose level, due to an insufficient amount of blood samples. However, based on results with CsA at comparable dose level, 5 μg/mL, it was expected that uptake of DR into PBL of some of the anti-psychotic drugs would be enhanced by 1 and 0.5 μg/mL.

By the use of the model cell lines L1210 MDR, L5178 MDR and KB-V-1 and the fluorescent indicator R123 and DR, we showed that the clinical anti-psychotic drugs may significantly modulate the uptake of substrates of P-gp into mdr cells. We have also tested this possibility in a pilot study, using PBL prepared from the blood of drug resistant leukemic patients. Resistance was observed on clinical experience and was verified on PBL by the effect of CsA on the uptake of DR. In these PBL cells we compared the effects of the anti-psychotic drugs with that of CsA for the uptake of DR, the drug used for the treatment of two of the patients. The *ex vivo* results indicate that treatment with some of the anti-psychotic drugs result in as much change in DR uptake as CsA does in two out of three patient's PBL. Our results point in the direction of necessary pharmacological studies when anti-psychotic drugs and cancer chemotherapeutic agents stet used together.

CONCLUSIONS

The growing number of cancer patients and the emerging multidrug resistance of cancer cells leads to the increasing ineffectiveness of anticancer chemotherapy. The majority of anticancer chemotherapy medicines do not inhibit multidrug resistance but can induce the expression of the *MDR* gene. Therefore physicians have to change the strategy of chemotherapy. One way is to improve effectiveness of chemotherapy is to inhibit one of the resistance mechanisms, such as the prevention of induction of the MDR gene, and to develop new types of drugs for inhibition of activity of P-gp.

We can summerise our results as follows:

- The mdr gene expression of the mouse lymphoma cells was reduced by some phenothiazines, such as PZ and TFP, when the cells were cultured in their presence.
- The activity of P-gp responsible for the multidrug resistance was blocked by some compounds, which have the following effects:
  - ⇒ the phthalimido- and chloroethyl-phenothiazines had a concentration-dependent, high mdr reversal effect.
  - ⇒ some new 3-benzazepines had a moderate effect.
  - ⇒ 6,12-dihydro-1-benzopyrano [3,4-b][1,4] benzothiazin-6-ones compounds were ineffective
- The antiproliferativ effect of three different cytostatic drugs was enhanced in the presence of fluphenazine used as a resistance modifier in our *in vitro* experiments.
- Minor modifications in the chemical structure, such as changes in stereosymmetry could modulate the mdr reversing effects of methothrimeprazine, butaclamol and clopenthixol isomers. Therefore, it is possible that the CNS inactive member of stereoisomer pairs can be used as a resistance modifier without any risk in patients suffering from drug resistant cancer.
- Some representative anti-psychotic medicines (imipramine, amitriptylin, fluphenazine), used in cancer patients undergoing chemotherapy and block the function of P-gp, were able to enhance the drug uptake of leukocytes from patients, suffering from leukemia. Blood cells of two treatment-resistant leukemic patients showed increased uptake of daunorubicin if treated *ex vivo* with the anti-psychotic drugs. Our results suggested that pharmacokinetical studies should be performed prior to concomitant clinical use of such drugs which block P-gp function.

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