UNIVERSITÉ DE LA MÉDITERRANÉE
FACULTÉ DE PHARMACIE DE MARSEILLE, FRANCE

ALBERT SZENT-GYÖRGYI MEDICAL UNIVERSITY
FACULTY OF MEDICAL SCIENCES, SZEGED, HUNGARY

PhD THESIS

Scientific field: MEDICINAL CHEMISTRY

Anikó HEVÉR

INHIBITION OF P-GLYCOPROTEIN MEDIATED EFFLUX AND MODULATION OF MDR-1 GENE EXPRESSION IN TUMOR CELLS BY NEWLY SYNTHESISED AZAHETEROCYCLIC DERIVATIVES

Presented and defended publicly on November 6th, 1998 with the aim to obtain the PhD degree from the Albert Szent-Györgyi Medical University, Szeged and the Université de la Méditerranée, Marseille.

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Prof. J. BARBE, Université de la Méditerranée, Marseille, France
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The work was achieved in the **Institute of Microbiology**, Albert Szent-Györgyi Medical University, Szeged; in the **Laboratoire de Génie Génétique et Biotechnologie, UPRES A CNRS 6032** and in the **GERCTOP UPRES A CNRS 6009,** Université de la Méditerranée, Faculté de Pharmacie, Marseille.

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... to the memory of my father,

... to my mother,

... to all my family,

... and to my friends ...

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CONTENT

I. INTRODUCTION

Resistance to chemotherapy is a major problem in clinical medicine. Drugs, used in bacterial, viral, parasitic infections as well as in cancer, are concerned by this phenomenon. One form of drug resistance is termed multidrug resistance (MDR). It means that a cell treated with a drug becomes capable to develop resistance to several other drugs, which are functionally and structurally unrelated. The outward transport of the drugs - using a membrane P-glycoprotein (P-gp) as a pump - is significantly enhanced.

Since about two decades, attention has been focused on the reversal of MDR and many chemosensitizers were prepared and tested. Among the chemosensitizers with heterocyclic structure, quinacrine (aminoacridine) gave interesting results, as it was able to reverse the resistance against Vinca alkaloids and doxorubicin about 1 to 10 fold at 1 μM to 50 μM doses (Ford and Hait, 1990). That is why we were interested in preparing and testing new acridine derivatives, namely 9-thioacridines (general formula in Figure 1A).

Pyridoquinolines are structurally related to the acridines mentioned above. Thus, we decided also to prepare and to test some pyridoquinoline derivatives (general formula in Figure 1B). Indeed, in these derivatives the same side-chain can be branched twice at the same carrier-ring, as in the acridines, while the planarity of the heterocyclic moiety is the same in both series.

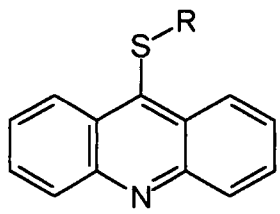


Figure 1A

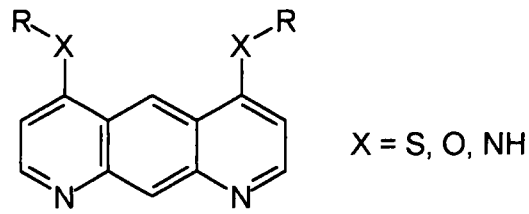


Figure 1B

The aim of our work was to determine whether the newly synthesised azaheterocyclic compounds are able to overcome the MDR. Theoretically, the reversal of the MDR can be achieved at two different levels: gene and protein. Most of the up to date discovered MDR reversing agents exert their effects on the protein level, blocking the exclusion of drugs by direct inhibition of the P-gp efflux pump. However, the same agents can influence also the expression of the MDR-1 gene in the same time. Verapamil, one of the best known chemosensitizer, not only inhibits the P-gp efflux pump, but promisingly also decreseases the MDR-1 gene expression in K562/ADR human leukemia cell line (Muller *et al.*, 1995). However, in the case of LS180-Ad50 and DLD-1 colon carcinoma cells, the same verapamil increases the gene expression (Herzog *et al.*, 1993). This phenomenon was also described for other chemosensitizers, like nifedipine, diltiazem, or cyclosporin A (Herzog *et al.*, 1993). All these observations emphasized for us the necessity to study the MDR modifying capability of compounds in various cell lines, and on protein and on gene level also.

The physical and chemical properties of many agents involved in MDR and its circumvention are quite different. Yet, a common « pharmacophoric group » - the minimum set of structural and functional features required for the modulator binding to P-gp - was suggested. Actually, this group contains two planar aromatic domains and a basic nitrogen atom. With a view to enrich our knowledge on the role of the molecular structure, we wished to study the MDR reversing ability in the series of thioacridines and pyridoquinolines. Hence, structure-activity relationships deduced from these investigations, should help us to prepare compounds with enhanced MDR reversal activity and to portray the drug-binding site on P-gp. It must be emphasized that P-gp is located inside the cellular membrane, so that only its

sequence is available at the present time. Thus, to investigate the binding of the drugs to P-gp, we used homologous protein with known three-dimensional structure.

Now, here are reported the synthesis and biological activity (reversion of the pump activity, changes in gene expression and protein level) of selected sample derivatives. Moreover, structure-activity relationships and a plausible mechanism of action (based on a molecular modelling preliminary study) is also depicted.

II. RECENT ADVANCES IN THE REVERSAL OF MULTIDRUG RESISTANCE IN CANCER

II. 1. MULTIDRUG RESISTANCE PHENOTYPE

Clinical resistance to chemotherapeutic drugs is a major problem in the treatment of cancer. Most metastatic cancers are either resistant originally to chemotherapy (intrinsic resistance), or first respond to chemotherapy but, later recur as cancers that have acquired chemotherapy resistance.

One form of drug resistance, termed multidrug resistance (MDR), is defined as the ability of cells exposed to a single drug to develop resistance to a broad range of structurally and functionally unrelated drugs, which include natural products such as anthracyclines, Vinca alkaloids, epipodophyllotoxins, colchicine and actinomycin D, but not alkylating agents and antimetabolites.

Since the initial reports of MDR in the late 1960s and early 1970s (Bech-Hansen *et al.*, 1976; Biedler and Riehm, 1970; Juliano and Ling, 1976; Kessel *et al.*, 1968; Ling and Thompson, 1973), numerous investigators have described tumor cell lines that display such a phenotype (Beck and Danks, 1991; Bellamy and Dalton, 1994; Nielsen and Skovsgaard, 1992). A common feature of many of these drug-resistant lines is an increased expression of the 170 kDa transmembrane P-glycoprotein (P-gp) (Gerlach *et al.*, 1986), which functions as an energy-dependent pump, exporting drugs out of cells and lowering the intracellular drug concentration to sublethal levels (Endicott and Ling, 1989).

In contrast to the « classical » P-gp associated MDR cells, the « atypical » MDR cells appear to posses other mechanism for resistance to multiple chemotherapeutic drugs, *e.g.*: changes in the expression or activity of enzymes involved in the glutathione detoxification pathway (especially glutathione S-transferase) (Green *et al.*, 1993) and alterations in the nuclear enzyme topoisomerase II (Beck *et al.*, 1994; Hochauser and Harris, 1993). Other mechanisms, such as decreased drug sensitivity (either by elevating levels of the target to overcome drug doses, or mutating the target, thus rendering the drug ineffective) (Schimke, 1984) or increased DNA repair (as a means to reverse cytotoxicity) (Pegg and Byers, 1992) may also be important.

The « non-P-gp » MDR phenotype is caused by the overexpression either of the multidrug resistance-associated protein (MRP) (Cole *et al.*, 1992; Cole and Deeley, 1993; Grant *et al.*, 1994; Kruh *et al.*, 1994; Zaman *et al.*, 1994), or the lung resistance-related protein (LRP) (Izquierdo *et al.*, 1996; Scheffer *et al.*, 1995; Scheper *et al.*, 1993; Slovak *et al.*, 1995). The 190 kDa MRP is predominantly located in the plasma membrane, while the 110 kDa LRP is closely associated with vesicular/lysosomal structures. Both proteins are related to active outward drug transport mechanisms, similar to the P-gp.

In the following we will focus our attention on the P-gp mediated MDR.

II.2. STRUCTURE AND FUNCTION OF P-GLYCOPROTEIN

II. 2. 1. SEQUENCE OF P-GLYCOPROTEIN

Despite the fact that P-gp was purified from multidrug resistant cells over a decade ago, there is no direct spectroscopic or crystallographic information on its structure. P-gp is an integral membrane protein of fairly large size and thus difficult to study by conventional solution methods. The occurrence of glycosylated forms of the protein leads to the existence of a variety of heterogeneous isoforms, further complicating attempts for detailed physical characterization.

Most of what is known about the structure of P-gp (and in fact about the structure of most membrane proteins) is based on analysis of amino acid sequences. Three independent groups simultaneously announced the sequence of the cDNA for P-gp in 1986 and analysed the predicted protein by standard computational methodologies (Chen *et al.*, 1986; Gerlach *et al.*, 1986a; Gros *et al.*, 1986).

The human P-gp is 1280 amino acid long and has a molecular mass of 141 kDa. The most notable characteristics of the P-gp is a division of the sequence into two homologous halves (43% amino acid homology and an additional 35% with functionally similar amino acids). 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.

The similarity of the two halves of the protein is even more pronounced in the comparison of their hydrophobicity profiles, which look nearly identical. Each half of P-gp consists of a short highly hydrophilic N-terminal region, a long hydrophobic region, and a long, relatively hydrophilic cytoplasmic C-terminal region.

Each half of the P-gp sequence also has a conserved consensus nucleotide binding region in the cytoplasmic hydrophilic domain. It is not known if both sites are functionally the same, although it has been shown that both must be present in order to allow full P-gp function (Rothenburg and Ling, 1989).

II. 2. 2. STRUCTURAL ORGANIZATION OF P-GLYCOPROTEIN

Both halves of the protein have six hydrophobic stretches that by hydropathy plots are suggesting of serving as transmembrane regions. It is notable that the sequence similarity between the two long hydrophobic stretches is not especially high, only the hydropathy plots. It is tempting to propose that the mature protein has twelve membrane spanning regions, and such a model for P-gp structure has become widely accepted (Figure 2).

The word « model » should be emphasized, because there is a little direct experimental evidence to support the conclusion drawn by sequence analysis, and in fact there is really no hard structural information on the molecular orientation of the protein in membranes other than the immunochemical localization of the C-terminus and the ATP binding domains in the cytoplasm (Kartner *et al.*, 1985).

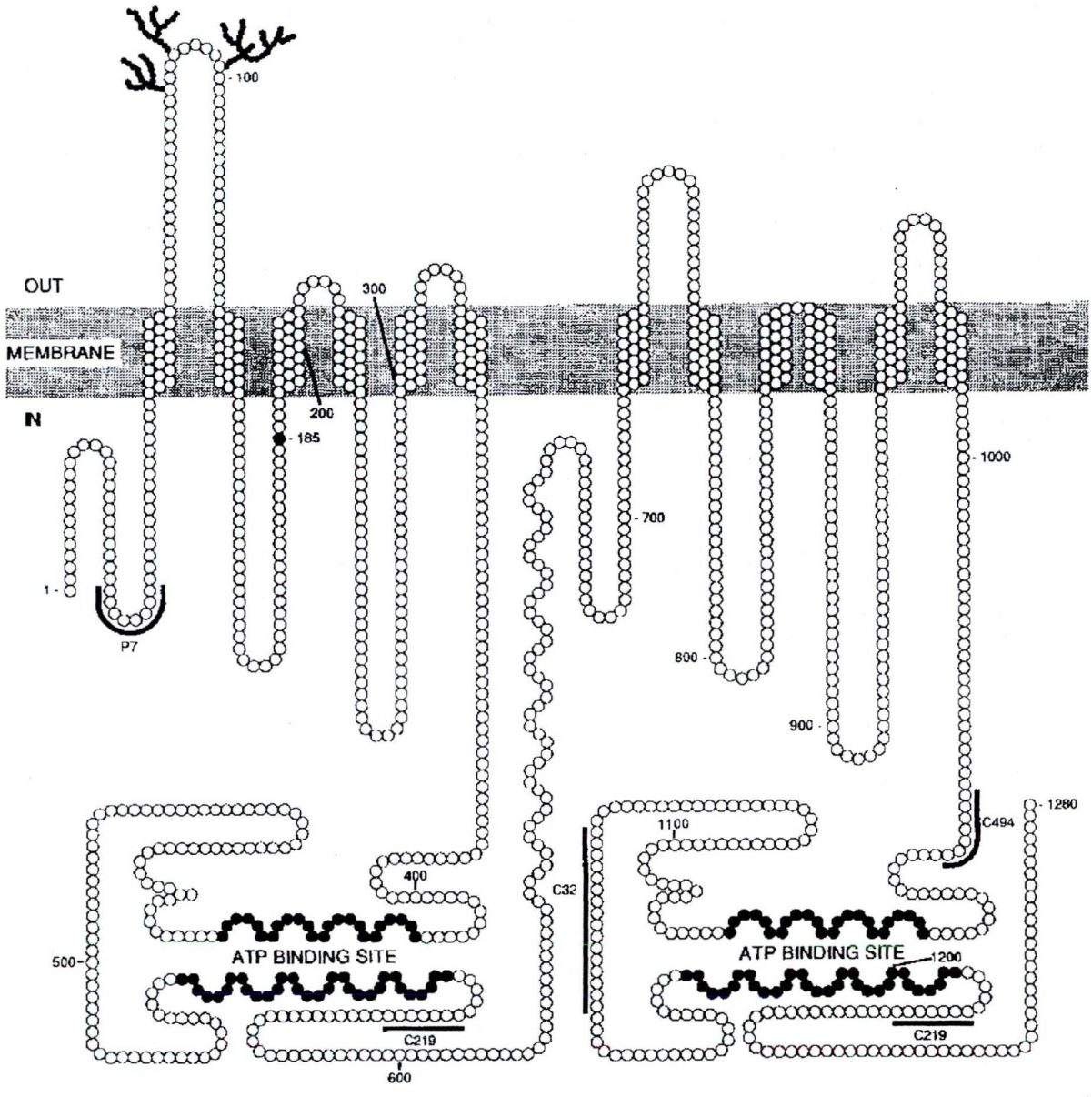


Figure 2: Model for topology of P-gp (from Juranka *et al.*, 1989). Each circle represents a single amino acid residue, whereas the small black-branched structures, restricted to the first extracellular domain, represent putative N-linked carbohydrates. Each half of P-gp has six membrane-spanning helices and each half contains consensus ATP-binding motifs, represented by filled circles. These ATP-binding regions have been localized to the cytoplasm using several monoclonal antibodies (C219, C494 and C32), their epitope sequences are delineated by a solid black line. A fourth polyclonal antibody (P7), raised against a small peptide, is also delineated by a solid black line.

It is important to note however, that although different analytical approaches to the prediction of transmembrane stretches often yield very different conclusions about the same sequence, in the case of P-gp both the Kyte-Doolittle (Kyte and Doolittle, 1982) and Eisenberg (Eisenberg et al., 1984) algoritms yield the same twelve domains, supporting the premise that the model structure may be a close representation of the real one.

II. 2. 3. GLYCOSYLATION OF P-GLYCOPROTEIN

Soon after P-gp was discovered (Juliano and Ling, 1976), it was reported to be a phosphoglycoprotein (Carlsen *et al.*, 1977).

P-gp is synthesised as a non-glycosylated precursor with an molecular size of 120-140 kDa, which is processed to the mature form with a half-life time $t_{1/2}$=1-2 hr in human cells or $t_{1/2}$=20-30 min in mouse cells (Greenberger *et al.*, 1988; Richert *et al.*, 1988). Mature P-gp has a molecular size that may range from 130-180 kDa depending on the type of cell and species in which it is expressed (Greenberger *et al.*, 1988; Richert *et al.*, 1988).

The primary structures of mammalian P-gp predicts three N-linked glycosylation sites that are located in the N-terminal half within the first extracellular loop. Asn-91, Asn-94 and Asn-99 have been confirmed as glycosylation sites within human P-gp (Schinkel *et al.*, 1993).

Many studies have been reported which suggest that glycosylation of P-gp is not essential for its basal drug transport function (Germann *et al.*, 1990; Kuchler and Thorner, 1992; Ling *et al.*, 1983; Raymond *et al.*, 1992; Sarkadi *et al.*, 1992). For example, treatment of

several multidrug resistant cell lines with tunicamycin (which inhibits processing of the P-gp precursor to the mature form) did not decrease their drug resistance (Beck and Cirtain, 1982; Chou and Kessel, 1981; Ichikawa *et al.*, 1991). However, treatment of a human colon carcinoma cell clone with tunicamycin resulted in reduced levels of cell surface-associated multidrug transporter, suggesting that glycosylation is required for efficient translocation of P-gp to the plasma membranes (Kramer *et al.*, 1995).

II. 2. 4. PHOSPHORYLATION OF P-GLYCOPROTEIN

Phosphorylation of P-gp has been observed in many multidrug resistant human and rodent cell lines (Germann *et al.*, 1995) and similar to native MDR gene products, recombinant P-glycoproteins are also phosphorylated (Germann *et al.*, 1990; Schurr *et al.*, 1989), suggesting that phosphorylation of P-gp may be universal.

Most of the research has concentrated on protein kinase C (PKC) as the major responsible kinase (Chambers *et al.*, 1990; Chambers *et al.*, 1992), but some evidence for a role of cAMP dependent protein kinase (PKA) also exists (Mellado and Horwitz, 1987).

Phosphoamino acid analyses of human P-gp have revealed the exclusive presence of phosphoserine (Center, 1983; Hamada *et al.*, 1987). In human P-gp, PKC phosphorylation sites are Ser-661, Ser-667 and Ser-671 (Chambers *et al.*, 1993; Chambers *et al.*, 1994), PKA phosphorylation site are Ser-667, Ser-671 and Ser-683 (Chambers *et al.*, 1994). These phosphorylation sites are confined to a central cytosolic segment that connects the two homologous halves of P-gp.

Several studies have demonstrated that brief exposure to phorbol ester protein kinase activators enhanced phosphorylation of P-gp, reduced intracellular drug accumulation, and increased drug resistance in a number of multidrug resistant cells (Chambers *et al.*, 1990; Chambers *et al.*, 1992; Yu *et al.*, 1991; Bates *et al.*, 1993; Aftab *et al.*, 1994). Conversely, treatment of multidrug resistant cells with the protein kinase inhibitors staurosporine and calphostin C reduced phosphorylation of P-gp and enhanced intracellular drug accumulation (Aftab *et al.*, 1994; Bates *et al.*, 1993; Chambers *et al.*, 1992; Ma *et al.*, 1991), supporting the hypothesis that the state of phosphorylation of P-gp may regulate its drug export function and modulate multidrug resistance.

However, it was also demonstrated that bryostatin I, which decreased P-gp phosphorylation, did not affect its drug efflux activity in multidrug resistant human breast cancer cells (Scala *et al.*, 1995) and that phosphorylation-defective P-gp variants were able to execute basal drug efflux activity (Germann *et al.*, 1996). Thus, it is probable that phoshorylation/dephoshorylation mechanisms do not play a crucial role in the establishment of P-gp mediated MDR.

II. 2. 5. ATP BINDING TO P-GLYCOPROTEIN

Early cell biology studies indicated that an ATP-driven process was the basis for the multidrug resistance phenomenon since reduced drug uptake and increased drug release in multidrug resistant cells were sensitive to poisons of mithocondrial respiration (*e.g.* azide), and reduced intracellular drug accumulation in MDR cells in the presence of azide could be sustained by adding ATP, but not with non-hydrolysable ATP analogues (Dano, 1973).

P-gp has been shown to bind the photoactivated ATP analogue ^{32}P-8-azido-ATP (Cornwell *et al.*, 1987; Cornwell *et al.*, 1991; Sarkadi *et al.*, 1992; Schurr *et al.*, 1989) and ^{32}P-2-azido-ATP (Al-Shawi and Senior, 1993). This labeling can be competed with ATP or GTP, but not with ADP or drug substrates (Cornwell *et al.*, 1987a), which is consistent with the finding that both ATP and GTP can provide energy for the transport reaction (Lelong *et al.*, 1992) and also suggesting that P-gp contains specific nucleotide binding regions different from the drug binding sites.

The drug efflux from MDR cells has been known to be ATP-dependent for a long time, but only recently has been characterised in detail the drug-stimulated ATPase activity of P-gp (Ambudkar, 1995; Scarborough, 1995; Senior *et al.*, 1995; Shapiro and Ling, 1995; Sharom, 1995). Studies involving membranes of MDR-1 infected insect cells (Homoloya *et al.*, 1993; Sarkadi *et al.*, 1994; Rao and Scarborough, 1994; Rao *et al.*, 1994), the plasma membrane of Chinese hamster ovary cells selected for high levels of P-gp expression (Al-Shawi and Senior, 1993; Al-Shawi *et al.*, 1994; Garrigos *et al.*, 1993; Sharom, 1995a), partially purified P-gp preparations (Doige et al., 1992; Doige et al., 1993), and purified, reconstituted P-gp (Ambudkar *et al.*, 1992; Naito and Tsuruo, 1995; Shapiro and Ling, 1994; Sharom *et al.*, 1993; Urbatsh *et al.*, 1994) have corroborated the high capacity ATPase activity of P-gp.

The hypothesis that ATP binding and hydrolysis are necessary for efflux is supported by studies involving in vitro mutagenesis of the putative ATP binding sites and transfection of these mutant cDNA clones. These studies have shown that mutation of one or both nucleotide-binding consensus sequences results in failure to confer drug resistance in transfectant cells that express the altered protein (Rothenburg and Ling, 1989). Moreover, P-gp half-molecules

expressed in Sf9 insect cell were found to exhibit ATPase activity, but drug stimulation was only observed when the half-molecules were expressed together, suggesting that interaction between both halves of P-gp is required for coupling of ATPase activity to drug binding (Loo and Clark, 1994).

II. 2. 6. DRUG BINDING TO P-GLYCOPROTEIN

The binding of drugs that are transported by P-gp to P-gp itself was first demonstrated using ^{3}H-vinblastine, which bound to membranes prepared from multidrug-resistant cells (Cornwell *et al.*, 1986). These studies were then extended to include the binding of agents, such as calcium channel blockers, which inhibit the multidrug transporter (Cornwell *et al.*, 1987a). That this binding was directly to P-gp was demonstrated with a photoaffinity analog of vinblastine (Cornwell *et al.*, 1986a), which binds to P-gp immunoprecipitated from multidrug-resistant Chinese hamster cells (Safa *et al.*, 1986).

Since these initial studies, a large number of substrates and substrate analogs of P-gp have been shown to be photoaffinity labels, including azidopine (Bruggemann *et al.*, 1989; Safa *et al.*, 1987; Yoshimura *et al.*, 1989), verapamil (Safa, 1988), iodomycin (Busche *et al.*, 1989), colchicine (Safa *et al.*, 1989; Safa *et al.*, 1990), azidoprazosin (Greenberger *et al.*, 1991), forskolin (Morris *et al.*, 1991), cyclosporin (Foxwell *et al.*, 1989) and others (reviewed by Beck and Qian, 1992; Safa, 1993).

Progress has been made in identifying the sites in P-gp labeled by these various photoaffinity labels. A carboxy-terminal site was found to be labeled by ^{3}H-azidopine in mouse

P-gp (Greenberger *et al.*, 1990; Yang *et al.*, 1988), whereas two azidopine labeled sites were found in human P-gp (Bruggemann *et al.*, 1989; Bruggemann *et al.*, 1992; Yoshimura *et al.*, 1989). The presence of these sites, one or more in the amino terminal part of P-gp and one or more in the carboxy terminus, has since been confirmed in mouse P-gp using azidoprazosin photoaffinity labelling (Greenberger *et al.*, 1991).

By proteolytic digestion and cyanogen bromide cleavage, labeled fragments of P-gp have been identified using antibodies to specific parts of P-gp (Bruggemann *et al.*, 1992). Both the amino and carboxy sites are labelled equally; one of these sites is in the region around transmembrane segments 5 and 6, and the other occupies an analogous site near transmembranes 11 and 12 (Bruggemann *et al.*, 1992; Greenberger *et al.*, 1991). With more complete digestions, and the use of an iodo-forskolin analog, as well as iodoazidoprazosin, the regions of labeling have been narrowed to the 5th or 6th transmembrane domain or the cytoplasmic domain immediately following the 6th transmembrane region and a region within the 12th transmembrane domain or the cytoplasmic domain immediately following the 12th transmembrane region.

Recent data showing that inhibition of azidopine labeling by vinblastine reduces labeling equivalently in both the amino- and carboxy-terminal halves of P-gp (Bruggemann *et al.*, 1992) suggests that these two binding sites are equivalent with respect to their ability to bind drugs and supports a model of P-gp in which both halves of the transporter come together to form a single transport channel.

Perhaps the most difficult to explain is the very broad specificity (or lack of specificity) of the P-gp. But there are precedents for this property in well-known proteins. Thus serum albumin (He and Carter, 1992; Jakoby *et al.*, 1995) and α1-acid glycoprotein (Eksborg *et al.*, 1982; Shibukawa *et al.*, 1994) bind a range of molecular types, including substrates and reversers of P-gp (Ayesh *et al.*, 1996; Toffoli *et al.*, 1995), seemingly as broad as does P-gp. Probably all the drugs share a single site for transport by P-gp, but there is more than one site on the transporter for binding.

II. 2. 7. MECHANISMS OF THE P-GLYCOPROTEIN MEDIATED DRUG EFFLUX

P-gp renders cells resistant to lypophilic cytotoxic drugs by serving as an active efflux pump, which removes various lipophilic drugs from the cells in an ATP-dependent manner (Gottesman and Pastan, 1993).

The mechanism of drug removal by P-gp can be explained in three different way:

I) The efflux pump function of P-gp is most often viewed as illustrated in Figure 3A. According to this model, drugs enter the cell by passive diffusion through the lipid bilayer, bind to P-gp on the cytoplasmic side of the membrane and then P-gp utilizes the energy of ATP hydrolysis to pump the drugs out of the cell.

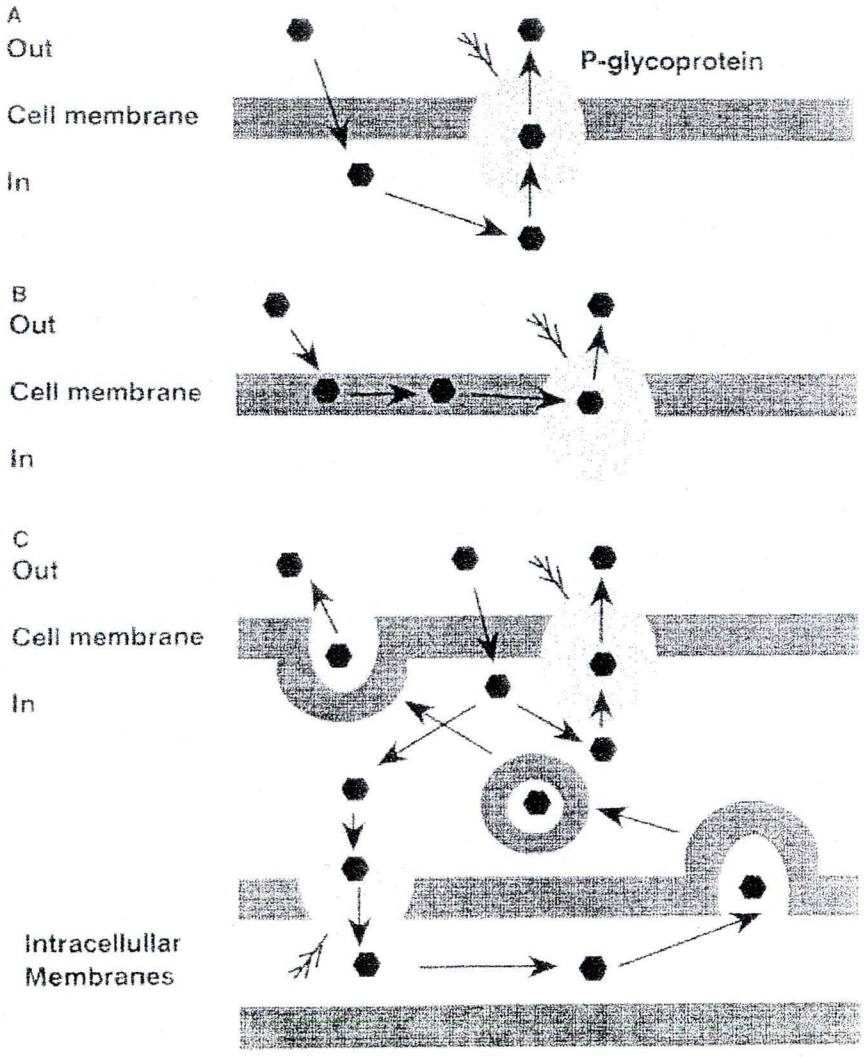


Figure 3: Models for P-gp mediated drug efflux (from Roninson IB, 1991a).

II) In the model illustrated in Figure 3B, drug binding to P-gp occurs not within the cell, but rather inside the lipid bilayer. This hypothesis was proposed by Gros (Gros,1986a). The hydrophobic nature of the drugs transported by P-gp makes it quite possible that the drugs spend a considerable period inside the membrane, where their lateral movement is likely to bring them into contact with P-gp. Then P-gp acts as a « membrane vacuum cleaner » (Raviv *et al.*, 1990), removing the drugs directly from the plasma membrane.

III) Another possibility is illustrated in Figure 3C. A slightly different version of this model was originally proposed by Beck (Beck, 1987) from the observations of increased number of vacuoles in MDR cells and localisation of anthracyclines to cytoplasmic vesicles in these cells. In this model, P-gp may be localized not only at the plasma membrane, but also on intracellular membranes, so that a drug entering the cell may be pumped by P-gp into the lumen of the intracytoplasmic membranes and subsequently removed by exocytosis. This hypothesis is consistent with immunohistochemical studies, which indicate that P-gp is found both in the plasma membrane and in the cytoplasm of MDR cells (Willingham *et al.*, 1987).

Recent evidence indicates that the P-gp is not a Cl^- channel, as proposed by Higgins and Sepulveda, but that P-gp activates an endogenous Cl^- channel (Borst *et al.*, 1993; Hardy *et al.*, 1995).

In an alternative model for an indirect mechanism of drug transport, it has been suggested that P-gp acts as an outwardly directed ATP channel, thus, generating an electrochemical ATP gradient which drives drugs across the plasma membrane (Abraham *et al.*, 1993).

II. 3. P-GLYCOPROTEIN IS A MEMBER OF THE ATP-BINDING CASSETTE SUPERFAMILY

P-gp shares extensive homology with numerous bacterial and eukaryotic transport proteins, belonging to the evolutionary conserved ATP-binding cassette (ABC) superfamily (Higgins, 1992; Juranka *et al.*, 1989). There are over 50 members of the ABC family and they

are involved in the transport of a variety of substrates, ranging from ions to large proteins and sugar polymers.

In addition to the P-gp, members of this family include several bacterial nutrient transporters (Higgins *et al.* 1985; Higgins *et al.*,1986; Higgins *et al.*,1990), a pigment transporter in *Drosophila melanogaster* (O'Hare *et al.*, 1984), a pump that appears to mediate chloroquine resistance in *Plasmodium falciparum* (pfmdr) (Foote *et al.*, 1990; Wilson *et al.*, 1989), a transporter for the alpha peptide mating factor of yeast (*Ste6*) (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989), two linked genes associated with transport of peptides into the endoplastic reticulum for class I antigen presentation (Tap-1 and Tap-2) (Monaco, 1992), the product of cystic fibrosis gene (CFTR) (Hyde *et al.* 1990; Riordan *et al.* 1989), the MRP (Cole *et al.*, 1992; Cole and Deeley, 1993), *etc.*

Recently an additional member of ABC superfamily has been identified: the sister of P-glycoprotein (SPGP), which has 61% of amino acid sequence identity with human MDR-1 (Childs *et al.*, 1995).

II. 4. THE P-GLYCOPROTEIN MULTIGENE FAMILY

DNA transfection studies have established a causative role for the MDR-1 gene in drug resistance. Gros demonstrated, that a full-length cDNA clone coding for the MDR-1 gene was sufficient to confer the MDR phenotype, including the expression of the P-gp, when transfected into drug sensitive cell lines (Gros *et al.*, 1986a).

Genetic analysis has also revealed the existence of more than one MDR gene in mouse, hamster and human (Gros *et al.*, 1991; Juranka *et al.*, 1989). The analysis of the 3' untranslated region of the various MDR genes has allowed the identification of equivalent isoforms between species (Ng *et al.*, 1989). Although each gene within a species encodes a unique 3' untranslated region, these differences are conserved across species. Thus, all currently identified P-gp isoforms can be grouped into one of three classes (Ng *et al.*, 1989) (Table 1).

Table 1: Classification of the P-glycoprotein genes (from Ng *et al.*, 1989).

	P-glycoprotein*		
Species	Class I	Class II	Class III
Hamster	pgp1	pgp2	pgp3
Mouse	mdr1a	mdr1b	mdr2
Human	MDR-1	—	MDR-3

*Human MDR-3 is also called MDR-2, mouse mdr1a is also called mdr3 and mouse mdr1b is also called mdr1.

Transfection studies indicate that only class I and class II P-gp can confer the MDR phenotype (Guild *et al.*, 1988; Ueda et al., 1987), whereas transfection and expression of the class III P-gp (both human and mouse) do not result in drug resistance (Gros *et al.*, 1988; Rothenburg and Ling, 1989).

The number of MDR genes in vertebrates has been estimated by Southern analysis using a conserved single exon probe (Ng *et al.*, 1989). Such an analysis revealed that primates (human, rhesus, monkey, orangutan), rabbit, chicken and fish contain two genes; rodents (hamster, mouse, rat) and cow contain three genes, and pig contains five genes. These observations suggest that P-gp is highly conserved in all vertebrates and likely plays a fundamental role in normal cell physiology.

Invertebrates also contain MDR genes. Two genes in *Drosophila melanogaster* and three genes in *Caenorhabditis elegans* (Rothenburg and Ling, 1989) have been detected by hybridization to mammalian probes. Furthermore, one gene has been cloned from *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1989) and two genes from *Plasmodium falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989).

II. 5. INTRON-EXON STRUCTURE OF THE MDR-1 GENE

The human MDR-1 gene is localized in chromosome 7, band q21.1 (Callen *et al.*, 1987; Chin *et al.*, 1989). Its partial genomic sequence is presented on Figure 4.

The MDR-1 gene includes 29 exons (numbered from -1 to 28) , 27 of which is in protein-coding sequence: 14 coding for the left and 13 coding for the right half of the protein (Roninson, 1991); and also 28 introns (numbered as the preceding exons), 26 of which is in protein-coding sequence.

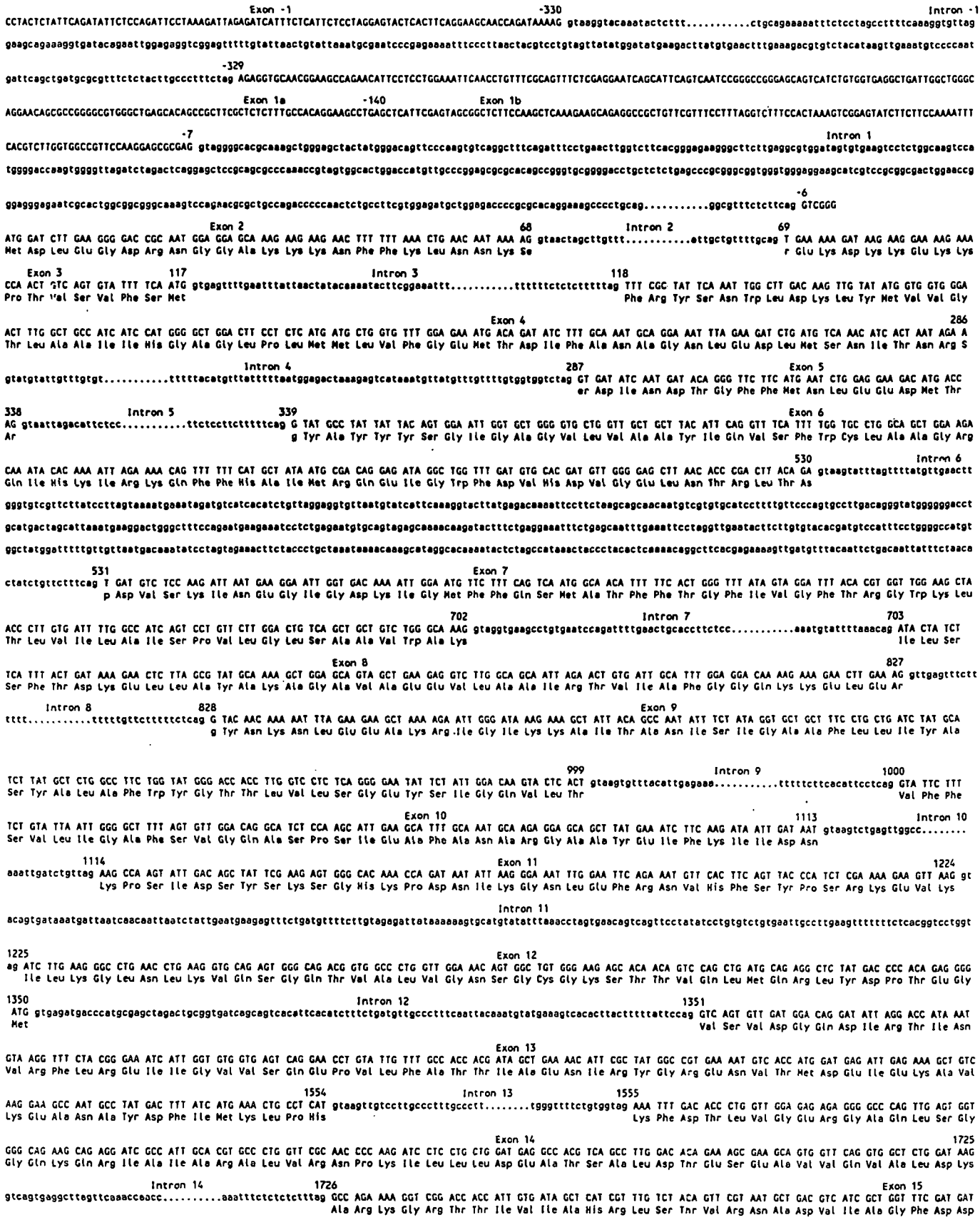


Figure 4: Genomic sequence of the human MDR-1 gene (from Chen *et al.*, 1990a).

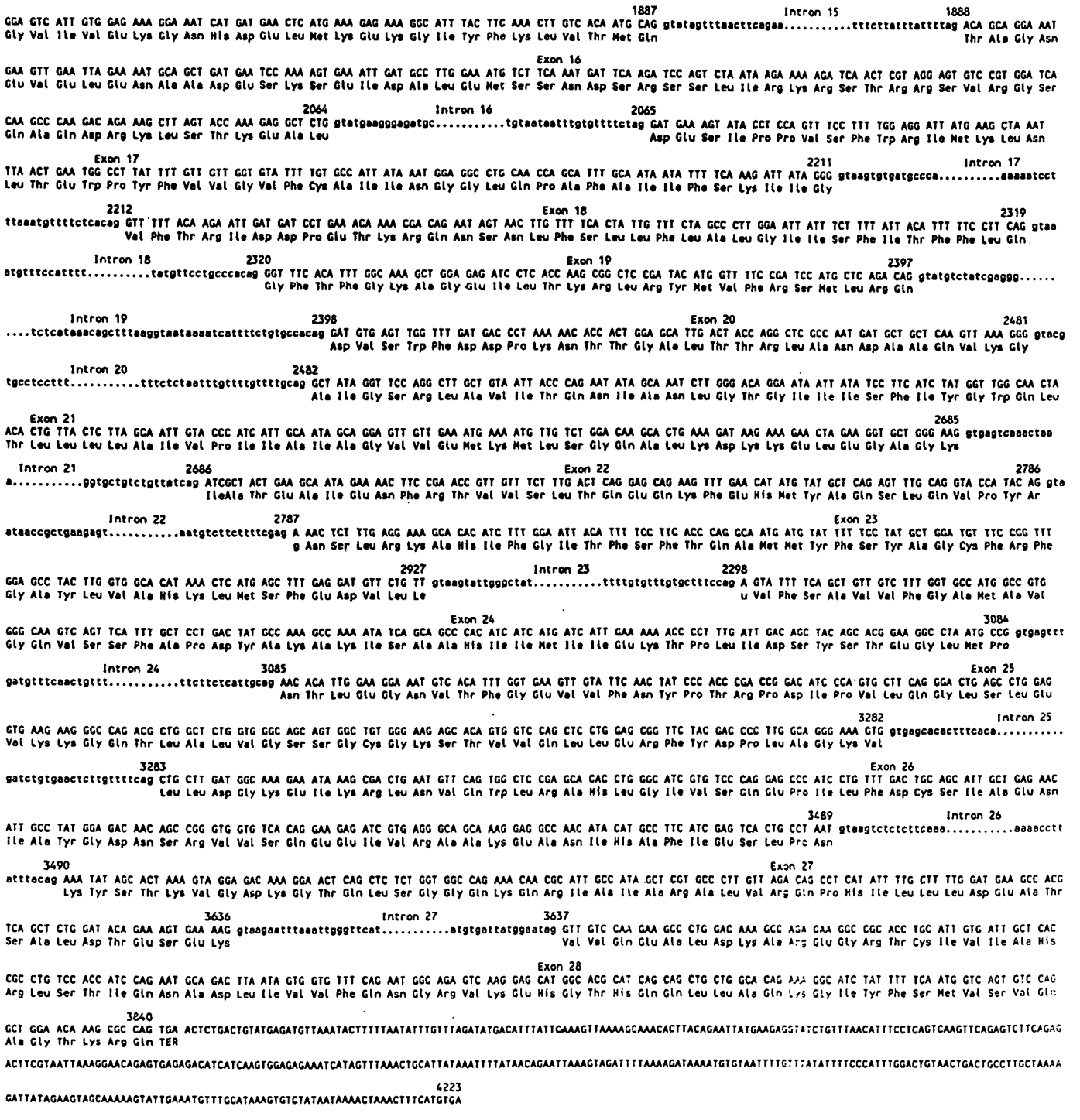


Figure 4: -continued

Among the introns located within the open reading frame, 19 interrupt this frame between the codons (type 0 introns), 1 intron interrupts the frame after the first nucleotide of a

codon (type 1 intron), and 6 introns occur after the second nucleotide of a codon (type 2 introns).

The mRNA can be transcribed from two different promoters (Ueda *et al.*, 1987a; Ueda *et al.*, 1987b), an upstream and a downstream promoter, with the downstream promoter preferentially expressed in most cell types (Ueda *et al.*, 1987a; Ueda *et al.*, 1987b). The upstream promoter is found at the beginning of exon -1, and the downstream promoter is located within exon 1, with the major transcription initiation site at nucleotide -140 (Ueda *et al.*, 1987b). The portion of exon 1 located 5' from the downstream promoter is designated exon 1a, and the 3' portion of this exon is called exon 1b. The ATG translation initiation codon is located within exon 2.

II. 6. ORIGIN OF THE MDR-1 GENE

Sequence homology between the N-terminal and C-terminal halves of P-gp suggested that this protein arose by duplication of a primordial gene (Chen *et al.*, 1986). This hypothesis predicts that introns are likely to be found at similar positions in the two halves of the protein-coding sequence, since almost all other known genes with an internal duplication show strong conservation of the intron positions between the duplicated domains (Traut, 1988).

However, it was found that only two or three pairs of introns in the MDR-1 gene are located at corresponding positions in both halves of protein (Chen *et al.*, 1990; Chen *et al.*, 1990a) (Figure 5).

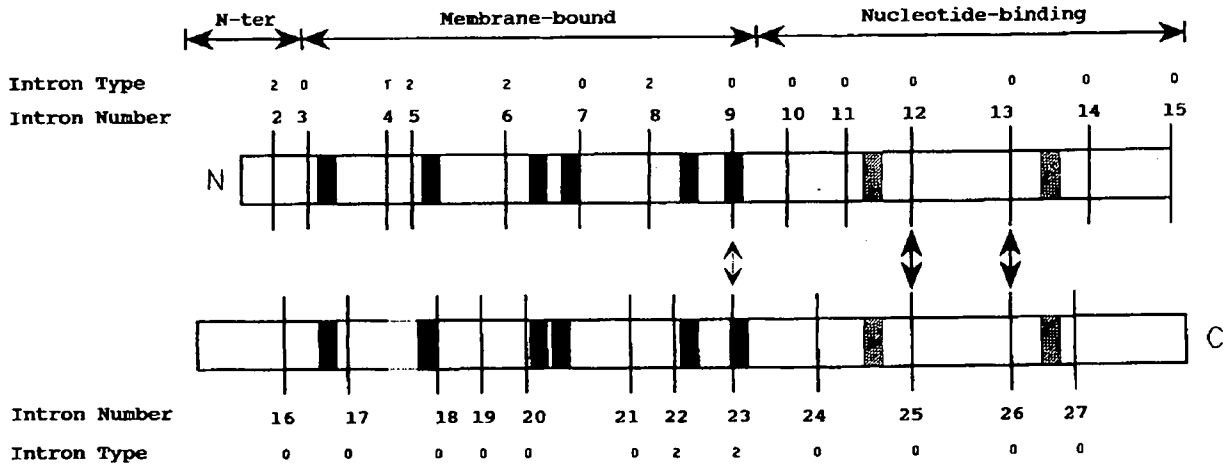


Figure 5: Positions of introns in the MDR-1 gene (from Chen *et al.*, 1990a).

Within the nucleotide-binding region, one pair of introns (introns 13 and 26) is matched precisely and another pair (introns 12 and 25) is shifted by one codon, with both introns belonging to type 0. Outside the nucleotide-binding domain, only one pair of introns (introns 9 and 23) is found at corresponding codons, but these two introns belong to different types. All the other introns appear to be misaligned.

Thus, it was proposed a new alternative hypothesis for the origin of P-gp (Chen *et al.*, 1990; Chen *et al.*, 1990a). According to this hypothesis, primordial proteins corresponding to the left and the right halves of P-gp were formed independently by fusion of closely related genes coding for the nucleotide-binding domain, with genes for different transmembrane proteins, which may or may not have been genetically related. Subsequent fusion of these two independently evolved primordial genes resulted in the formation of P-gp.

II. 7. EXPRESSION OF MDR-1 GENE IN NORMAL HUMAN TISSUES

A very high level of P-gp expression was found in human adrenal cortical cells, the brush border of renal proximal tubule epithelium, the lumenal surface of biliary hepatocytes, small and large intestinal mucosal cells and pancreatic ductules (Cordon-Cardo *et al.*, 1990; Fojo *et al.*, 1987; Sugawara *et al.*, 1988).

P-gp is also expressed at lower levels in lung, heart, prostate, stomach and muscle (Bellamy, 1996; Juranka *et al.*, 1989), in the placenta and secretory glands of the pregnant endometrium (Arceci *et al.*, 1988), in capillary endothelial cells of the testis and brain (Cordon-Cardo *et al.*, 1989; Hegmann *et al.*, 1992; Thiebaut *et al.*, 1989; Tsuji *et al.*, 1992; Fakla *et al.*, 1998); in NK cells (Wilisch *et al.*, 1993); in CD34 positive marrow stem cells (Chaudhary and Roninson, 1991); T- and B- lymphocytes and monocytes (Gupta and Gollapudi, 1993).

II. 8. SPECULATIONS ON THE NORMAL FUNCTION OF THE MDR-1 GENE

The availability of monoclonal antibodies C219 and MRK16, which recognize P-gp, made it possible to localize P-gp directly in cultured cells and in frozen sections of normal tissues. P-gp is found generally in polarized epithelial cell layers, where it generally localize to the apical (or luminal) surface of the cell. This localization, together with analysis of knockout mice (disrupted in mdr1a or/and mdr1b, or mdr2 genes) (Borst and Schinkel, 1996; Schinkel *et al.*, 1994), indicates that a major function of P-gp is the protection of organism against many of the toxic xenobiotics to which they can potentially be exposed in nature. P-gp confers

protection by limiting the uptake of compounds from the gastrointestinal tract, and by stimulating excretion of compounds in the liver, kidney and intestine. Moreover, P-gp in the blood-brain barrier and other blood-tissue barriers protects sensitive organs from exposure to toxic compounds that have entered the bloodstream.

Although we cannot exclude additional physiological functions for P-gp (see points 3-8. in Table 2) (Borst *et al.*, 1993; Chong *et al.*, 1993; Coon *et al.*, 1991; Field *et al.*, 1995; Hardy *et al.*, 1995; Klimecki *et al.*, 1994; Muesch *et al.*, 1990; Rubartelli *et al.*, 1990; Ueda *et al.*, 1992; Zhang *et al.*, 1995; Zhang and Casey, 1996), these are not vital, since the MDR-1 deficient mice are viable and fertile, and do not display obvious phenotypic abnormalities other than hypersensitivity to drugs (Borst and Schinkel, 1996; Schinkel, 1997).

Table 2: Possible physiological functions of P-gps in mammals and the rationale for these speculations (from Borst and Schinkel, 1996).

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 *Ste6*, *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**

II. 9. EXPRESSION OF MDR-1 GENE IN HUMAN CANCERS

Increased levels of P-gp transcript have been detected in all forms of human cancers: leukemias, lymphomas, sarcomas and carcinomas (Goldstein *et al.*, 1989; Goldstein, 1996) (Table 3).

Table 3: Expression of the MDR-1 gene in human tumours (from Goldstein, 1996).

1.	**High expression of the MDR-1 gene in untreated tumours:**	
	Colon	Pancreatic carcinoma
	Renal	NSCLC-NE
	Hepatoma	Carcinoid
	Adrenocortical carcinoma	Multiple myeloma
	Pheochromocytoma	CML-Blast Crisis
2.	**Occasionally high expression of the MDR-1 gene in untreated tumours:**	
	ALL (adult)	
	AML (adult)	
	Non-Hodgin's lymphoma	
	Neuroblastoma	
	Astrocytoma	
	CLL	
3.	**Low or no expression of the MDR-1 in untreated tumours:**	
	Breast	Mesothelioma
	NSCLC	Ovarian
	Bladder	Prostate
	CML-Chronic Phase	Sarcoma
	Oesophageal	SCLC
	Gastric	Thymoma
	Head and neck	Thyroid
	Melanoma	Wilms'
4.	**High MDR-1 gene expression in tumours relapsing after treatment:**	
	Non-Hodgin's Lymphoma	Breast
	Neuroblastoma	ALL (childhood)
	CML-Blast Crisis	Phaeochromocytoma
	ALL (adult)	CLL
	Multiple mueloma	

Keys: CML=chronic myelocytic leukaemia; SCLC=small cell lung cancer; NSCLC-NE=non-small cell lung cancer with neuroendocrine properties; ALL=acute lymphoblastic leukaemia; ANLL=acute non-lymphocytic leukaemia; AML=acute myeloblastic leukaemia; CLL=chronic lymphocytic leukaemia.

In a number of instances, an increased level of P-gp was observed after a relapse from chemotherapy, when compared with tumor biopsies obtained before treatment (Carulli *et al.*, 1988; Epstein *et al.*, 1989; Ma *et al.*, 1987; Nooter *et al.*, 1990; Rothenberg *et al.*, 1989). But in other cases (usually in tumors derived from tissues known to normally overexpress P-gp), a relatively high level of P-gp was seen even before chemotherapy (Fojo *et al.*, 1987; Holmes *et al.*, 1989; Kakehi *et al.*, 1988; Kieth *et al.*, 1990; Schwartsmann *et al.*, 1989).

Measurement of P-gp or MDR-1 expression in tumor samples is likely to be beneficial, as tumors found to express P-gp have been shown to have a poor prognosis (Chan *et al.*, 1990; Grogan *et al.*, 1993; Salmon *et al.*, 1989; Weinstein *et al.*, 1991).

II. 10. REGULATION OF THE MDR-1 GENE EXPRESSION

Treatment of human colon cancer cell lines with differentiating agents (*e.g.* DMSO or sodium butyrate) (Mickley *et al.*, 1989), with P-gp antagonists (*e.g.* verapamil, nifedipine, nicardipine, diltiazem and cyclosporin) (Herczog *et al.*, 1993) or with reserpine and yohimbine analogs (Bhat *et al.*, 1995) has been shown to increase the MDR-1 gene expression.

Cytotoxic agents (Chaudhary and Roninson, 1993), UV irradiation (Uchiumi *et al.*, 1993), heat shock and arsenite (Chin *et al.*, 1990), lowered extracellular pH and increased osmotic strength (Wei *et al.*, 1994), as well as transfection with oncogenes (Chin *et al.*, 1992; El Rouby *et al.*, 1993; Zastawny *et al.*, 1993) and with human immundeficiency virus-1 (Gollapudi and Gupta, 1990) also increase the expression of the MDR-1 gene in both rodent and human cell lines.

The increased MDR-1 gene expression may be regulated at different levels, which include an increase in gene copy number (Bradley *et al.*, 1988), increased rates of transcription (Bradley *et al.*, 1989; Shen *et al.*, 1986), and possibly control at the translational and posttranslational levels (Bradley *et al.*, 1989).

The down-regulation of the MDR-1 gene expression by a pharmacological agent was demonstrated for the first time by Muller (Muller *et al.*, 1994; Muller *et al.*, 1995). This agent was the verapamil which inhibits the function of the P-gp, and simultaneously decreases the MDR-1 gene expression. The decreased gene expression can be the consequence of the decreased transcriptional rate, which can be due to the reduced activity of the MDR-1 proximal promoter.

II. 11. PHARMACOLOGICAL REVERSAL OF THE MULTIDRUG RESISTANCE

With the realization that chemotherapeutics resistant cells contain an efflux pump for these drugs, it became clear that reversal of pumping would lead to increased levels of chemotherapeutics in the cell and hence increased cell killing and that such reversing agents (termed « chemosensitisers » or « resistance modifiers ») could have great clinical importance.

The first report of the pharmacological reversal of MDR came from Skovsgaard *et al.* in 1980 (Skovsgaard, 1980), who showed that N-acetyldaunorubicin, a simple competitor of the daunorubicin extrusion, delivered together with daunorubicin, gave improved survival of mice bearing drug-resistant ascites tumors. The next discovery by Tsuruo et al. in 1981

(Tsuruo *et al.*, 1981), that a widely used pharmaceutical, verapamil, could reverse drug resistance both in vitro and in vivo in an MDR murine leukemia cell line, gave added emphasis to research on MDR reversing agents. In the following years hundreds of reversers have been identified (Hevér *et al.*, 1998; Molnàr *et al.*, 1995; Molnàr *et al.*, 1997; Nacsa *et al*, 1998; Nonnenmacher *et al.*, 1997; Varga *et al.*, 1996).

The majority of chemosensitisers described to date may be grouped into seven broad categories (Ford, 1996) (Table 4).

Table 4: Selected pharmacological agents with ability to reverse MDR (from Ford, 1996).

1.	**Calcium channel blockers:** R-verapamil (5-10μm), Dexniguldipine (0.1-1μM), Gallopamil (5μM), Ro11-2933 (2-6μM), PAK-200 (5μM)
2.	**Calmodulin antagonists:** Trifluoperazine (3-5μM), Fluphenazine (3μM), Trans-Flupenthixol (3μM)
3.	**Protein kinase C inhibitors:** Calphostin C (250nM), Staurosporine (200nM), CGP 41251 (150nM), NPC 15437 (60μM), Safingol (20-50μM)
4.	**Steroidal agents:** Progesterone (2μM), Tamoxifen (2-10μM), Toremifene (5-10μM), Megestrol acetate (5μM)
5.	**Cyclic peptides:** Cyclosporin A (0.8-2μM), SDZ PSC 833 (0.1-1μM), SDZ 280-446 (0.1-1μM), FK506 (3μM), Rapamycin (3μM)
6.	**Vinca alkaloid analogues:** Vindoline (20-50μM), Thaliblastine (2μM)
7.	**Miscellaneous compounds:** S 9788 (1-3μM), GF120918 (0.02-0.1μM), Tolyporphin (0.1-0.5μM), BIBW 22 (1μM), Dipyridamole (5-10μM), Quinidine (10μM), Terfenadine (3-6μM), Reserpine (5μM), Amiodarone (4μM), Methadone (75μM)

Concentrations in parenthesis are those shown to have effect in reversing MDR *in vitro*.

II.12. STRUCTURE-ACTIVITY RELATIONSHIPS AMONG CHEMOSENSITIZERS

Many studies have attempted to find structural or physicochemical features of the P-gp inhibitors that might account for their effectiveness and thus lead to the design of better chemosensitizers (Motohashi *et al.*, 1996; Motohashi *et al.*, 1997).

Thus, Zamora *et al.*, studying a wide range of compounds, including indole alkaloids, lysosomotropic agents, and amines, concludes that lipid solubility at physiological pH, cationic charge and molar refractivity in the range of 9-16 are the important physical properties for modulators of MDR. Some structural similarities also may play a role, as quinoline derivatives (e.g. primaquine, quinacrine), which possess conjugated ring structures attached to substituted amino side groups, display significant anti-MDR activity, while acridine, which completely lacks an amino side group, retains only partial activity at a 10-fold higher concentration (Zamora *et al.*, 1988).

Pearce *et al.*, studying a series of reserpine and yohimbine analogs, emphasized the importance of the presence of the aromatic rings and basic nitrogen atoms in these compounds in determining the effectiveness of the reversers. They conclude that the aromatic ring represented by the benzoyl moiety is likely to be necessary for an effective MDR pharmacophore (Pearce *et al.*, 1989).

Nogae *et al.* studied 24 dihydropyridine analogues, measuring their effects on reversing MDR, on cytotoxic accumulation and on their ability to reverse photoaffinity labeling of P-gp

by an azidovindesine reagent. No structural relationships between this ability and the reversers themselves were uncovered other than a tendency for the strongest reversers to be the most hydrophobic (Nogae *et al.*, 1989).

A number of dihydropyridine analogs were also investigated by Yoshinari *et al.* Many of the more active of these compounds possess one or more tertiary amine side groups branched on the dihydropyridine ring (Yoshinari *et al.*, 1989).

Gosland *et al.* identified several cephalosporin antibiotics with chemosensitizing activity. The most active of the five drugs studied, cefoperazone, was the only one to contain N-ethyl piperazine ring structure (Gosland *et al.*, 1989).

The results of Ford *et al.* with phenothiazines show that substitutions on the phenothiazine tricyclic ring that increased hydrophobicity of the ring, such as halogens at position C2, increase also anti-MDR activity, whereas those that decrease hydrophobicity, such as hydroxyl groups, decrease activity. Specific structural features of the amino side chain are also important: tertiary amines (chlorpromazine) were more potent chemosensitizers than primary or secondary amines, particularly those with para-methyl (trifluoperazine) or ethyl (flupenazine) substitutions were more effective than noncyclic, aliphatic amines. Finally, the distance between the amino group and the phenothiazine ring was proved to be important, with a four-carbon alkyl bridge being more effective than shorter chains (Ford *et al.*, 1989; Ford and Hait, 1990).

Similarly to the phenothiazines, thioxanthenes with halogenated tricyclic rings and piperazinyl amino side groups were effective chemosensitizers (Ford *et al.*, 1990). The distance

between the amino group and the thioxanthene ring remained an important determinant for the activity. It seems also that trans-isomers of thioxanthenes are more effective chemosensitizers than cis-isomers.

In a study conducted with 115 bis(phenylalkyl)amines, Ramu and Ramu found that effective reversers in this series had two or three phenyl rings connected through one or two bridges to secondary or tertiary amine groups and also possessed carbonyl and/or dimethoxyphenyl functions (Ramu and Ramu, 1992).

Weaver *et al.* investigated a wide range of compounds of quite different types, including blockers of calcium, potassium, proton and sodium channels, as well as immunosuppressive agents. No relation was found between the ability of these drugs to block any specific ion channel and their ability to act as reversers of P-gp function, nor could they find any structural features of the reversers that correlated with their activity. The list of effective reversers includes neutral molecules and molecules bearing a positive charge at neutral pH (Weaver *et al.*, 1993).

Klopman *et al.*, by a literature search identified 137 different reversers and applied to these a comprehensive structure-activity study. A series of structural elements in this list of compounds appeared to enhance the effectiveness of a reverser (« biophores ») and a series of structural elements diminished reversing activity (« biophobes »). Using this tabulation of elements, they identified new compounds that would have a high ratio of biophores to biophobes and would be expected to be effective reversers. Seven such compounds were identified, of which four turned out, by direct testing, to be effective reversers. None of these

was, however, as effective as the well-known reverser verapamil, a result which somewhat diminishes the impact of this study (Klopman *et al.*, 1992).

Lee *et al.* and Alvarez *et al.* were searching for effective reversers using the data base of the National Cancer Institute (NCI) about more than 30000 chemical compounds. They identified many already-known substrates (such as vinblastine, daunorubicin, colchicine, etoposide), but found many compounds previously unsuspected. A small core of compounds were both effective substrates and effective reversers, but the large majority of compounds in the data base fell exclusively into one or the other of these classes. Again, little can be said about any structure-activity relationships. The only firm generalization that can be made is that all the effective compounds are lipophilic (Lee *et al.*, 1994; Alvarez *et al.*, 1995).

In summary, on the basis of studies mentioned above we can point out only two particular structural features common to most active anti-MDR pharmaceuticals: a hydrophobic, conjugated planar ring and a substituted, preferably cyclic, tertiary amino group.

II. 13. CLINICAL STUDIES

The first clinical trial performed was a Phase I/II study published in 1984 using oral verapamil (Presant *et al.*, 1984) (Figure 6). Ozols *et al.* (Ozols *et al.*, 1987) were the first to use intravenous (IV) verapamil in the treatment of patients with drug-resistant cancer. In the late 1980s, other chemosensitizers went into clinical trials. They included trifluoperazine (Miller *et al.*, 1988), followed by quinidine (Szumowski *et al.*, 1989), tamofixen (Cantwell *et al.*, 1989) and cyclosporin (Verweij *et al.*, 1990).

MDR reversing effect of 0.02μM *in vitro* (Hyafil *et al.*, 1993). Phase I clinical trials have been undertaken.

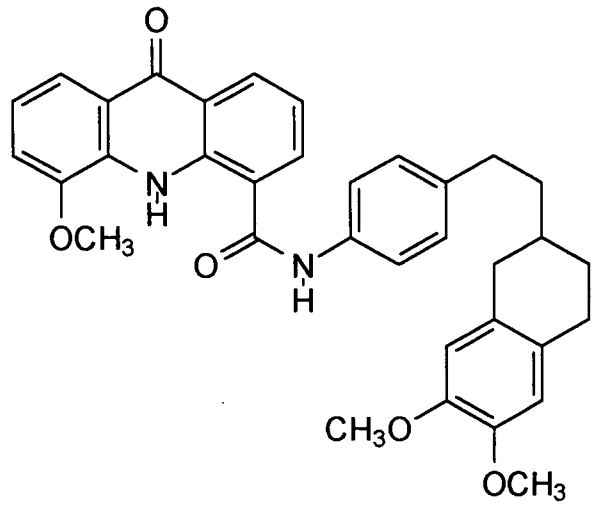


Figure 7: Chemical structure of the compound GG 918.

Another novel approach that might turn out to have clinical use is combined chemosensitization. Synergism in reversing MDR *in vitro* have been shown *e.g.* with verapamil in combination with quinidine (Lehnert *et al.*, 1991) or cyclosporin (Hu *et al.*, 1990). Clinical studies are in progress.

II. 14. NOVEL TREATMENT STRATEGIES

High-dose chemotherapy in conjunction with the insertion of MDR-1 gene itself into human bone marrow cells by using retroviral vector (McLachlin *et al.*, 1990) represents

another possibility to overcome drug resistance. Because myelotoxicity is the usual dose-limiting toxicity associated with many chemotherapeutic drugs, making the normal bone marrow resistant to the toxic effects may enable patients to tolerate higher therapeutic doses of drugs (Mickisch *et al.*, 1992).

A new strategy to overcome drug resistance due to P-gp is to specifically target overexpressing cells with anti-MDR-1 monoclonal antibodies, anti-MDR-1 antisense oligodeoxynucleotides (ODNs) or anti-MDR-1 ribozymes.

MRK-16, a monoclonal antibody developed against an external P-gp epitope, has been shown to inhibit tumor formation and to reduce tumor volume when administered to mice bearing MDR human ovarian xenografts or alone (Pearson *et al.*, 1991), or conjugated with Pseudomonas toxin (Fitzgerald *et al.*, 1987), or in combination with human interferon alfa (Fogler *et al.*, 1995). However, such results should be interpreted with caution. The MRK-16 antibody is specific for the human P-gp and therefore may target MDR-1 expressing cells in normal tissues as well as in tumor cells, thus leading to unacceptable toxicities. The issue of delivery of the antibody to the tumor is also important and may present a problem. Studies carried out in animal models have primarily utilized intraperitoneally grown tumors treated with intraperitoneally administered antibodies. The distribution of the anti-P-gp antibodies will be different if given intravenously, and their efficacy may be diminished in animals bearing tumors established at distal sites. Although these problems remain to be solved, the promise of such an approach still bears its continuance.

Vasanthakumar and Ahmed were the first to demonstrate that a 15-base ODN (nucleotide -9 to +6) completely inhibit P-gp synthesis in the K562/III erythroleukaemia cells

resistant to daunorubicin (Vasanthakumar and Ahmed, 1989). Other groups have also demonstrated antisense ODN related modulation of the MDR phenotype (Nakashima *et al.*, 1995; Quattrone *et al.*, 1994; Thierry *et al.*, 1993). Further studies are needed on the ODN stabilitiy and effective delivery to the target cancer cells.

Scanlon *et al.* have been at the forefront of utilizing ribozyme technology to reverse drug resistance (Scanlon *et al.*, 1991; Scanlon *et al.*, 1994), followed by others (Holm *et al.*, 1994; Kiehntopf *et al.*, 1994; Kobayashi *et al.*, 1994). They have successfully employed anti-MDR-1 and anti-FOS ribozymes to reverse MDR in human tumor cell lines. Although ribozyme technology represents a very promising and novel approach to reverse drug resistance, an effective means of delivery to the tumor must be developed prior to the initiation of clinical trials (Rossi, 1995).

III. CHEMISTRY

III. 1. MATERIAL AND METHODS

Starting compounds were obtained from chemical companies (Aldrich, Lancaster and Acros).

Alkylation was usually achieved under phase transfer catalysis conditions. This method is a very convenient one, successfully used for alkylating acridines (Galy *et al.*, 1980; Galy *et al.*, 1981; Galy *et al.*, 1987; Mahamoud *et al.*, 1982), quinolines (Kayirere *et al.*, 1998) and pyridoquinolines (Matias *et al.*, 1996). However, when triethylbenzylammonium chloride is used as a catalyst, thiobenzyl derivatives are obtained as side products. Owing to this, compounds were alkylated either with tetrabutylammonium bromide as catalyst, or without any catalyst.

The crudes obtained were purified by crystallisation from usual solvents.

Compounds prepared were identified by Nuclear Magnetic Resonance. Spectra were recorded at room temperature on a Brucker ARX 200 spectrometer using tetramethylsilane as internal reference. Melting points were measured on a Köfler bench. Elemental analyses were performed on a Technicon CHN analyser.

III. 2. RESULTS

III. 2. 1. THIOACRIDINES

Dibenzopyridine, also quoted as « acridine » (Figure 8.), is a planar tricyclic compound which was extracted from tar-coal for the first time in 1870 (Graebe and Caro, 1870).

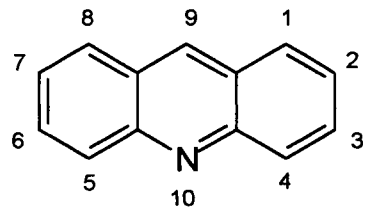


Figure 8: General formula and numbering of acridine.

Derivatives are usually prepared from anthranilic acids and anilines, used as starting compounds (Ullmann, 1907). Cyclisation of intermediates with phosphorus oxichloride leads to the acridinones, **1**. Thioacridinones, **2**, are then obtained treating **1** with phosphorus pentasulfide (Smolders *et al.*, 1982).

Synthetic pathways are summarised in Figure 9.

Alkylation of thioacridinones, using phase transfer catalysis conditions (PTC), gives acridinic thioethers **3**, but depending on the nature of alkylating agents, several methods can be used.

These methods are listed in Figure 10.

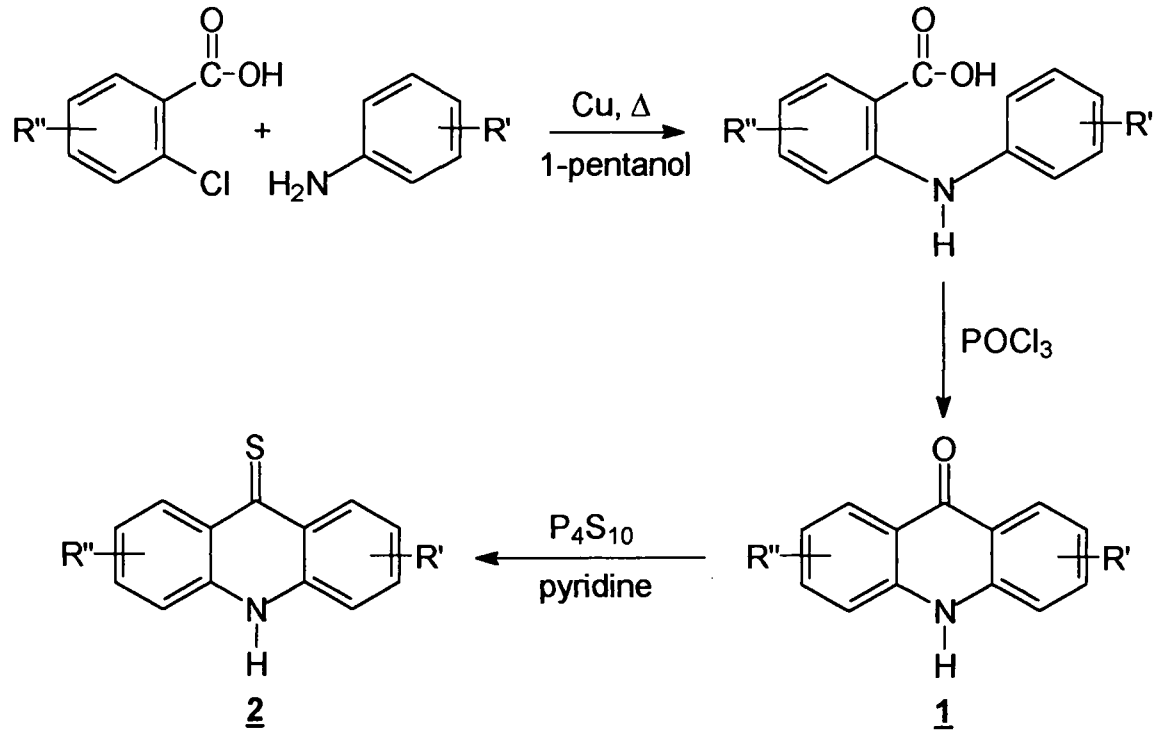


Figure 9.

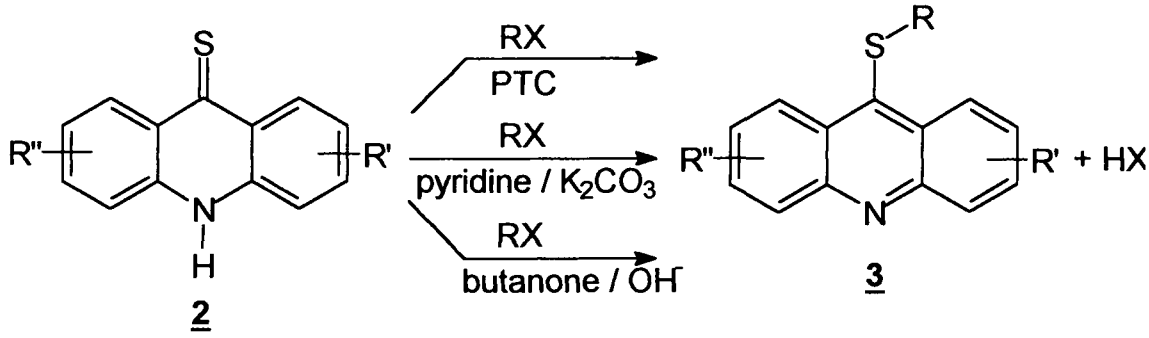


Figure 10.

In spite of the presence of two possible sites of alkylation, *e.g.* the sulfur atom and the nitrogen one, thioethers are the sole compounds obtained whatever are the conditions selected (Galy *et al.*, 1981). This is clearly demonstrated using ^{13}C Nuclear Magnetic Resonance (Faure *et al.*, 1983), because the chemical shifts of the C=S carbons (195-197 ppm) strongly differ from those of the C-SR carbons (about 145 ppm).

It must be noted that thioethers **3** can be oxidised using aqueous oxygen peroxide. However, depending upon the reaction time, sulfoxides **4** or sulfones **5** are prepared in this way (Figure 11). Yet, sulfones **5** were obtained in a restricted number of derivatives, because of the required constraints. Indeed, heating is not allowed and pH of the mixture must be strictly maintained to the neutral value for fear of hydrolysis which would lead back to acridinones **1**.

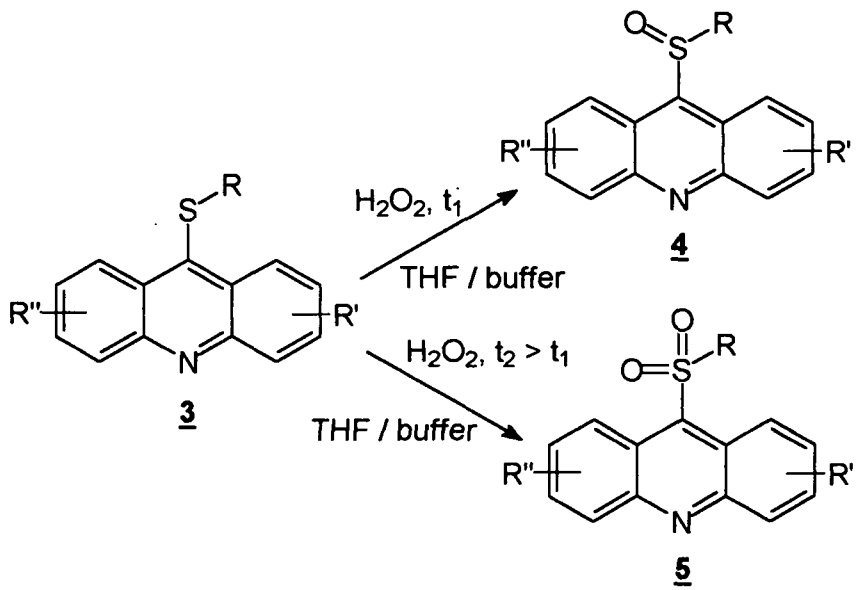


Figure 11.

Finally, bis-acridine derivatives **6** and **7** which are of interest from a biological point of view (LePecq *et al.*, 1975; LePecq and Roques, 1976; Fico and Canellakis, 1977), because of

the increased number of pharmacophoric groups, are prepared from the thioacridinones **2**. With respect to this, the bis α,α'-bromoacetamidodiphenyl (Rahal, 1991), prepared from α,α'-dinitrodiphenyl in a two step procedure, is used as connecting bridge, as depicted in Figure 12.

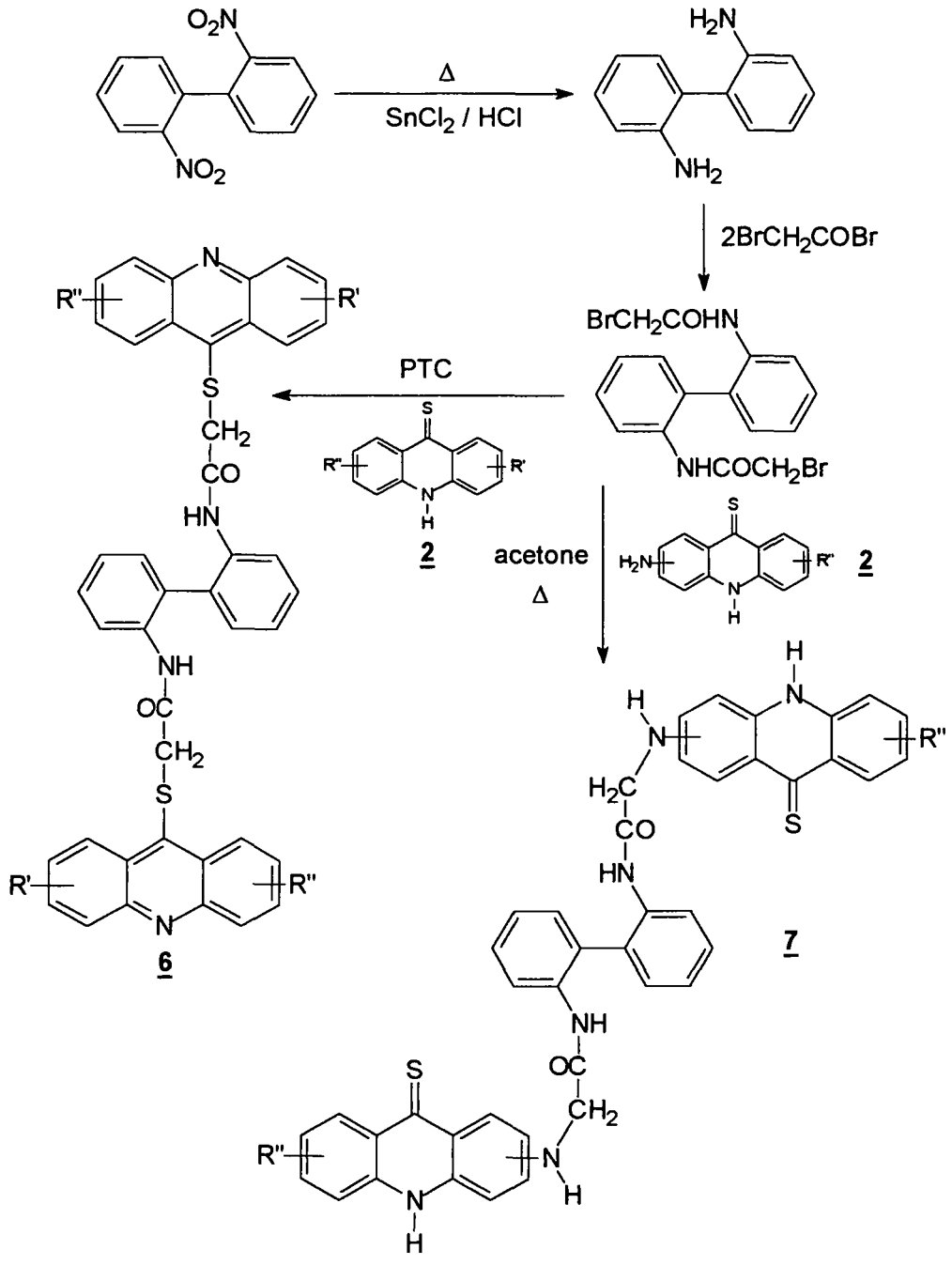
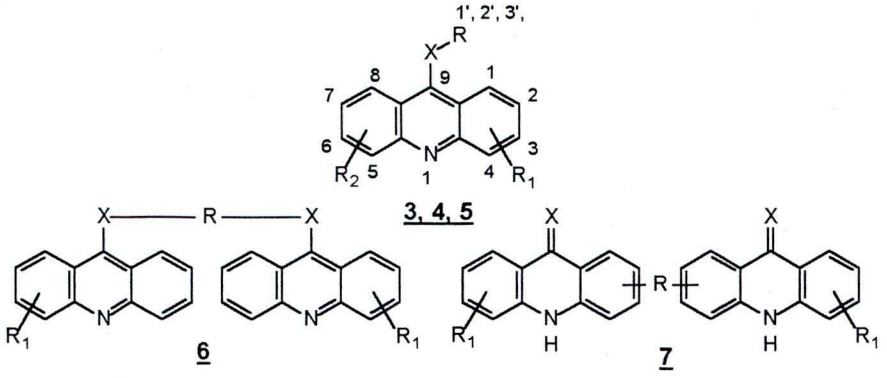


Figure 12.

Thioacridines prepared are listed in **Table 5.**

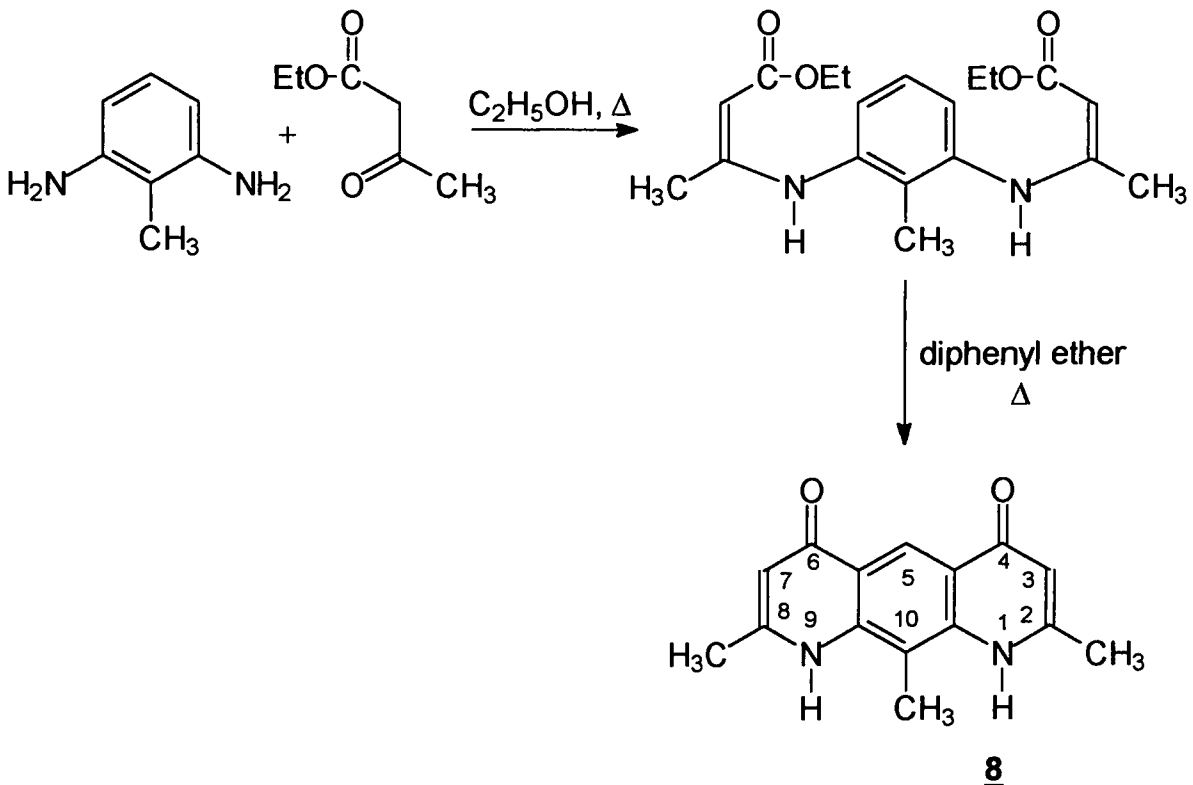


Compounds	X	R_1	R_2	R
3a	S	1,4-dimethoxy	-	ethyl
3b	S	-	-	2'-chloroethyl
3c	S	3-chloro	-	2'-chloroethyl
3d	S	2-methoxy	7-methoxy	2'-chloroethyl
3e	S	2-methoxy	7-methoxy	2'-hydroxyethyl
3f	S	3-chloro	-	2',3'-epoxypropyl
3g	S	2-methoxy	7-methoxy	2',3'-epoxypropyl
3j	S	-	-	4'-aminophenyl
3k	S	2-methoxy	6-chloro	4'-aminophenyl
3l	S	3-amino	-	4'-nitrobenzyl
3m	S	2-methoxy	7-methoxy	4'-nitrobenzyl
3n	S	3-amino	-	2'-(2'-diethylamino)ethyl
3p	S	2-methoxy	7-methoxy	2'-(2'-diethylamino)ethyl
4a	SO	2-methoxy	7-methoxy	2'-chloroethyl
4b	SO	2-methoxy	7-methoxy	benzyl
4c	SO	2-methoxy	7-methoxy	2'-(2'-diethylamino)ethyl
5a	SO_2	2-methoxy	7-methoxy	ethyl
5b	SO_2	2-methoxy	7-methoxy	ethenyl
6a	S	-	-	3,3'-(bis-α,α'-acetamidobiphenyl)
6b	S	3-amino	-	3,3'-(bis-α,α'-acetamidobiphenyl)
7	S	-	-	3,3'-(bis-α,α'-aminacetamidobiphenyl)

III. 2. 2. PYRIDOQUINOLINES

Within the frame of researches devoted to antibacterial and antitumor agents, attention was focused during the last two decades on the synthesis of pyridoquinolines (Antonello *et al.*, 1993; Croisy-Delcey and Bisagni, 1983; Hall *et al.*, 1977; Molock and Boykin, 1983). However, the processes suggested are usually time and material consuming. Thus, a novel two step procedure was proposed with a view to readily prepare the title compounds. With respect to this, 2,6-diaminotoluene reacts with ethylacetylacetate at moderate temperature before a thermal cyclisation under nitrogen pressure leads to the expected 2,8,10-trimethylpyrido[3,2-g]quinoline-4,6-dione **8** in almost quantitative yields (Matias, 1997).

Synthetic pathways are portrayed in Figure 13.



8

Figure 13.

Compound **8** is then alkylated under phase transfer catalysis (PTC) conditions to give the 4,6-dialkoxypyrido[3,2-g]quinolines **9**. Thiation of **8** with phosphorus pentasulfide leads to the homologous dithione **10**, which is used without purification because of its insolubility in pure or mixed usual solvents. Alkylation of **10** gives the 4,6-dialkylthiopyrido[3,2-g]quinolines **11**.

These synthetic processes are summarized in Figure 14.

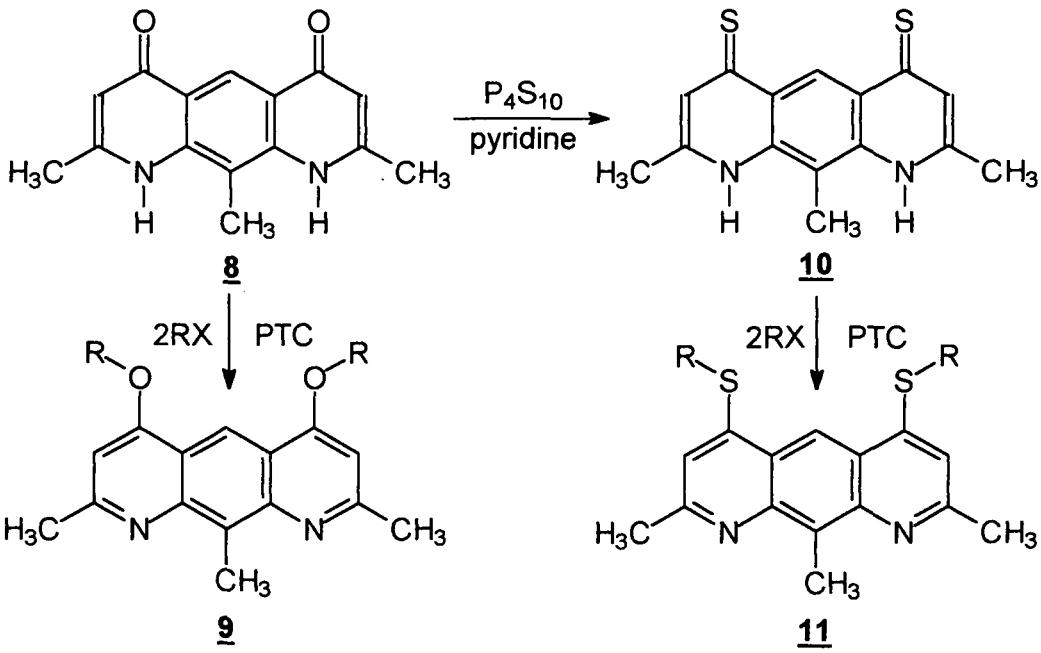


Figure 14.

Compounds 9 and 11 were characterized by Nuclear Magnetic Resonance spectroscopies, which clearly demonstrate that alkylation only leads to O- and S- substituted derivatives, whilst no N-alkylated derivatives are identified.

Finally, the dialkylaminoalkylamino 4,6-disubstituted derivatives 12 were prepared from the dihaloethoxy pyridoquinoline as intermediate (Figure 15), according to the method described by LaMontagne (LaMontagne *et al.*, 1977) in the case of simple quinoline derivatives.

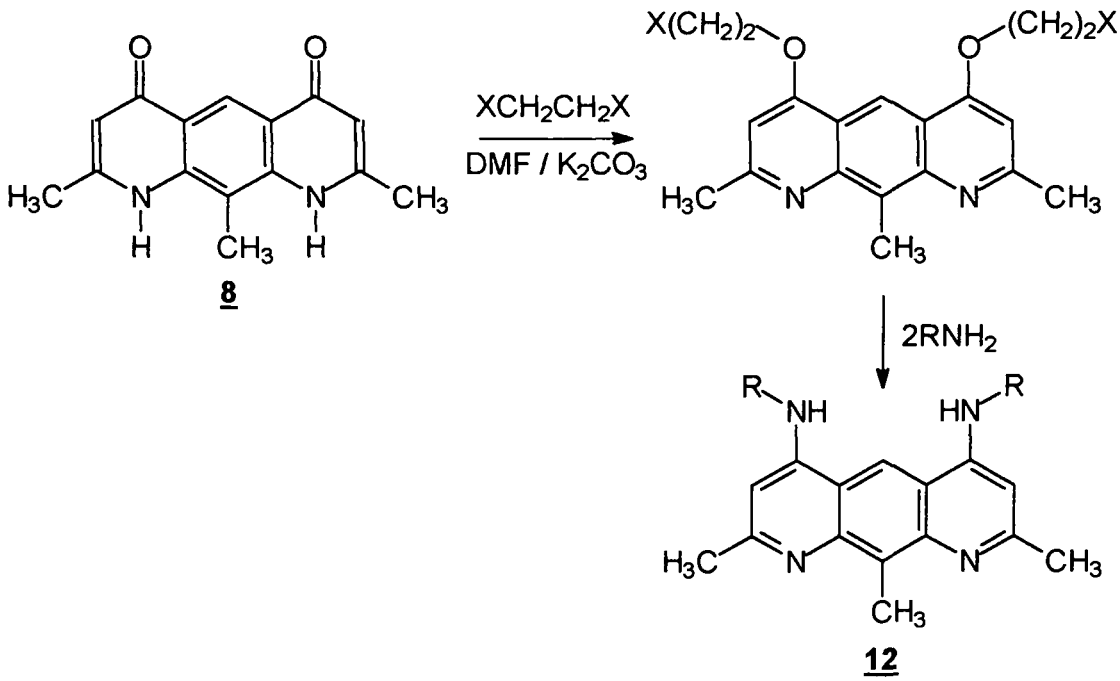
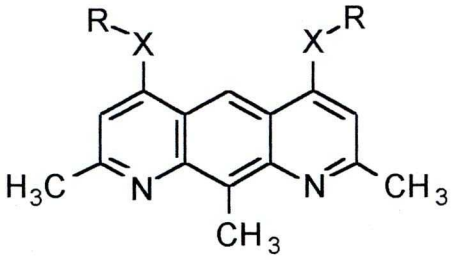


Figure 15.

Pyridoquinolines prepared are listed in **Table 6**.



Compounds	X	R
9a	O	dimethylaminoethyl
9b	O	diethylaminoethyl
9c	O	diisopropylaminoethyl
9d	O	dimethylaminopropyl
9f	O	pyrrolidinoethyl
9g	O	piperidinoethyl
9h	O	piperidinopropyl
9i	O	morpholinoethyl
11a	S	dimethylaminoethyl
11b	S	diethylaminoethyl
11c	S	diisopropylaminoethyl
11f	S	pyrrolidinoethyl
11g	S	piperidinoethyl
11h	S	piperidinopropyl
11i	S	morpholinoethyl
11m	S	piperazinopropyl
12a	NH	dimethylaminoethyl
12b	NH	diethylaminoethyl
12c	NH	diisopropylaminoethyl
12e	NH	diethylaminopropyl
12i	NH	morpholinoethyl
12k	NH	morpholinopropyl

IV. BIOLOGY

IV. 1. MATERIAL AND METHODS

IV. 1. 1. CELLS

1) The L5178Y mouse T-lymphoma parent cell line was infected with the pHa MDR1/A retrovirus as previously described by Pastan (Pastan *et al.*, 1988).

The L5178 MDR cell line and the L5178 Y parent cell line were grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics (and 60 ng/ml colchicin for the MDR cell line).

2) The K562/ADR multidrug resistant cell line was isolated (Tsuruo *et al.*, 1986) by adaptation to adriamycin from the K562 human chronic myelogenous leukemia parental cell line (Lozzio and Lozzio, 1975).

The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine and antibiotics (and 100 nM adriamycin for the K562/ADR cell line).

IV. 1. 2. RHODAMINE 123 (R123) UPTAKE ASSAY

The L5178 and K562 cells (2×10^6 cells/ml) were resuspended in serum-free medium and distributed (0.5 ml aliquots) to Eppendorf tubes. Compounds to be tested were added at different concentrations and the samples were incubated for 10 min at room temperature.

Then the indicator R123 was added to the samples at a final concentration of 5.2 μM and the cells were incubated 20 min at 37°C; washed twice and resuspended in 0.5 ml phosphate-buffer saline (PBS) for analysis.

The fluorescence of the cell populations was measured by flow cytometry using a Beckton Dickinson FACScan instrument. Since R123 is a substrate of P-gp (Kessel, 1989; Canitrot and Lautier, 1995; Petriz and Garcia-Lopez, 1997), there was a significant difference in fluorescence between MDR and parental cells. Untreated MDR cells accumulate only a low level of R123. Verapamil was used as a reference drug (Weaver et al., 1993).

The fluorescence mean intensities (FL) were determined for the treated cells and were compared to these of untreated cells. The percentage of the multidrug resistance reversion (% MDR Rev.) was calculated as follows :

$$\% \text{ MDR Rev.} = \frac{\text{FL(MDR treated)} - \text{FL(MDR untreated)}}{\text{FL(Parent untreated)} - \text{FL(MDR untreated)}} \times 100$$

IV. 1. 3. CYTOTOXICITY STUDY

K562/ADR cells (1×10^5 cells / ml) were grown for 24 hr in the continuous presence of increasing concentrations of selected compounds.

Cell viability was determined by flow cytometryc analysis using propidium iodide.

IV. 1. 4. RNA EXTRACTION

Total cellular RNA was extracted by the Chomczyinski and Sacchi method (Chomczyinski and Sacchi, 1987) using the RNAXEL Kit (Eurobio).

1 ml RNAXEL (Eurobio) was added to 1-5 x 10^6 cells for 5 min at 4°C, then 160 µl chloroform 5% isoamyl alcohol for further 5 min at 4°C. Cells were centrifuged during 15 min with 12000g, at 4°C.

The water phase (upper phase), which contains exclusively the RNA, was transfered in new tubes. 0,5 volume isopropyl alcohol and 0,05 volume RNABIND (Eurobio) were added to cells and were centrifuged during 1 min with 12000g at 4°C. The supernatant was eliminated; the pelet was washed twice with 1 ml 75% ethanol and dried to eliminate all the remaining ethanol.

The pelet was then resuspended in 0,1 volume ultra-purified water, centrifuged 1 min. The supernatant was transfered in new tubes and the quantity of the RNA was estimated by spectrophotometry.

IV. 1. 5. MDR-1 GENE EXPRESSION STUDY BY RT-PCR

The quantitative analysis of MDR-1 gene was performed by reverse transcription-polymerase chain reaction (RT-PCR).

First, the K562/ADR cells (1×10^5 cells / ml) were treated with non toxic doses of selected compounds for 24 hr at 37°C.

Total cellular RNA was then extracted and about 1 μg of it was used for reverse transcription reaction with random primers.

MDR-1 and the internal control β2 microglobuline (β2m) were amplified with Taq polymerase (Appligen).

The sequences of the primers used are:

MDR-1 (Sense): 5' GCCTGGCAGCTGGAAGACAAATACACAAAAT 3'

MDR-1 (Antisense): 5' GAAGATAGTATCTTTGCCCAGACAGCAGC 3'

β2m (Sense): 5' CCGACATTGAAGTTGACTTAC 3'

β2m (Antisense): 5' ATCTTCAAACCTCCATGATG 3'

PCR was carried out in a Perkin Elmer system 2400. The reaction conditions included an initial cycle of denaturation at 93°C for 2 min, followed by 20 cycles of denaturation for MDR-1 and 23 cycles for β2m at 92°C for 10 sec, annealing at 52°C for 30 sec and extension at 72°C for 45 sec with increments of 20 sec each cycle and one final cycle of extension at 72°C for 7 min at the end.

The amplified products were separated by electrophoresis on 2% agarose gel. The DNA bands were visualized by ethidium bromide staining, and the image was digitalized.

MDR-1 expression was normalized to β2m transcript and was noted as Relative Expression Level (REL):

$$\mathbf{REL} = \frac{\textbf{Densitometric value of MDR-1}}{\textbf{Densitometric value of } \beta\textbf{2m}}$$

IV.1. 6. IMMUNOLOGICAL DETECTION OF P-GLYCOPROTEIN

$5x10^5$cells were washed in phosphate-buffered saline (PBS) and incubated in 50µl of human serum (diluted 1:1 in PBS) for 30 min at 4°C.

The cells were washed again, collected by centrifugation and incubated for 30 min at 4°C with or without 7.5 µg/µl of the primary monoclonal antibody UIC2, which is known to recognize an external epitope of the P-gp (Mechetner *et al.*, 1997; Boutonnat *et al.*, 1998).

After three washes with PBS-1 % BSA, a 30 min incubation was performed at 4°C in the dark with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin (Fab)2 fragment at a 1:20 dilution. This incubation was followed by three washes in PBS-1% BSA and fixation in PBS-1% paraformaldehyde.

The samples were analysed on a FACScan flow cytometer (Becton-Dickinson). The results were expressed as relative fluorescence intensities FL (REL), resulting from differences between the mean fluorescence intensity FL obtained with UIC2 and without it.

$$\textbf{\% FL (REL)} = \frac{(\textbf{FL with UIC2})_{\textbf{treated}} - (\textbf{FL without UIC2})_{\textbf{treated}}}{(\textbf{FL with UIC2})_{\textbf{control}} - (\textbf{FL without UIC2})_{\textbf{control}}} \times \mathbf{100}$$

IV. 2. RESULTS

IV. 2. 1. INHIBITION OF THE P-GLYCOPROTEIN EFFLUX PUMP

IV. 2. 1. 1. SCREENING FOR POSSIBLE MODULATORS OF MDR AMONG NEW THIOACRIDINE DERIVATIVES ON L5178 AND K562/ADR CELLS

New thioacridine derivatives were first screened for their ability to reverse the P-gp function in the L5178 resistant cells at two concentrations (10 and 20 μM). Of the 21 compounds tested, 18 were found to be active in reversing the MDR by inhibition of the rhodamine 123 efflux (Table 7). The reversion varied from 1% (**3g** or **5b**) to 87% (**6a**) for 10 μM and from 2% (**4b**) to 93% (**3n** and **6a**) for 20 μM. Two drugs (**3e** and **5a**) were completely inactive even at the higher concentration.

The efficacy of the same drugs were also investigated on K562/ADR cells (Table 8). We did not find any activity at 10 and 20 μM (data not shown). At 40 μM and 80 μM the highest reversion was obtained with compound **3l** (54%) and compound **3p** (82%). Six drugs (**3d**, **3e**, **3j**, **3m**, **5a**, **6a**) were inactive at 40 μM and three (**3d**, **3e**, **5a**) at 80 μM.

Table 7: Effect of thioacridine derivatives on the P-gp function in L5178 cells.

Compounds	%MDR Rev.	
	10 μM	20 μM
3a	4	16
3b	2	26
3c	4	7
3d	0	2
3e	0	0
3f	6	73
3g	1	5
3j	49	75
3k	24	38
3l	59	67
3m	2	4
3n	27	93
3p	25	58
4a	3	23
4b	2	5
4c	7	23
5a	0	0
5b	1	4
6a	87	93
6b	58	70
7	46	74

Table 8: Effect of thioacridine derivatives on the P-gp function in K562/MDR cells.

Compounds	%MDR Rev.	
	40 μM	80 μM
3a	3	4
3b	1	1
3c	13	13
3d	0	0
3e	0	0
3f	4	25
3g	6	28
3j	0	4
3k	2	24
3l	54	67
3m	0	22
3n	24	21
3p	35	82
4a	4	9
4b	1	14
4c	0	29
5a	0	0
5b	18	20
6a	0	2
6b	1	3
7	5	16

IV. 2. 1. 2. SCREENING FOR POSSIBLE MODULATORS OF MDR AMONG NEW PYRIDOQUINOLINE DERIVATIVES ON L5178 AND K562/ADR CELLS

The effect of 22 new pyridoquinoline derivatives on the function of the P-gp was tested first at 10 and 20 μM concentrations in L5178 cells. Because some of the compounds (**11f**, **11g**, **11i**) could already completely block the function at 10 μM, they were further tested at lower concentrations.

The pyridoquinolines of the series **9** were inactive at 0.4 μM and 1μM, but at 2 μM we found 7% reversion with **9a** and 10% with **9d** compounds (Table 9). The pyridoquinolines of the series **11** were active even at 1 μM, except **11m**. The best activity was observed with compound **11i** which even at the lowest tested concentration (0.4 μM) showed 11% reversion (Table 10). The derivatives of the series **12** were the less active among the tested pyridoquinolines. **12a**, **12e**, **12i**, **12k** were absolutely inactive even at highest concentration (20 μM). The **12c** was the only compound with real reversal effect (18% reversion), but only at 20 μM (Table 11).

The effect of the tested new pyridoquinoline derivatives on the P-gp function in the K562/ADR cells (Table 12) was in the same range, as in the L5178 cells. The compounds **11** showed strong reversion varying from 2% (**11c**) to 54% (**11i**) for 40 μM and from 4% (**11c**) to 63% (**11i**) for 80 μM. The compounds **9** were almost inactive or just slightly active, as **9g** (5% reversion at 80μM) and the compounds **12** were inactive.

Table 9: Effect of pyridoquinoline ethers on the P-gp function in L5178 cells.

Compounds	%MDR Rev.					
	0.4 µM	1 µM	2 µM	4 µM	10 µM	20 µM
9a	0	3	7	19	38	57
9b	0	1	2	3	4	6
9c	0	0	0	2	5	49
9d	0	2	10	44	50	54
9f	0	1	3	9	31	88
9g	1	2	4	12	43	84
9h	0	0	0	1	7	63
9i	0	1	1	2	10	41

Table 10: Effect of pyridoquinoline thioethers on the P-gp function in L5178 cells.

Compounds	%MDR Rev.					
	0.4 µM	1 µM	2 µM	4 µM	10 µM	20 µM
11a	1	3	12	55	82	100
11b	3	9	18	42	90	100
11c	3	3	3	8	30	31
11f	2	9	41	100	100	100
11g	1	8	25	77	100	100
11h	2	8	25	64	92	100
11i	11	32	100	100	100	100
11m	0	0	2	6	25	68

Table 11: Effect of pyridoquinoline amines on the P-gp function in L5178 cells.

Compounds	%MDR Rev.					
	0.4 μM	1 μM	2 μM	4 μM	10 μM	20 μM
12a	0	0	0	0	0	0
12b	0	0	0	2	2	3
12c	0	0	2	5	10	18
12e	0	0	0	0	0	0
12i	0	0	0	0	0	0
12k	0	0	0	0	0	0

Table 12: Effect of pyridoquinoline derivatives on the P-gp function in K562/ADR cells

Compounds	%MDR Rev.		Compounds	%MDR Rev.		Compounds	%MDR Rev.	
	40 µM	80 µM		40 µM	80 µM		40 µM	80 µM
9a	--	--	11a	5	23	12a	0	0
9b	0	0	11b	20	58	12b	0	0
9c	0	0	11c	2	4	12c	0	0
9d	--	--	11f	21	42	12e	0	0
9f	--	--	11g	10	23	12i	0	0
9g	0	5	11h	35	47	12k	0	3
9h	0	3	11i	54	63			
9i	0	2	11m	--	--			

IV. 2. 2. INHIBITION OF THE MDR-1 GENE EXPRESSION

IV. 2. 2. 1. EVALUATION OF THE MDR-1 RNA EXPRESSION AFTER EXPOSURE OF K562/ADR CELLS TO SOME SELECTED THIOACRIDINES

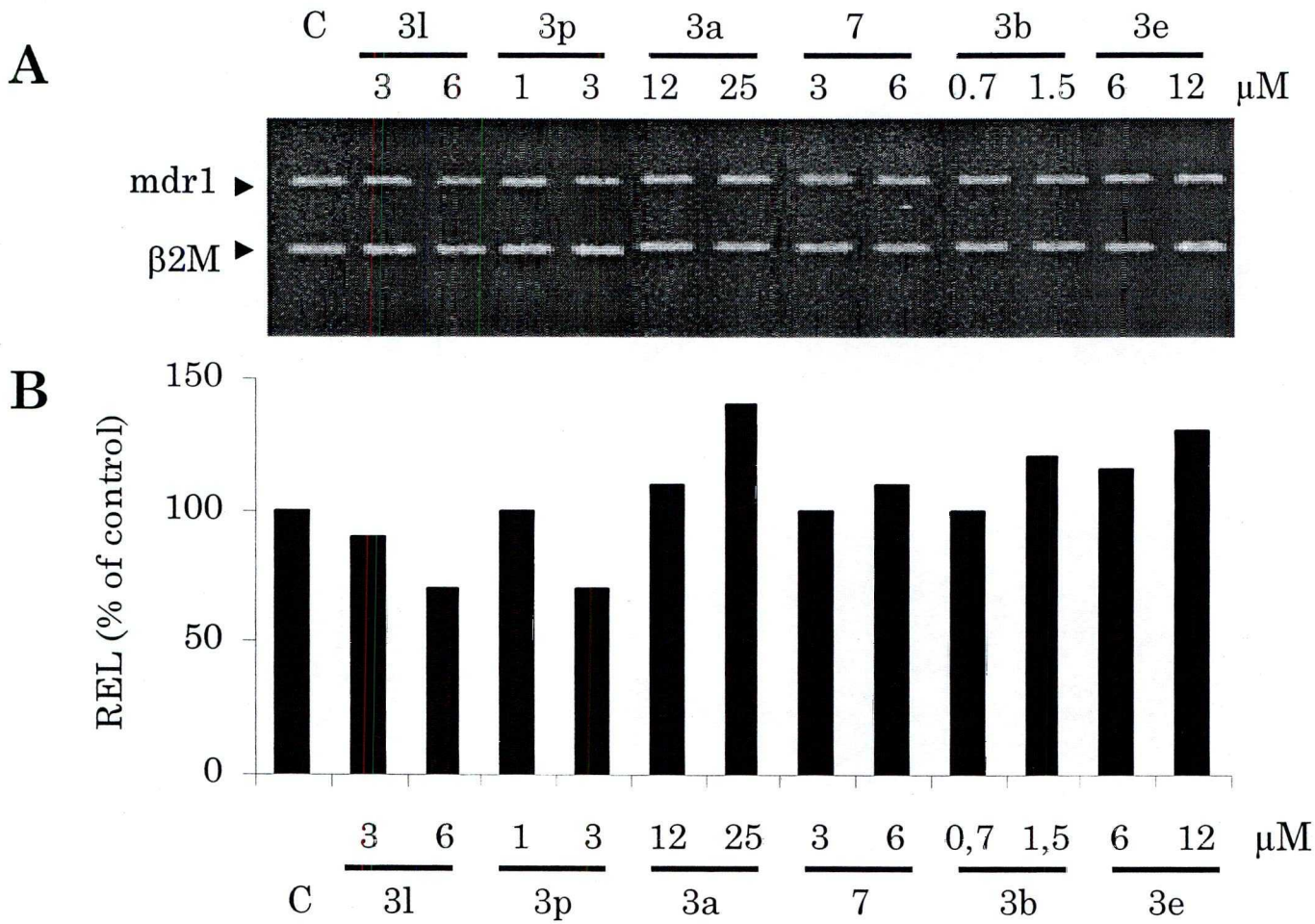
Six representative of the previously tested compounds (two active, two medium active and two inactive reversers of the P-gp function) were analyzed for their possible effect on the MDR-1 gene expression. Initial experiments attempted to establish their highest non-toxic doses for a 24 hr treatment (Table 13). With **3l**, **3p**, **3a**, **7**, **3b** and **3e** these doses were 6 μM, 3 μM, 25 μM, 6 μM, 1.5 μM and 12 μM respectively.

It can be seen in Figure 16, that depending on the drug, treatment produces a 30% decrease (**3l** and **3p**), a 30% increase (**3e**) or 40% increase (**3a**) in MDR-1 gene expression in K562/ADR cells. In some cases (**3b** and **7**) no modifications were found at the gene expression level.

Table 13: Cytotoxicity of the thioacridine derivatives in K562/ADR cells.

Compounds	% of dead cells							
	0	1.5	3	6	12	25	50	100 µM
3l	15	8	9	13	40	90	100	100
3p	12	9	11	34	36	99	99	100
3a	15	10	10	10	12	10	17	24
7	15	8	10	12	20	100	100	100
3b	15	7	23	29	47	52	74	80
3e	16	4	6	8	8	30	32	44

Figure 16: Effect of thioacridine derivatives on the MDR-1 gene expression in K562/ADR cells.



A: Representative RT-PCR analysis of MDR-1 gene expression
B: Representation of the results obtained after quantification of the digitalized gel image
REL quantitates the level of MDR-1 mRNA normalized to β2 M (mean of 10 RT-PCR realized after 3 independant treatments)

IV. 2. 2. 2. EVALUATION OF THE MDR-1 RNA EXPRESSION AFTER EXPOSURE OF K562/ADR CELLS TO SOME SELECTED PYRIDOQUINOLINES

For the MDR-1 gene expression studies from the pyridoquinolines series, we selected the following representative derivatives: **11h** and **11i** as active compounds on the reversal of the P-gp function, **11c** and **9g** as medium active compounds, **12i** and **12e** as inactive compounds.

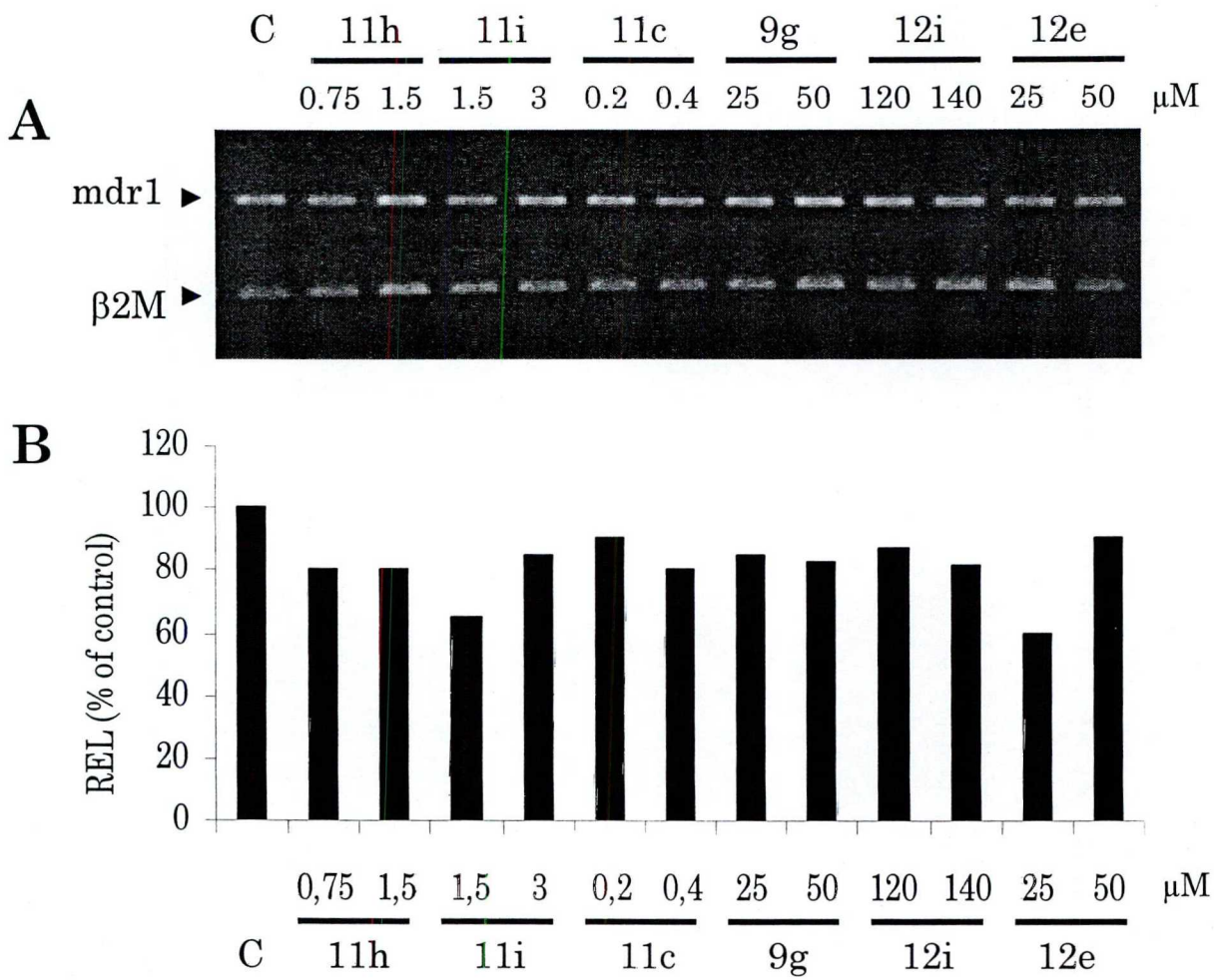
Treating the K562/ADR cells for 24 hr with different concentrations of these compounds, the highest non-toxic doses were: 1.5 μM for **11h**, 3 μM for **11i**, 0.4 μM for **11c**, 50 μM for **9g**, 140 μM for **12i** and 50 μM for **12e** (Table 14).

Exposing the cells to the non-toxic doses of compounds, we obtained 20% decrease with **11h**, 35% decrease with **11i** and 40% decrease with **12e**. No significant effect was found with **11c**, **9g** and **12i** (Figure 17).

Table 14: Cytotoxicity of pyridoquinoline derivatives in K562/ADR cells.

Compounds	% of dead cells													
	0	**0.2**	**0.4**	**0.75**	**1.5**	**3**	**6**	**12**	**25**	**50**	**100**	**120**	**140**	**160µM**
11h	17	-	-	-	15	61	95	97	98	98	99	-	-	-
11i	17	-	-	-	14	15	20	30	63	74	85	-	-	-
11c	7	6	6	9	23	31	44	73	83	87	99	-	-	-
9g	14	-	-	-	6	8	8	7	10	11	16	19	20	24
12i	12	-	-	-	7	7	9	9	10	11	11	12	12	18
12e	14	-	-	-	8	12	11	11	13	13	17	24	25	25

Figure 17: Effect of pyridoquinoline derivatives on the MDR-1 gene expression in K562/ADR cells.



A: Representative RT-PCR analysis of MDR-1 gene expression
B: Representation of the results obtained after quantification of the digitalized gel image
REL quantitates the level of MDR-1 mRNA normalized to β2 M (mean of 10 RT-PCR realized after 3 independant treatments)

IV. 2. 3. CHANGES IN THE P-GLYCOPROTEIN LEVEL ON THE CELL SURFACE

IV. 2. 3. 1. EFFECTS OF THIOACRIDINES ON THE QUANTITY OF P-GLYCOPROTEIN IN K562/ADR CELLS

We treated the cells with non-toxic doses of some selected thioacridines for 24 hr, similarly as we did for the MDR-1 gene expression studies. After this treatment we quantified the amount of P-gp on the cell surface by immunostaining method.

In the case of all tested thioacridines, there was an increase in the P-gp level. However, we have to admit that this increase was significant only when cells were treated with compound **3l** (40% increase), while with compounds **3p** and **3e** we only found a 10% increase (Table 15).

Table 15: Effect of thioacridines on the P-gp level.

Compounds	FL with UIC2	FL without UIC2	% FL (REL)
3l (6μM)	529	83	144
3p (3μM)	392	48	111
3e (12μM)	398	45	114
control	353	43	-

IV. 2. 3. 2. EFFECTS OF PYRIDOQUINOLINES ON THE QUANTITY OF P-GLYCOPROTEIN IN K562/ADR CELLS

For the studies on the quantity of P-gp we selected compounds **11h**, **11i** and **12e** from the pyridoquinolines series.

A 24 hr treatment of K562/ADR cells with non-toxic doses of these derivatives results in a slight increase with **11h** (10% inrease) and **11i** (20% increase), or in a slight decrease with **12e** (10% decrease) (Table 16).

Table 16: Effect of pyridoquinolines on the P-gp level.

Compounds	FL with UIC2	FL without UIC2	% FL (REL)
11h (0.75µM)	395	39	110
11i (1.5µM)	420	31	120
12e (25µM)	318	30	89
control	365	41	-

V. STRUCTURE-ACTIVITY RELATIONSHIP STUDIES AND MOLECULAR MODELLING

V. 1. MATERIAL AND METHODS

Ionization constants and partition coefficients were calculated from the PALLAS software (Compudrug Chemistry Ltd., Budapest, Hungary).

Molecular modelling was performed on a ESV10/32 work station from Evans and Sutherland, using the Sybyl software from Tripos Associates.

Docking was achieved using the crystal structure of the ligand (Karolak *et al.*, unpublished results) and that of the ATP site of adenylate kinase (Dreusicke *et al.*, 1988) as starting geometries. Amino acid mutation (deletion/insertion), needed to portraying the three dimensional structure of the ATP site of the P-gp 170, were made according to the structural analysis published by Hydes (Hydes *et al.*, 1990).

Molecular mechanics calculations (Tripos Force Field) at the default level set-up were used for the minimisation of the energy.

V. 2. RESULTS

V. 2. 1. STRUCTURE-ACTIVITY RELATIONSHIPS

Although studied compounds belong to two closely related series, effectively, acridine and pyridoquinoline derivatives are both planar aromatic azatricyclic compounds, nevertheless the former are monoaza derivatives with only one side chain, while the later are diaza derivatives with two side chains. This fact has an influence on the various chemical and physical properties of the compounds in terms of basicity, lipophilicity, molecular volume, conformational flexibility, *etc*.

However, there are some definite common features which allow to do the assumption of a common mechanism of action for both series, despite there are some drastic different characteristics within each of these series.

On one hand, structural similarities can be pointed out as shown in Figure 18.

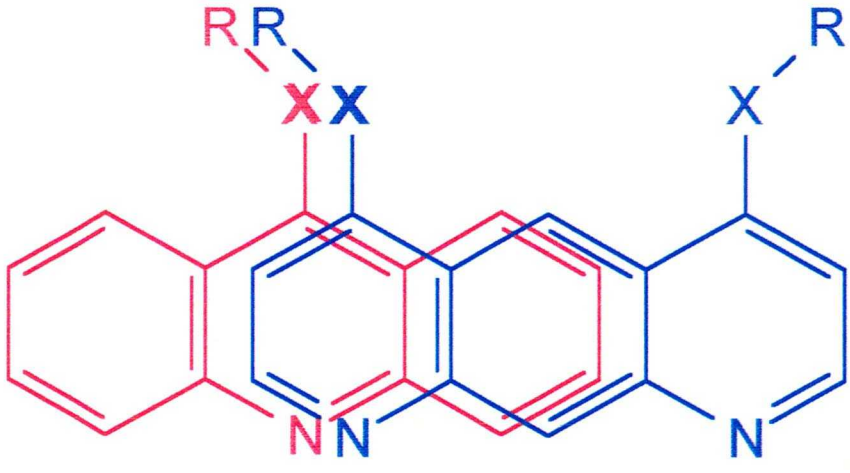


Figure 18: Structural similarities between thioacridines and pyridoquinolines.

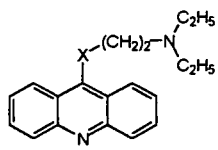
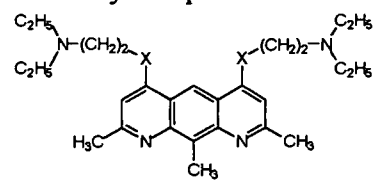
On the other hand, as differences, we can mention:

- in the thioacridine series: Three compounds are bis-derivatives, while for the rest of them which are mono-derivatives, the type of side-chain is basically dissimilar. Some compounds are branched with an alkylaminoalkyl group, while some others are branched either with an arylalkyl or an aryl one, and at the last, some of them are thioethers, some are sulfoxides, some others are sulfones.

- in the pyridoquinoline series: All the compounds are mono-derivatives alkylaminoalkyl side chained, but they separate themselves into three sub-groups according to the nature of the connecting atom between the aromatic nucleus and the side chain, namely, ethers, thioethers and amines.

Some properties picked out of the different compounds are compared in Table 17.

Table 17: Comparison between the physicochemical properties of selected azatricyclic compounds.

Series : ⇨ Formulas : ⇨	Acridine 	Pyridoquinoline 		
X	S	S	O	NH
basicity (pKa)	9.29	9.59	9.52	10.41
lipophilicity (LogP)	4.46	4.99	4.23	3.56
molecular volume in $Å^3$	179	248	233	241

Due to that, at first we shall discuss separately the structure-activity relationships (SAR) among thioacridines and among pyridoquinolines before the two series together, as azatricyclic compounds be gathered and SAR deduced for the whole.

SAR are based on the structural-, physical- and chemical properties of the compounds and their ability to inhibit the function of the P-gp in L5178 tumor cells (because the range of this inhibition effect is on a wider scale in the case of L5178 cells, than in the case of K562/ADR cells). Due to the insufficient number of tested drugs on MDR-1 gene expression, we cannot establish a correlation between the chemical structure and the gene expression modification by compounds.

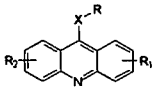
V. 2. 1. 1. STRUCTURE-ACTIVITY RELATIONSHIPS AMONG THIOACRIDINES

As a rule, sulfoxides and sulfones are poorly active derivatives. Where the parent sulfide is active, derivatives oxygenated on the sulfur atom are notably less active (**4c**<**3p**).

As regards the side chain, protonatable nitrogen is generally required for a good activity, insofar as compounds **3k**, **3p**, **3n** are almost the ones to be really active. This is clearly shown in the following sequences: **3e**~**3d**<**3g**<<**3p** and **5a**<**4b**~**5b**<**4c**. But this feature seems to be not absolutely necessary, as compound **3l** is also active, although its protonable nitrogen is not located in the side chain but directly branched to the nucleus.

Added to this, substituents directly branched on the heterocyclic moiety usually decrease the activity, apart from the protonatable ones (**3l**). However, no definitive conclusion can be drawn, because of the restricted number of substituents tested; although it is a proved fact that substitution in both phenyl rings is of a few interest. Actually, this is unambiguously demonstrated by results collected in Table 18.

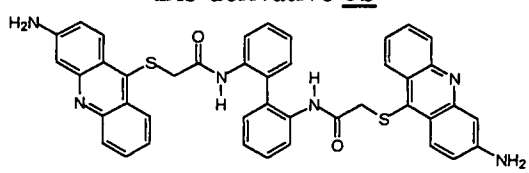
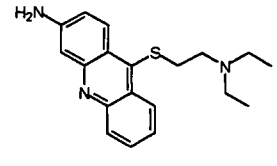
Table 18: Number of compounds <> and % of measured activity at 10μM concentration of drugs (min-max).

Subst.	No subst.	1,4-dimethoxy	2,7-dimethoxy	2-methoxy-6-chloro	3-chloro	3-amino
	<2> (2-49)	<1> (4)	<10> (0-25)	<1> (24)	<2> (4-6)	<2> (27-59)

Moreover, this is confirmed comparing in Table 7 the following compounds: **3c** to **3d** (4% and 0% reversal effects at 10μM), **3f** to **3g** (6% and 1% reversal effects at 10μM), **3k** to **3j** (49% and 24% reversal effects at 10μM), **3l** to **3m** (59% and 2% reversal effects at 10μM) and **3n** to **3p** (27% and 25% reversal effects at 10μM, but 93% and 58% at 20μM).

About the bis-derivatives we have to notice that they distinguish from the rest of the series, because they have no protonatable nitrogen on the side chain and possess highly different physicochemical properties. Thus, when we shall discuss SAR in the thioacridines studied, we shall exclude the three bis-compounds (**6a**, **6b**, **7**), because neither their physicochemical properties nor their structural properties are of the same order than for the other derivatives, as shown in Table 19.

Table 19: Differences between the properties of two related bis- and mono-derivatives.

Compounds : ⇨ Formulas : ⇨ Properties ⇩	Bis-derivative **6b**	Mono-derivative **3n**
		
Max molecular extent (Å)	23.2	10.8
Molecular volume ($Å^3$)	407	179
Molecular weight	686	310
Log P	10.2	3.60

V. 2. 1. 2. STRUCTURE-ACTIVITY RELATIONSHIPS AMONG PYRIDOQUINOLINES

As a rule, the thioderivatives **11** are the most active compounds tested whilst the amino derivatives **12** are almost inactive. The oxoderivatives **9** show intermediate activity. This is clearly demonstrated by the following sequences: **11a**>**9a**>**12a** and **11b**>**9b**>**12b** at the 20μM concentration, and **11i**>**9i**>**12i** at the 2μM concentration of drugs.

Added to this, active compounds can be ranked in the same order whatever is the series, **11** or **9**. The main difference to be noted is the magnitude of the effect which is greater with compounds **11** than with compounds **9**. This leads to admit the prominent role of the sulfur atom in the binding of the ligand to the active site.

As regards the side chains (see Figure 19), ethyl group is better than the propyl one as shown in the following sequences: **11i**>**11f**>**11g**,**11h**>**11m** at 2μM concentration and **11g**>**11h**>**11m** at 10μM concentration of drugs. However, heterocyclic substitutions are more favourable than alkyl substitutions. This is demonstrated by the sequence: **11i**>**11f**>**11g**>**11b**>**11a**<**11c**>**11m** at 2 μM concentration of drugs.

The abnormality noted in the position of **11m** in this sequence could be due to the localization of the cationic charge on the piperazinyl nitrogen, which is the farthest from the sulfur atom branched with the substituent. Indeed, in this condition, the ionic bond between the cationic nitrogen of the ligand and the anionic charge carried on the acidic moiety of the target as depicted below in the chapter devoted to molecular modelling, is not permitted. According

to this, the activity is expected to decrease, as actually observed. Added to this, pKa and logP values for this derivative are very close to those of the inactive amino subseries.

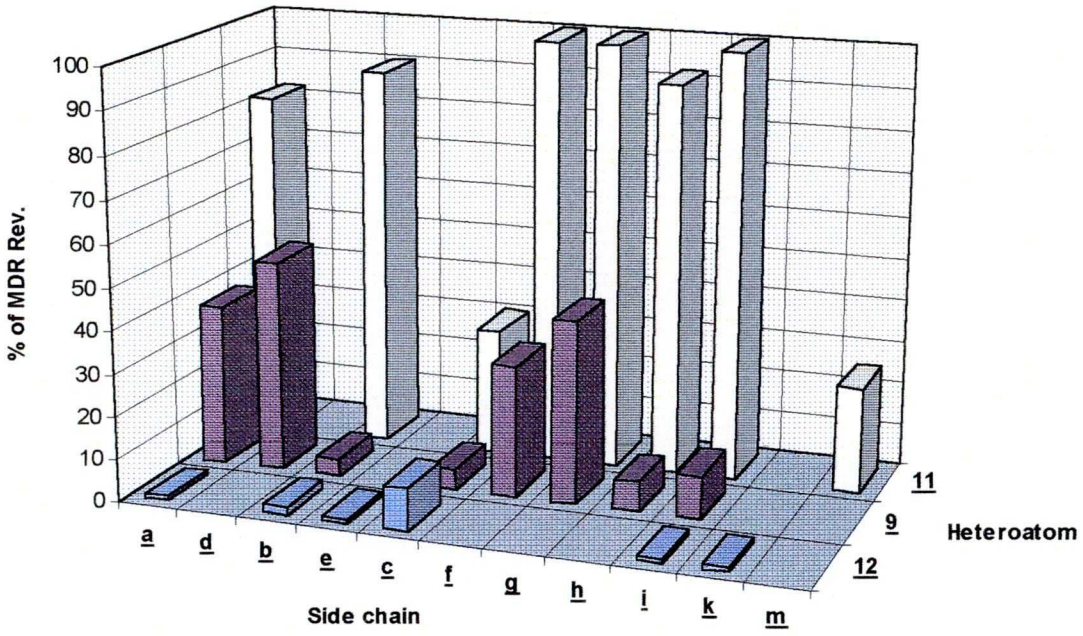


Figure 19: Activity *vs* type of side chain Side chain: a = dimethylaminoethyl, d = dimethylaminopropyl, b = diethylaminoethyl, e = diethylaminopropyl, c = diisopropylaminoethyl, f = pyrrolidinoethyl, g = piperidinoethyl, h = piperidinopropyl, i = morpholinoethyl, k, m = piperazinopropyl. Heteroatom: 9 = sulfur, 11 = oxygen, 12 = nitrogen.

We have to emphasize that the activity due to amino functions substituted with a little sized group (methyl) is favoured by a long alkyl chain (propyl), while that due to cyclic amines which are of important size are favoured by a shorter alkyl chain (ethyl).

Moreover, the diethylaminoethylether **9b** and the diisopropylaminopropylether **9c** show abnormally low activities.

There are no significant differences between pyrrolidinyl and piperidinyl nuclei and the presence of an oxygen atom in morpholino compounds seems only change slightly the activity.

V. 2. 1. 3. COMPARATIVE STRUCTURE-ACTIVITY RELATIONSHIPS BETWEEN THIOACRIDINES AND PYRIDOQUINOLINES

As illustrated in Figure 20, we can discriminate the compounds studied between three groups:

-First, 7 derivatives with activity more than 80%

-Second, 13 derivatives with activities between 24% and 59%

-Third, 23 derivatives with activity less than 10%

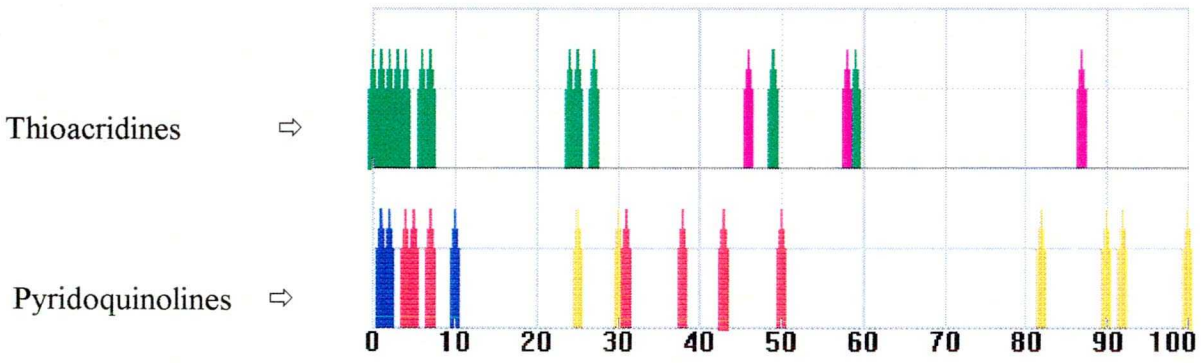


Figure 20: Statistical distribution of activities in the compounds studied. Keys for colors: magenta = acridinyl bis-derivatives (**6** and **7**), green = acridinyl mono-derivatives (**3**, **4** and **5**), yellow = pyridoquinolinyl thioethers (**11**), red = pyridoquinolinyl ethers (**9**), blue = pyridoquinolinyl amines (**12**).

V. 2. 1. 3. 1. TOPOLOGICAL FEATURES

As previously mentioned, the bis-derivatives are not included in the discussion. As regards the 40 remaining compounds, several structural features must be taken into account, as shown in Figure 21.

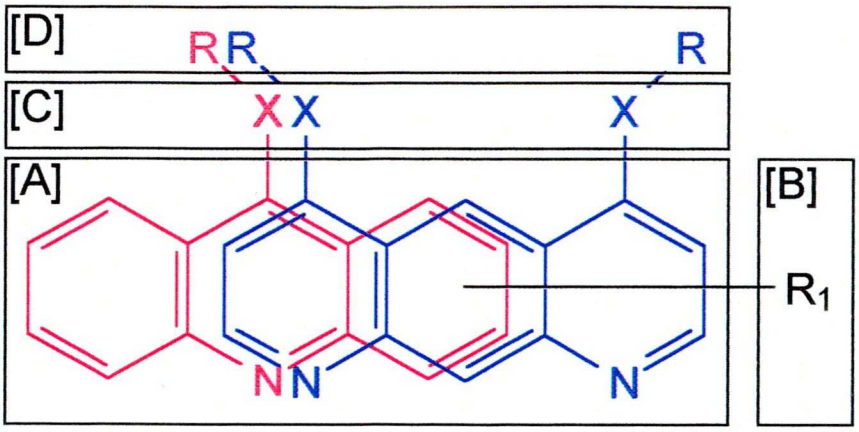


Figure 21: Molecular cutting out for SAR.

Thus, have to be considered:

- the heterocyclic moiety [A]
- the substituent(s) R_1 [B]
- the connecting atom or atom group X [C]
- the side chain R [D]

[A] Heterocyclic moiety:

According to the results gathered in Table 20, the pyridoquinoline nucleus seems to be markedly more convenient than that of acridine. This is demonstrated comparing for example at 10μM concentration the activity of **3n** (27%) to that of **11b** (90%) which are from a topological point of view, the most similar compounds among those tested. However, at 20μM concentration, activities of the selected compounds are not so different (93% *vs* 100%). Thus, no definite conclusion can be drawn because the differences observed could only depend on the presence of two side-chains in the pyridoquinolinic derivatives, while there is just one in the acridinic derivatives.

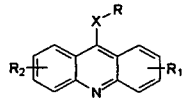
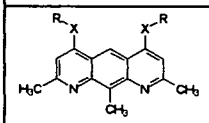
[B] Substituents R_1:

The lack of similarity in these substituents, when acridines are compared to pyridoquinolines, do not allow any common discussion.

[C] Connecting atoms or atom groups:

Results summarized in Table 20 clearly demonstrate also that the sulfur atom is undoubtedly the best connecting atom, whatever is the heterocyclic moiety. this is demonstrated by an activity level greater than 50%, which is only observed with thioethers.

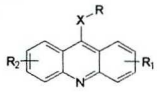
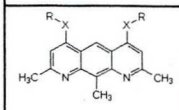
Table 20: Number of compounds <>and % of measured activities at 10µM concentration of drugs (min-max).

X ⇨ Chemical series ⇩	NH	O	S	SO	SO_2
			<13> (0 - 59)	<3> (2 - 7)	<2> (0 - 1)
	<6> (0 - 10)	<8> (4 - 50)	<8> (25 - 100)		

[D] Side chain R:

As shown in Table 21, activity is dramatically increased when compounds are branched with an alkylaminoalkyl side-chain. However, activity is not so bad with thioarylacridines (see Table 22; where optimal activity is observed, the vertical and horizontal columns are shadowed), like **3j**, **3l** and **3k** (75%, 38% and 67% reversion at 20µM concentration). This is a little bit disturbing, but could be more understandable if there is more than one active site in the target.

Table 21: Number of compounds <> and % of measured activity (min-max) at 10µM concentration of drugs.

X group ⇨	NH	O	S			SO			SO2
Chain ⇨ Nucleus ⇩	ami	ami	div	ami	aro	div	ami	aro	div
			<7> (0-6)	<2> (7-27)	<4> (2-59)	<1> (3)	<1> (7)	<1> (2)	<2> (0-1)
	<6> (0-10)	<8> (4-50)		<8> (25-100)					

Keys: -div for diverse: ethyl, chloroethyl, hydroxyethyl, epoxypropyl;
-ami for alkylaminoalkyl with protonatable nitrogen;
-aro for aromatic: phenyl or benzyl.

Table 22: Activity (in %) measured at 10µM (min-max) and 20µM [] concentration of acridines.

Subst.	No subst.	1,4-dimethoxy	2,7-dimethoxy			2-methoxy 6-chloro	3-chloro	3-amino
X group	S	S	S	SO	SO_2	S	S	S
div	(2)	(4)	(0-1)	(3)	(0-1)		(4-6)	
ami			(25) [58]	(7)				(27) [93]
aro	(49) [79]		(2)	(2)		(24) [38]		(59) [67]

V. 2. 1. 3. 2. PHYSICOCHEMICAL FEATURES

Physicochemical features, like logP and pKa, were tested independently *vs* activity, but no significant correlations were observed.

Yet, as shown in Figure 22, compounds could be divided in two clusters, [1] and [2], on the condition that the couple of parameters log P and pKa be used simultaneously. The area [1] includes all the alkylaminoalkyl derivatives (*e.g.* the whole series of pyridoquinolines and the thioacridines **3n**, **3p**, **4c**), while the area [2] contains the thioacridines with a non-alkylaminoalkyl side chain.

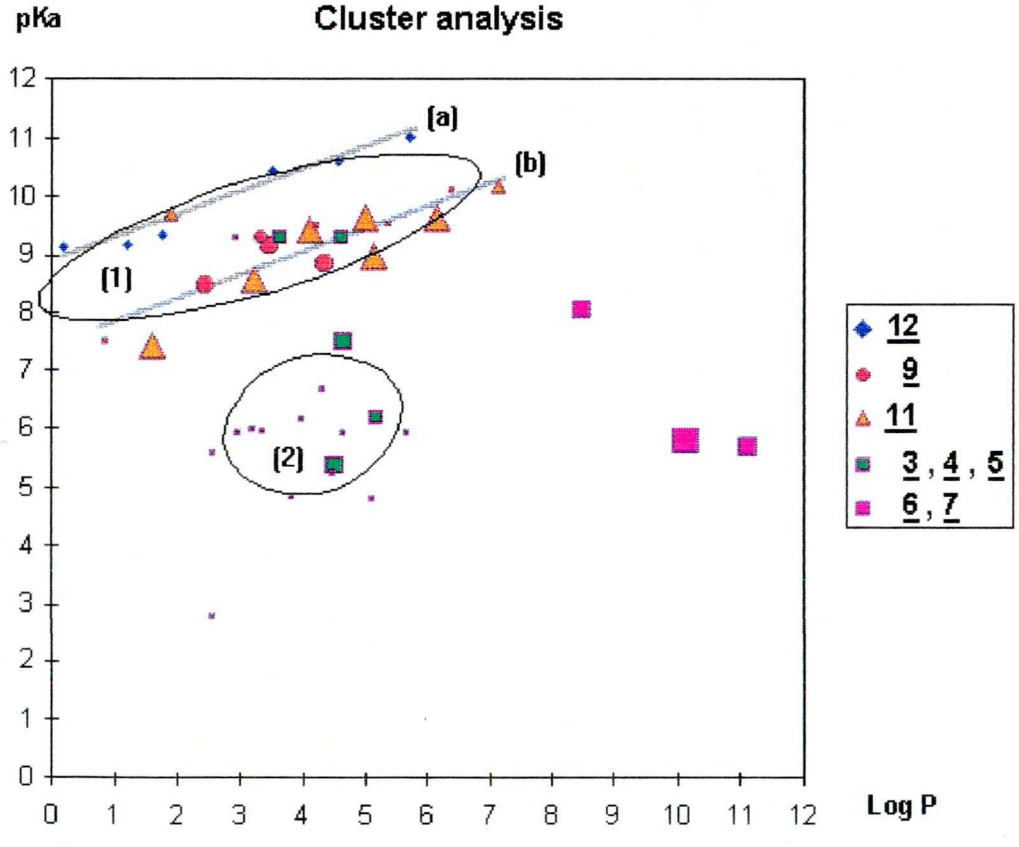


Figure 22: Distribution of pKa *vs* logP ([1], [2], [a], [b]: see in the text).

In addition, on the one hand, there is a sub-clustering separation in cluster [1] based on the number of amino groups.

Thus, we can distinguish:

- Derivatives with at the same time a tertiary amino group and a secondary amino group (**11m** and **12a** to **12k**) with a good correlation between pKa and log P : pKa = 0.37 logP +8.89 with R^2 = 0.95. This sub-cluster of compounds, called [a] in Figure 22, contains only poorly active derivatives.

- Derivatives with only a tertiary amino group (**3n**, **3p**, **4c**, **9a** to **9i** and **11a** to **11i**) with a good correlation between pKa and log P ; pKa = 0.42 logP +7.29 with R^2 = 0.82. This sub-cluster, called [b] in Figure 22, groups together active and inactive derivatives.

On the other hand, derivatives with various side-chains, including the aromatic ones, are gathered in cluster [2]. The sole active compounds (**3j**, **3l**) in this group have the same value of logP (approximately 4.5)

Finally, one can observe that bis-derivatives of thioacridines, which are highly active compounds, possess pKa similar to the mono-derivatives, while their logP is approximately twice that of mono-derivatives.

Consequently, we are forced to admit that no fully convincing correlations can be proposed at the present time.

V. 2. 2. INTERACTION OF LIGAND/ACTIVE SITE BY MOLECULAR MODELLING

Considering the suggested correlation between the chemical structures and the MDR reversion effects, the results previously discussed allow us to propose a hypothetic model for interactions between azatricyclic drugs, like thioacridines and pyridoquinolines, and the protein under consideration.

The sequence of the P-gp is known, its secondary structure was predicted and there are some hypothesis about its tertiary structure. It is generally assumed that this protein is constituted of two main parts : a multi-helix transmembrane domain with a role of active efflux channel and a nucleotide binding site giving the required amount of energy to the efflux pump.

Though these two domains could be used as a target for an inhibitor of the P-gp, no particular region of these two parts has been really identified as an active site for none of the known MDR reversal agents. Because the chemosensitisers belong to completely dissimilar pharmacological and chemical classes of drugs, it should not be surprising that they interact with different and unrelated active sites according to a multi-site mechanism as indicated by enzymatic analysis of P-gp.

Among the possible active sites already listed, attention was recently focused on the ATP site (Gottesman and Pastan, 1988; Safa *et al.*, 1990; Orlowski *et al.*, 1996; Garrigos *et al.*, 1997), because the cellular efflux is energy-dependent (Dano, 1973) and the active site of an ATPasic blocking action could be the proper fixing site for the nucleotide.

Owing to the lack of actual knowledge about the structure (yet, the three dimensional structure of the P-gp has not been solved till now) and the functionallity of the transmembrane domain, add to the unfortunate fact that in this place there is no acidic residue able to give a ionic pair with the protonatable nitrogen of reversal agents (Hait and Aftab, 1992), it is not possible to modelise any tricyclic derivatives-active site interactions at this level. The residues identified as having a role in the specificity of the recognition of anticancer drugs to efflux them out of the cell are not necessarily directly implied in their mechanism of action.

The similarities between the predicted secondary structures of members of the ATP-binding cassette (ABC) superfamily of transport systems (including the P-gp), and the previously determined structure of adenylate kinase, has been emphasised (Hydes *et al.*, 1990). As the crystal structure of the porcine cytosolic adenylate kinase cocrystallised with two sulphate ions (acting for the phosphate group of the ATP) (Dreusicke *et al.*, 1988) is known, we decided to adopt it as a target for the docking of the prepared compounds to the site. With respect to this, it must be noted that the concerned segments in the selected kinase are similar to those in the P-gp, where they are quoted as nucleotide binding sites NB1 and NB2 (Chen *et al.*, 1990; Yoshimura *et al.*, 1989). Moreover, there is two acidic residues in this site, which are capable to give an ionic pair with the studied drugs.

So, our challenge has been only to verify if the hypothesis of a binding of the reversal tricyclic agent in the nucleotide binding site was structurally reasonable.

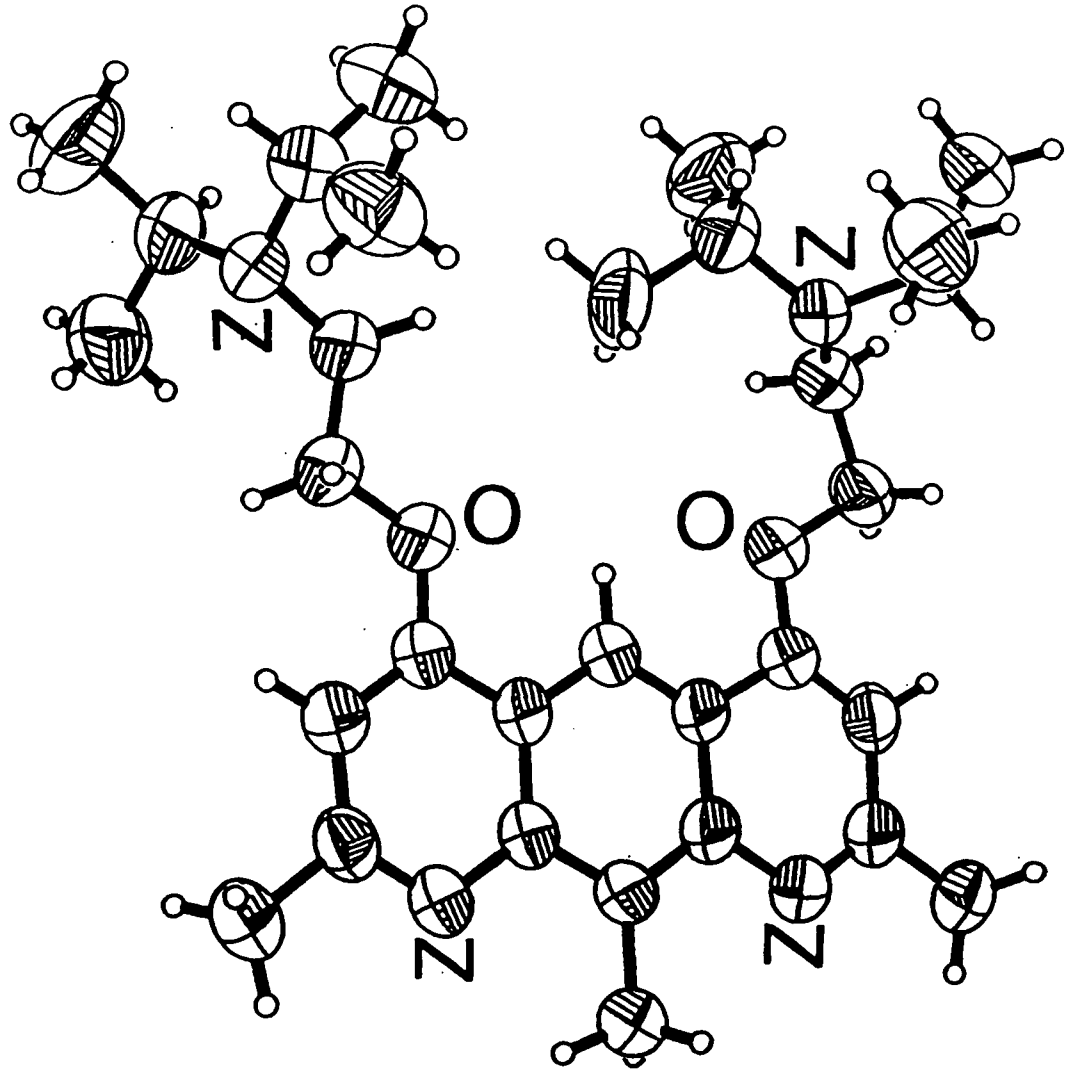


Figure 23: Crystal structure of compound **11c**.

Compound **11c** was selected for the molecular modelling study because X-ray structure of this derivative was successfully determined (Karolak *et al.*, unpublished results) (Figure 23). Thus, basic structural parameters of the ligand became available. However, because the crystal structure did not conveniently dock to the active site, the geometry of the ligand was minimised.

Actually, it is well admitted that the active conformation in the biological medium could be different from the solid state structure on conditions *i)* that energy levels of the conformers taken into consideration be in the same range with that of the crystal structure and *ii)* that energy barriers between conformers be not an insuperable constraint as well. Owing that, the resulting structure for the docking was compared to the solid state one after mapping conformational energy *vs* torsion angles.

With respect to this, twelve rotational motions around chemical bonds can be defined in the selected compound, as shown in Figure 24. Owing to the sterical hindrances which can dramatically restrict the rotation, the most critical pivots are those named 1 and 2 in Figure 24. The bonds concerned in are between the extracyclic heteroatom and the heterocyclic moiety.

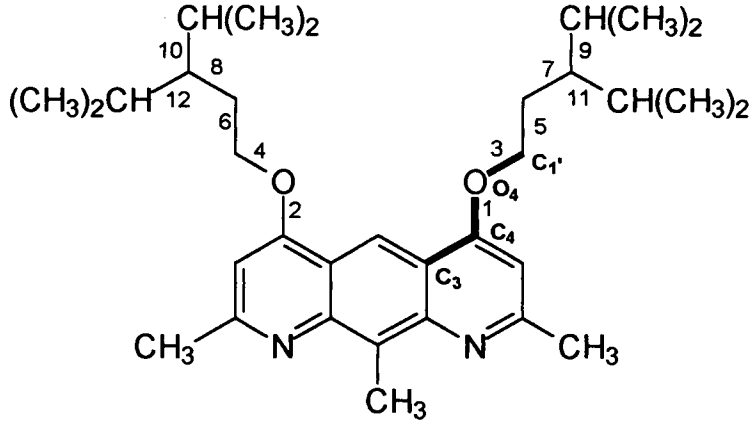


Figure 24: Molecular rotational pivots (1-12).

However, because of the molecular symmetry, only the results obtained with one of these pivots, namely 1 with **C3-C4-O4-C1'** as torsion angle are portrayed in Figure 25.

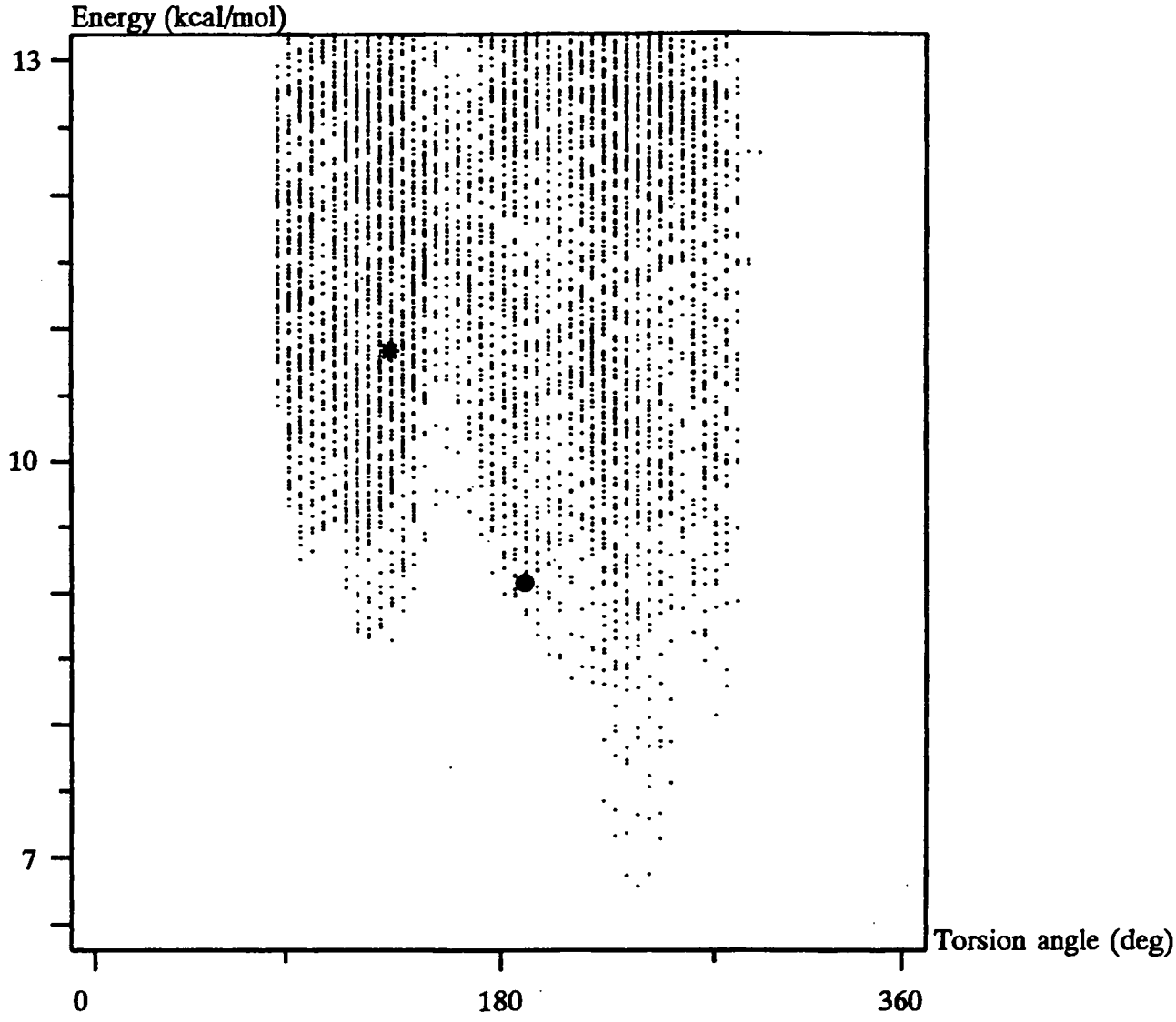


Figure 25: Conformational energy *vs* the **C3-C4-O4-C1'** torsion angle. Each point (·) depicts a conformation of the studied pyridoquinoline. For a given value of the torsion angle, the different spots vertically printed in line correspond to conformations with different energies; they are due to the other rotational motions in the molecule. Crystal (✸) and docking (●) conformations are pointed out.

As energy levels are only 1.74 kcal/mol different from the crystal structure to the computerized docking conformation and energy barrier between these conformers is only about 2 kcal/mol high, the conditions mentioned above are validated. Thus, the docking structure portrayed in Figure 26 can be taken into consideration.

Now, three pharmacophoric groups can be characterised in the pyridoquinolines *i)* the cationic extracyclic nitrogen capable to give ionic bonds, *ii)* the extracyclic heteroatom capable to give hydrogen bonding, and *iii)* the cyclic moiety capable to give hydrophobic interactions.

There are just a few opportunities for ionic bonding in the selected region of the protein: the sole acidic function to be considered is that of aspartic acid 1200. If the ligand is assumed to bind to this aspartic acid through the positive nitrogen atom, hydrophobic bonding with threonine 1203 and hydrogen bonding with either cysteine 1074 (sulfur derivatives) or serine 1077 (oxo- and amino derivatives) can be portrayed (Figure 26).

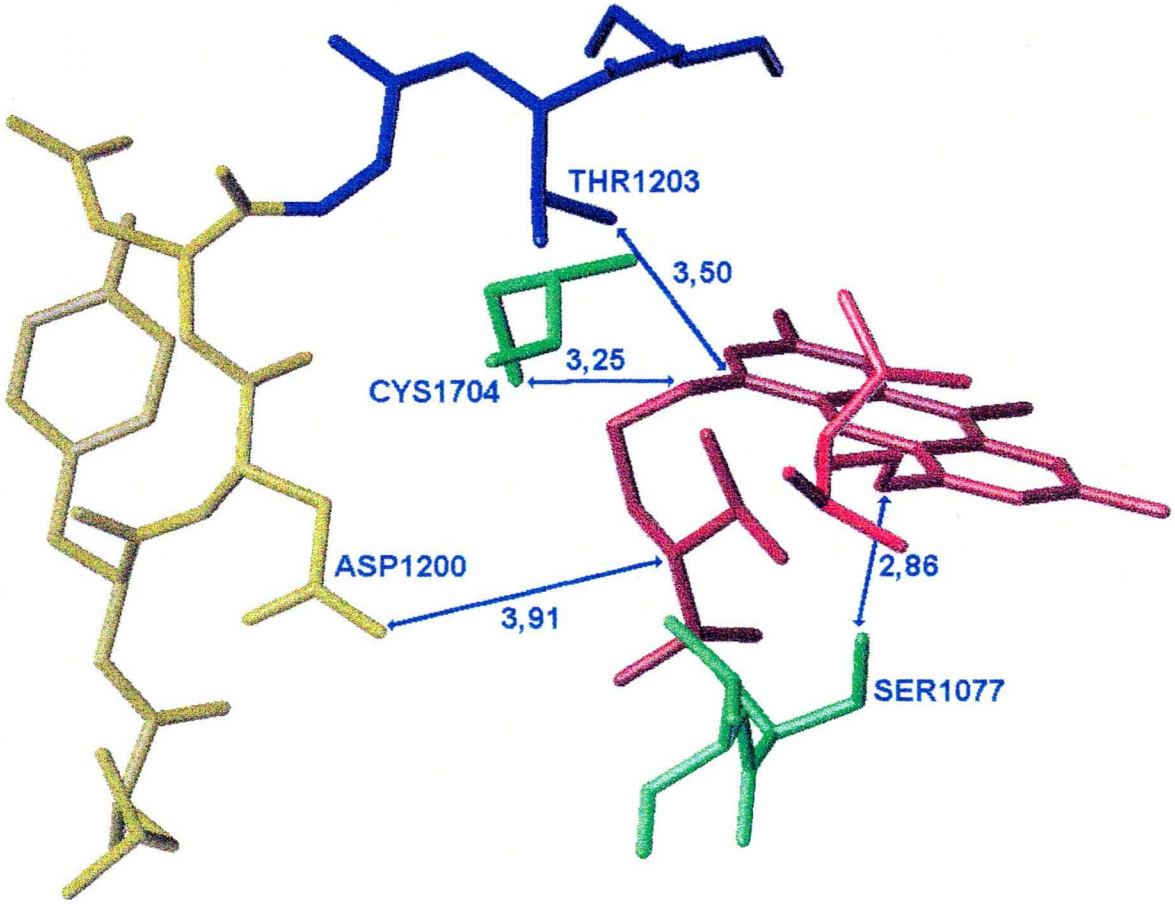


Figure 26: The main aminoacids of the active site in interaction with the ligand. The different groups of aminoacids are coloured according to the type of the involved bonds: ionic bond in yellow, hydrogen bond in green and hydrophobic bond in blue.The pyridoquinoline ligand is in red.

Based on the informations obtained from the docking, three conclusions must be drawn:

I) The amino acids which were taken as a part of the active sites are effectively located in the NB2 region of the protein which is considered as the most convenient one (Dano, 1973; Chen *et al.*, 1990; Yoshimura *et al.*, 1989; Sharom *et al.*, 1995b).

II) The ligand fits closely with the sulphate ions (or phosphate ions) co-crystallised into the kinase, as shown in Figure 27 and 28.

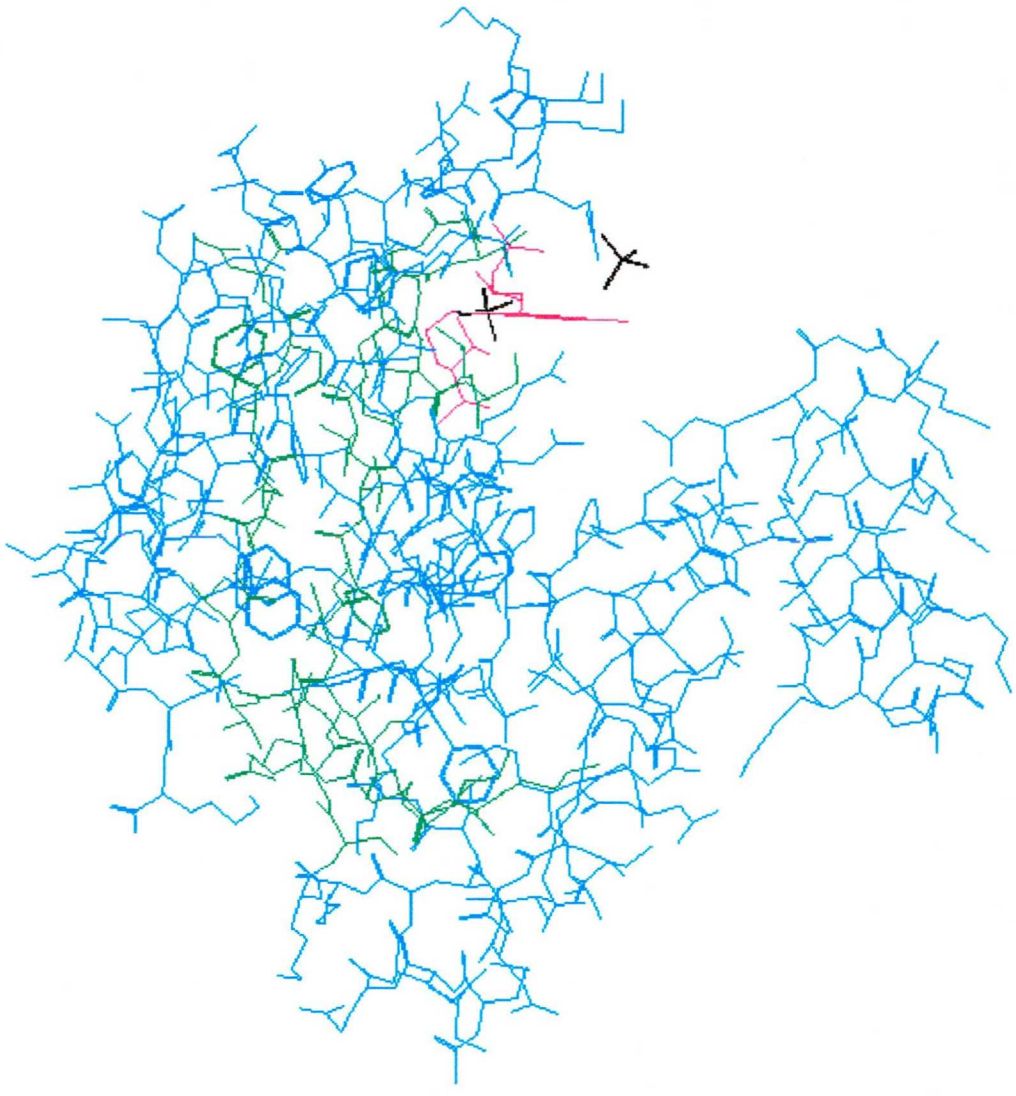


Figure 27: The complete ATPase domain of the modelised P-gp (blue) with pyridoquinoline (red) at the ATP binding site (green) and the two co-crystallised sulfate ions (black).

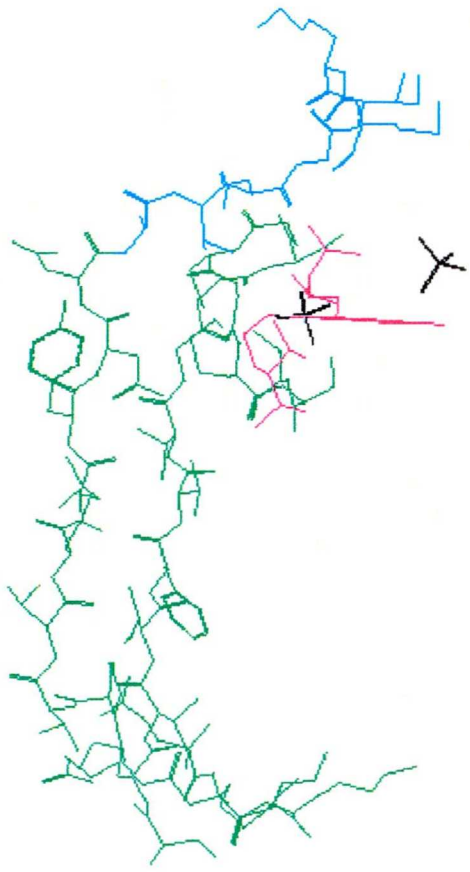


Figure 28: The ATPase domain reduced to the ATP binding site.

III) Third, hydrogen bonding in case of thioderivatives **11**, involves the cysteine 1074 whilst hydrogen bonding in case of oxoderivatives **9**, involves the serine 1077. However, one or the other side chain branched on the heterocyclic moiety of the ligand, is concerned in these hydrogen bondings depending on the chemical series **11** or **9** considered, as shown in Figure 26.

By the way, one can remind that hydrogen bonding of O-H ... O type is stronger than hydrogen bonding of N-H ... O type, as illustrated by the usual length of these bonds in crystals

(2.63 Å *vs* 3.04 Å). All these comments are in agreement with the decrease in activity from compounds **11** to compounds **12** through compounds **9**, as mentioned above.

In a same way, docking of a thioacridine, namely **3n**, can be achieved at the same position with similar interatomic interactions.

However, it must be kept in mind that the mechanism of binding proposed for pyridoquinolines to the ATP site in the modelised P-gp, still remains a model based on theoretical assumptions and has to be confirmed with subsequent investigations.

VI. DISCUSSION

We have shown that among a new series of azaheterocyclic derivatives (thioacridines and pyridoquinolines) there are compounds which inhibit the P-gp function. We observed this effect in both L5178 mouse T-lymphoma cell line and in K562/ADR human chronic myelogenous leukemia cell line. However, in the case of human cells, the reversing activity was observed at higher concentrations than in mouse cells (40-80 μM instead of 10-20 μM). This phenomenon can be linked to our observation that the cell surface is two times larger and the quantity of the P-glycoprotein in the membrane three times higher in the K562/ADR cells compared to L5178 cells.

In the thioacridines series the highest inhibition of the efflux pump was shown with compounds **3l** and **3p**, in both cell lines. These derivatives are in L5178 cells more effective P-gp inhibitors than verapamil, since at 10μM they inhibit the P-gp function by 70% (**3l**) or 25% (**3p**) while only 10 % was acheived with 40 μM verapamil. Moreover, in K562/ADR cells this inhibitory effect was about 70% (**3l**) and 80% (**3p**) at 80μM, while we have to increase the concentation of verapamil to 160μM to achieve only 10% of inhibition. Compounds **6a**, **6b** and **7** are also very active, but this activity strongly decreases from L5178 to K562/ADR cell line. At the present time, there is no explanation for this observation and thus, concerning these bis-derivatives, further investigations are needed. Finally, there are also compounds (**3e** and **5a**) which are completly inactive in both L5178 and K562/ADR cells.

Pyridoquinolines, namely pyridoquinoline thioethers **11**, are excellent agents for reversing the P-gp function in L5178 cells. Almost all of them have good reversal effect already at 2μM concentration (from 12% to 100% reversion) and at 20μM concentration they block completely the efflux pump. In the case of K562/ADR cells, they are also very active,

since most of them are able to reverse the P-gp function for more than 10% at 40 μM concentration, while verapamil gives 10% of reversion only at 160μM concentration. The amino derivatives **12** are inactive in both cell lines, except **12c**, which has 10% reversal activity at 10μM concentration, but only in L5178 cells. The oxoderivatives **9** show intermediate activity between thio- and amino derivatives. In L5178 cells they are already active from 4μM concentration, but in K562/ADR cells they are almost all inactive even at 80μM concentration.

So, on the basis of P-gp function reversing ability, compounds can be ranked in three groups:

I) inactive or almost inactive compounds

(*e.g.* **3d**, **3e**, **5a**, **12**),

II) moderately active compounds

(*e.g.* **3a**, **3c**, **5b**, **4b**),

III) very active compounds

(*e.g.* **3l**, **3p**, **11**).

Comparing the P-gp function inhibitory effects of the azatricyclic compounds with their chemical structure, it is evident that the best derivatives are the thioacridines and pyridoquinoline thioethers. Amino group on the side chain or at least on the tricyclic moiety is also very important for good P-gp reversing activity. Probably our compounds bind to the ATPase part of the P-gp, as we demonstrated by molecular modelling, but this model has to be confirmed with further investigations.

Another approach to reverse the MDR phenotype is to down-regulate the MDR-1 gene expression. For this purpose we selected some thioacridines and pyridoquinolines, active (**3l**, **3p**, **11h**, **11i**), slightly active (**3a**, **7**, **9g**, **11c**) or non active (**3b**, **3e**, **12i**, **12e**) on Pgp function in K562/ADR cells in order to investigate their influence on MDR-1 gene expression.

It was previously described by Muller *et al.* (Muller *et al.*, 1995) that some P-gp inhibitors can modify the MDR-1 gene expression by action on its promoter. The L5178 cells, resistant by transfection with pHa-MDR1 were then not appropriate for our study, since the MDR-1 gene is not under the control of its own promoter. Thus for the MDR-1 gene expression studies we selected the multidrug resistant K562/ADR cells, isolated by adaptation to adriamycin.

Compounds **3l**, **3p**, **11h** and **11i** (P-gp inhibitors) decrease the expression level of the MDR-1 gene by respectively 30, 30, 20 and 35%, at non-toxic dose. This result is in agreement with those previously obtained with verapamil in the same cell line (Muller *et al.*, 1995). However, Herzog *et al.* described an increase in the MDR-1 mRNA level after treatment of colon carcinoma cells by verapamil (Herzog *et al.*, 1993). For this reason, it should be useful to study the concentration dependent effect of our compounds in cell lines other than K562/ADR.

Verapamil, which was found to be less toxic than **3l**, **3p**, **11h** and **11i**, can be used at higher concentration leading to a better decrease in MDR-1 expression : 50% with 30µM verapamil compared to 30% with 6µM **3l** or 3µM **3p**, 20% with 1.5µM **11h** and 35% with 1.5µM **11i**. This does not mean that these azatricyclic derivatives are less effective than

verapamil, but their therapeutic index is more limited. Further studies merit consideration to determine whether our compounds down-regulate the MDR-1 gene by action on the promoter as described for verapamil (Muller *et al.*, 1995) or if different mechanisms are involved.

Compounds **3e** and **3a** increase the expression level of the MDR-1 gene by 30-40%. Similar phenomenon was already described with some other compounds (*e.g.* reserpine and yohimbine analogs) (Bhat *et al.*, 1995). As the reserpine and yohimbine analogs are P-gp inhibitors, thus, the explanation given by Bhat *et al.* (Bhat *et al.*, 1995) that the increase in MDR-1 gene expression can be the consequence of a functional blockage of P-gp with a positive feed-back, is acceptable in the case of **3a**, but not in the case of **3e**. The more so as, it has already been reported that cytotoxic drugs which are not P-gp substrates are also able to enhance the MDR-1 and P-gp induction (Choudhary and Roninson, 1993). Additional investigation is needed to understand the mechanism of this increase. Anyway neither **3e** cannot be taken in the consideration as chemosenzitisers, as it does not block the efflux pump and in addition it increases the MDR-1 gene expression, nor **3a** which block slightly the P-gp function, but also increases the MDR-1 gene expression.

Compounds **7**, **9g** and **11c** are able to block slightly the function of the P-gp, but they do not have any effect on the MDR-1 gene. Derivatives such as **3b** and **12i**, which do not have significant influence neither at the protein, nor at the gene level, are of no interest in MDR studies.

Pyridoquinoline **12e** can be promising compound. It is true that **12e** is not an inhibitor of the P-gp, but it can decrease the expression of the MDR-1 gene by 40% and in addition is not toxic even at 50μM concentration.

On the basis of their effect on the P-gp function and on the MDR-1 expression, our compounds can be divided in five groups. Thus, there are compounds which:

I) inhibit the P-gp function

decrease the MDR-1 gene expression

(3l, 3p, 11h, 11i)

II) inhibit the P-gp function

not decrease the MDR-1 gene expression

(7, 9g, 11c)

III) not inhibit the P-gp function

decrease the MDR-1 expression

(12e)

IV) not inhibit the P-gp function

increase the MDR-1 expression

(3a, 3e)

V) not inhibit the P-gp function

not decrease the MDR-1 gene expression

(3b, 12i)

We can note that the most promising compounds are those which belong to the first group and after also those of the second and third groups, while the azatricyclic derivatives of the fourth and fifth groups can not be candidates for becoming MDR modulators.

From the work of Muller *et al.* (Muller *et al.*, 1995), we know that verapamil which is P-gp antagonist, decreases the MDR-1 gene expression and also the quantity of P-gp on the

cell surface of K562/ADR cells. It was also demonstrated by Herzog *et al.* (Herzog *et al.*, 1993) that the P-gp antagonists verapamil and nifedipine increased the MDR-1 gene expression and there was also increase in the level of P-gp in the case of LS 180-AD50 and LS 180Vb2 cells.

So, we were interested to know what is about the P-gp level after treating the K562/ADR cells with some selected thioacridine (**3l**, **3p**, **3e**) and pyridoquinoline (**11h**, **11i**, **12e**) derivatives. The treatment with **3l**, **3p**, **3e**, **11h** and **11i** resulted in the increase of P-gp level, while treatment with **12e** resulted in the decrease of P-gp level. These compounds can be grouped in three categories, if we consider their effects on the P-gp function, on the expression of MDR-1 gene and also on the level of P-gp on the cell surface:

I) inhibit the P-gp function
decrease the MDR-1 gene expression
increase the P-gp level
(**3l**, **3p**, **11h**, **11i**)

II) not inhibit the P-gp function
decrease the MDR-1 gene expression
decrease the P-gp level
(**12e**)

III) not inhibit the P-gp function
increase the MDR-1 gene expression
increase the P-gp level
(**3e**)

Our hypothesis is that the compounds **3l**, **3p**, **11h** and **11i** block the P-gp function, stay attached to the P-gp and thus inhibit the degradation of the protein. The increased P-gp level by the way of negative feed-back decreases the MDR-1 gene expression. However, we can not rule out the possibility that besides the drugs, which stay attached to the P-gp, some drugs may also enter into the cell, reach the nucleus and act directly on the MDR-1 gene.

Indeed, we can consider the compounds belonging to the second (**12e**) and third (**3e**) groups, as the same group, according their possible mechanism of action. These compounds are not inhibitors of the P-gp function. They probably act directly on the MDR-1 gene: **12e** decreases the MDR-1 gene expression, leading to a decrease in P-gp level; while **3e** increases the MDR-1 gene expression resulting in an increased level of P-gp.

Further studies are needed to confirm these hypothesis.

VII. CONCLUSIONS AND PERSPECTIVES

New azatricyclic derivatives (*e.g.* acridines and pyridoquinolines) have been prepared and tested as chemosensitizers. It can be noted that within the series studied there are compounds which can efficiently block the P-gp efflux pump and/or decrease the MDR-1 gene expression.

As regards the P-gp efflux pump blocking effects, the following results must be emphasized:

* Pyridoquinolines are better inhibitors of the P-gp function, than acridines.

* In order to achieve a good P-gp efflux pump blocking effect, the best heteroatom for the attachment of the side chain to the tricyclic nucleus both in the case of pyridoquinolines and acridines is the sulfur one.

* Extracyclic amino group is suitable for the P-gp inhibitory effect.

Moreover, molecular modelling shows that there is a theoretical possibility for the studied compounds to bind into the ATPase site of the Pgp. Distances for the ligand-site interactions have been calculated. They are in the usual range of hydrogen bonding, ionic bonding and hydrophobic interactions. Thus, one of the active sites for the inhibitors of the P-gp could be this domain.

With a view to investigate the influence on MDR-1 gene expression, few compounds were selected. Their gene expression decreasing or increasing effects have been compared to their P-gp efflux inhibitory effects. The most promising compounds are undoubtly those which inhibit the P-gp function and decrease the MDR-1 gene expression, like **3l**, **3p**, **11h** and **11i**. However, some compounds without P-gp inhibitory capability, as **12e**, can still be considered as MDR modulators, because they decrease both gene expression and P-gp level on the cell surface.

Taking into account all these results, new derivatives with selected substituents acting either as electron-donating atoms (or groups) or electron-withdrawing atoms (or groups) have to be prepared and tested in the future for the improvement of SAR. The binding into the active site must be confirmed with structurally closely related compounds (quinolines, naphthyridines, acridines, etc.) branched with one or two, similar or different side chains, in order to clarify the role and the function of the nucleus and the chain respectively in the binding. Additional experiments with new compounds without protonable extracyclic nitrogen must also be achieved, with a view to make clear the capability of such compounds to bind in another active site.

Finally, understanding of the molecular mechanisms involved in the effect of thioacridines and pyridoquinolines on the gene and protein expressions will require to determine whether there are sequence specific interactions between DNA and compounds (exonuclease assay), and also whether the regulation of the MDR-1 gene is transcriptional, posttranscriptional, translational or posttranslational (chloramphenicol acetyl transferase assay, run-on assay, determination of the half-life time of mRNA and protein).

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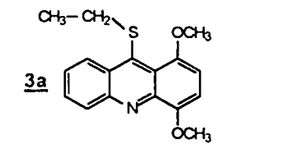
IX. ANNEX

Thioacridines



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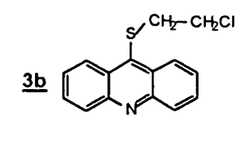

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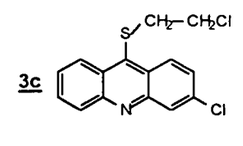

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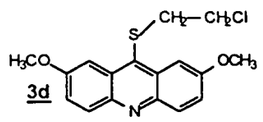

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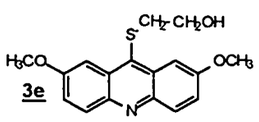

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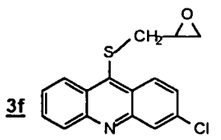

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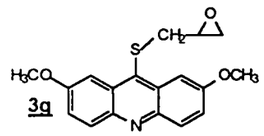

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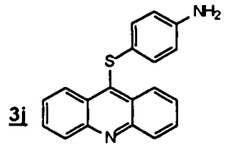

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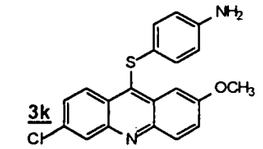

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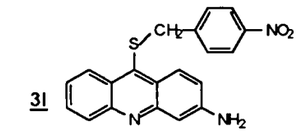

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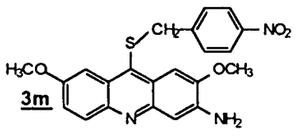

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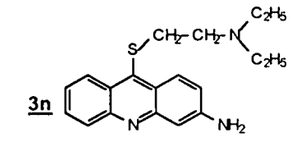

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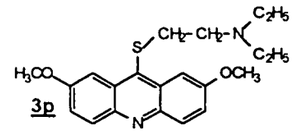

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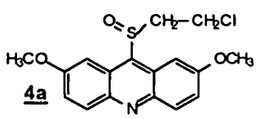

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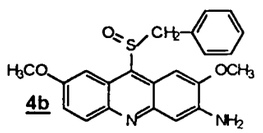

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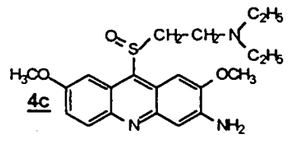

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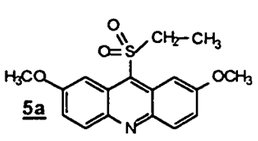

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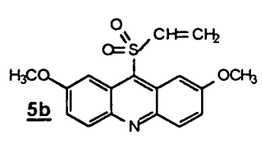

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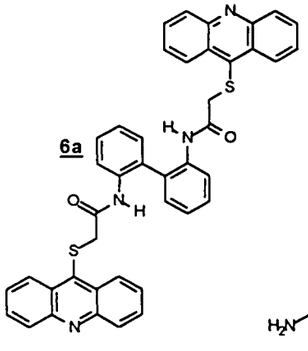

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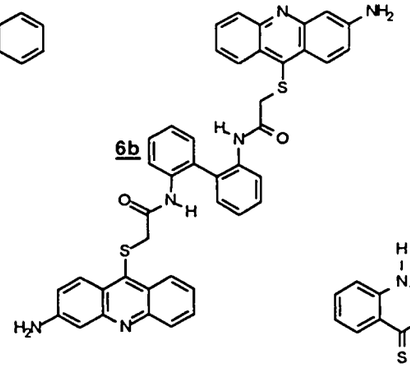

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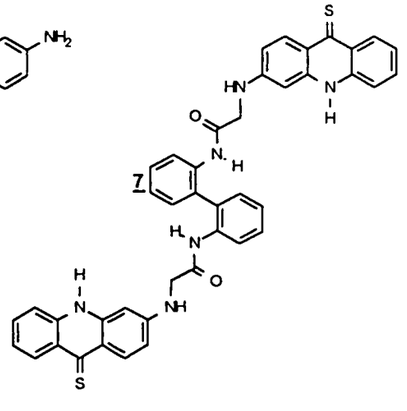

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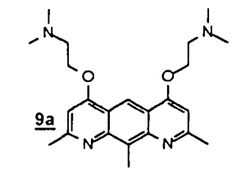
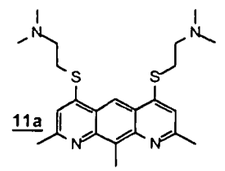
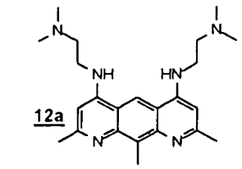
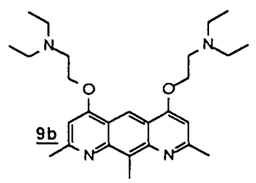
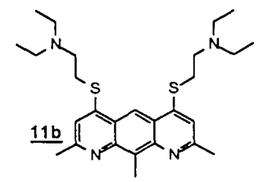
Pyridoquinolines

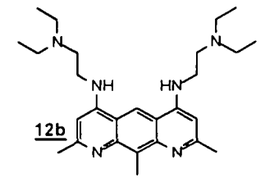


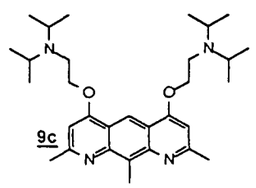


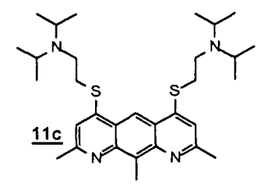


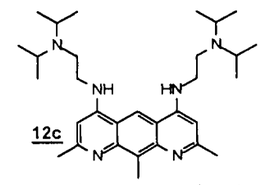


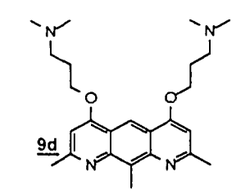


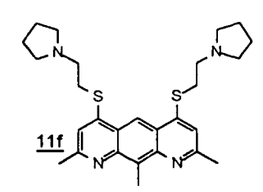


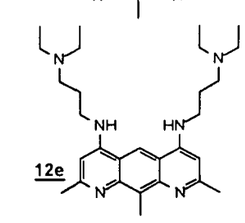


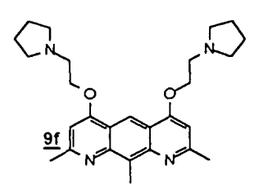


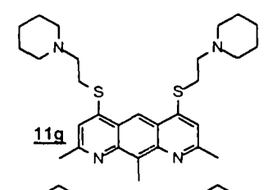


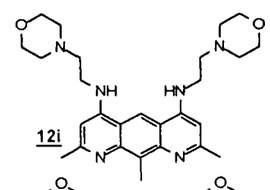


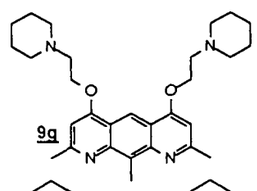


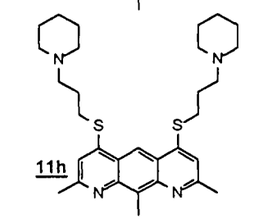


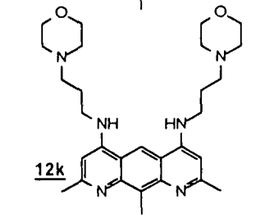


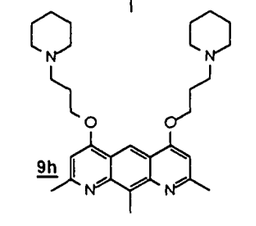


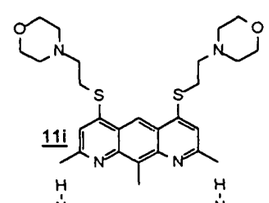


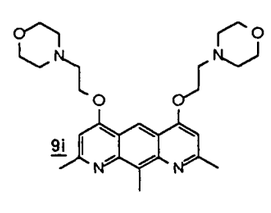


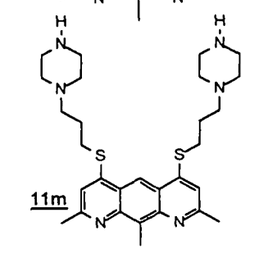












INHIBITION OF P-GLYCOPROTEIN MEDIATED EFFLUX AND MODULATION OF MDR-1 GENE EXPRESSION IN TUMOR CELLS BY NEWLY SYNTHESISED AZAHETEROCYCLIC DERIVATIVES

(SUMMARY)

Clinical resistance to chemotherapeutic drugs is a major problem in the treatment of cancer. One form of drug resistance, termed **multidrug resistance (MDR)**, is defined as the ability of cells exposed to a single drug to develop resistance to a broad range of structurally and functionally unrelated drugs, which include natural products such as anthracyclines, Vinca alkaloids, epipodophyllotoxins, colchicine and actinomycin D. This phenomenon is often associated with the overexpression of a 170 kDa membrane protein, known as **P-glycoprotein (P-gp)**, encoded by the human **MDR-1 gene** and acting as an efflux pump, transporting the antitumor agents outside from the cells.

Since about two decades, attention has been focused on the reversal of MDR and many chemosensitizers were prepared. Among the heterocyclic derivatives tested, quinacrine (aminoacridine) gave interesting results, as it was able to reverse the resistance against vinca alkaloids and doxorubicin about 1 to 10 fold at 1 μM to 50 μM doses. That is why we were interested in testing new acridine derivatives, namely **9-thioacridines**. With a view to enrich our knowledge on the role of pharmacophoric substituents, we decided also to test some **pyridoquinolines**, which are structurally related to the acridines mentioned above.

We studied the effect of 21 thioacridine and 22 pyridoquinoline derivatives on the **P-gp function** in MDR **mouse T-lymphoma cell line L5178** and in MDR **human leukemia cell line K562/ADR** by **rhodamine 123 (R123) fluorescence uptake assay,** since R123 is a substrate of P-gp. The fluorescence mean intensities were determined for the treated cells by

flow cytometry and were compared to those of untreated cells. We have shown that among a new series of azaheterocyclic derivatives there are compounds which inhibit successfully the function of the P-gp. In the case of L5178 cells, of the 21 thioacridines 8 were active (the reversion varied from 24% to 87%) and of the 22 pyridoquinolines (thioethers, ethers and amines) 14 could inhibit the function of P-gp at 10 μM (the reversion varied from 10% to 100%). However, we had to increase the concentration of the compounds in the case of K562/ADR cells to 40-80 μM to achieve significant MDR reversing activity. This phenomenon can be linked to our observation that the cell surface is two times larger and the quantity of the P-gp in the membrane three times higher in K562/ADR cells compared to L5178 cells.

Comparing the P-gp function inhibitory effects of the tested azaheterocyclic compounds with their chemical structure, we have found some **structure-activity relationships**. The best derivatives are the thioacridines and pyridoquinoline thioethers. In both cases, the most favorable heteroatom for the attachment of the side chain to the tricyclic nucleus is the sulfur one. Amino group on the side chain seems to be also very important for a P-gp inhibitory activity.

Considering the correlations between the chemical structures and the MDR reversing effects, we proposed a hypothesis for the interaction of thioacridines and pyridoquinolines with the P-gp. By **molecular modelling**, using the crystal structure of the ligand and of the ATP site of adenylate kinase (homologous with the ATP site of the P-gp), we demonstrated that probably our compounds bind to the ATPase part of the protein. Ionic bonding with aspartic acid 1200, hydrophobic bonding with threonine 1203 and hydrogen bonding with either cysteine 1074 or serine 1077 can be portrayed in the ATPase domain 2 of the modelised P-gp. However, this model is based on theoretical assumptions and has to be confirmed with subsequent investigations.

Another approach to reverse the MDR phenotype is the down regulation of the **MDR-1 gene expression**. For this purpose we selected some representative thioacridines and pyridoquinolines (four active, four slightly active and four inactive compounds on the P-gp function) in order to investigate their influence on the MDR-1 gene expression in K562/ADR cells. First we determined the **cytotoxicity** of these derivarives by **MTT test** and by **propidium iodide test**. Then, cells were treated for 24 hr with the highest non-toxic doses of compounds. After the **RNA extraction** and **reverse transcription**, the MDR-1 gene was amplified by **RT-PCR**, followed by **agarose gel electrophoresis.** The level of the P-gp on the cell surface was also determined by **immunostaining method**, using **UIC2 monoclonal antibody**. We have found that those selected thioacridines and pyridoquinolines, which strongly blocked the function of the P-gp, decrease significantly (about by 30-40%) in all cases the expression of the MDR-1 gene. We could not detect significant changes in the quantity of the P-gp, except in one case with a 40% increase. This increase was maybe due to the inhibition of the protein degradation. Compounds with slight or no effect on the P-gp function, did not influence the MDR-1 gene expression or they increased it and thus these derivatives can not be candidates for becoming MDR modulators. In contrast, there was one compound without P-gp inhibitory effect which can still be a promising MDR reversal agent, as it decreased the MDR-1 gene expression and also the P-gp level. We plan further investigations to determine whether the regulation of the MDR-1 gene is transcriptional and/or translational.

INHIBITION DE L'EFFLUX P-GP DÉPENDANT ET MODULATION DE L'EXPRESSION DU GÈNE MDR-1 DANS LES CELLULES TUMORALES PAR DE NOUVEAUX DÉRIVÉS AZAHÉTÉROCYCLIQUES

(RÉSUMÉ)

La chimiorésistance aux médicaments est un problème majeur dans le traitement des cancers. Une des formes de résistance est celle appelée **résistance multiple (MDR)** car concernant simultanément de nombreux médicaments. Dans ce cas une cellule exposée initialement à un seul médicament développe une résistance croisée à d'autres médicaments qui sont structurellement et fonctionnellement non apparentés, comme les anthracyclines, les Vinca-alcaloïdes, les épipodophyllotoxines, la colchicine et l'actinomycine D. Ce phénomène est associé à la surexpression d'une protéine membranaire, appelée **P-glycoprotéine (P-gp)**, codée par le **gène MDR-1**, agissant comme une pompe à efflux et transportant ainsi les agents antitumoraux à l'extérieur de la cellule.

Depuis deux décennies, la réversion du phénotype MDR a suscité de nombreux travaux et plusieurs agents reversants ont ainsi été préparés. Parmis les dérivés hétérocycliques testés, la quinacrine (aminoacridine) à donné des résultats intéressants car elle s'est avérée capable de réverser la résistance aux Vinca-alcaloïdes et à la doxorubicine d'un facteur 10 et ce à des doses comprises entre 1 μM et 50 μM. C'est pourquoi, nous nous sommes intéressée à un type particulier de dérivés acridiniques, les **9-thioacridines**. Dans le but d'approfondir nos connaissances sur le rôle des substituants pharmacophoriques, nous avons décidé de tester egalement quelques **pyridoquinolines** parce que structuralement reliées aux acridines mentionnées précédemment.

Nous avons donc étudié l'effet de 21 thioacridines et 22 pyridoquinolines sur la **fonction de la P-gp** dans la **lignée MDR L5178 provenant de lymphocytes T de souris** et dans la **lignée MDR érythroleucémique K562/ADR humaine**. Nous avons utilisé la technique **d'incorporation et de rétention de la rhodamine123 (R123) fluorescente** car celle-ci est un substrat de la P-gp. L'intensité moyenne de fluorescence des cellules traitées a été déterminée par **cytométrie en flux** et comparée ensuite à celle de cellules non traitées. Nous avons montré qu'il y avait des composés parmi ceux étudiés, qui inhibaient avec succès la fonction de la P-gp. Dans le cas des cellules L5178, parmi les 21 thioacridines testées, 8 sont actives avec une réversion variant de 24% à 87%, et parmi les 22 pyridoquinolines (thioéthers, éthers et amines) 14 peuvent inhiber la fonction de la P-gp à 10 μM avec une réversion variant de 10% à 100%. En revanche, dans les cellules K562/ADR, il a fallu augmenter la concentration de ces composés à 40-80 μM pour atteindre une réversion significative de la MDR. Ce phénomène pourrait être lié à notre observation montrant que la surface membranaire est deux fois plus grande et que la quantité de P-gp dans la membrane est trois fois plus élevée dans les cellules K562 /ADR que dans les cellules L5178.

En comparant l'effet inhibiteur de la fonction de la P-gp et la structure chimique des composés azahétérocycliques, nous avons pu établir quelques **relations structure-activité**. Les meilleurs dérivés sont les thioacridines et les thioéthers pyridoquinoliniques. C'est dire que l'hétéroatome qui paraît le plus favorable à l'activité est le soufre; le groupement amino sur la chaîne latérale semble aussi être très important.

Considérant alors tant la structure moléculaire que l'effet reversant, nous avons proposé une hypothèse d'interaction des thioacridines et des pyridoquinolines avec la P-gp. Par **modélisation moléculaire**, en nous basant sur la structure cristallographique d'un ligand et

celle d'une adénylate kinase (homologue du site ATP de la P-gp), nous avons montré que nos composés pouvaient se fixer sur le site ATPasique de la protéine. La fixation mettrait en jeu une liaison ionique avec l'acide aspartique 1200, une interaction hydrophobe avec la thréonine 1203 et des liaisons hydrogène avec la cystéine 1074 ou la serine 1077. Cependant, le modèle reste encore hypothétique et devra être confirmé par d'autres investigations.

Une autre approche pour réverser le phénotype MDR est basée sur la régulation négative de l'**expression du gène MDR-1**. Nous avons donc cherché à connaître le comportement de nos substances dans ce domaine. Pour cela, nous avons sélectionné quelques thioacridines et pyridoquinoléines représentatives (4 composés actifs , 4 légèrement actifs et 4 composés inactifs sur la fonction de la P-gp) dans le but d'étudier leurs influences sur l'expression du gène MDR-1 dans les cellules K562/ADR. Tout d'abord, nous avons déterminé la cytotoxicité de ces dérivés en utilisant le **test MTT** et le **test à l'iodure de propidium**. Les cellules sont traitées pendant 24h avec la dose la plus élevé de ces composés, qui ne soit pas cytotoxique. Après **extraction des ARNs** totaux et **transcription inverse**, le produit du gène MDR-1 est amplifié par la méthode de **RT-PCR** suivie par **électrophorèse sur gel d'agarose**. La quantité de P-gp présente sur la surface cellulaire à été aussi déterminée par la **méthode d'immunomarquage** utilisant **l'anticorp monoclonal UIC2**. Nous avons montré que les thioacridines et les pyridoquinoléines sélectionnées qui bloquent fortement la fonction de la P-gp, diminuent significativement (environ 30-40%) l'expression du gène MDR-1. Nous n'avons pas pu détecté de changement significatif du taux de P-gp à l'exception d'un seul cas où l'augmentation est de 40%. Cette augmentation est peut être due à une augmentation de la stabilité ou bien à une stabilité accrue de la protéine. Les composés qui ont un effet faible ou nul sur la fonction de la P-gp n'ont pas d'influence sur l'expression du gène MDR-1, si non ils l'augmentent. Dans ces conditions, ces dérivés ne peuvent pas être des

candidats pour devenir des modulateurs de la MDR. En revanche, il y a un composé qui bien que n'ayant pas d'effet inhibiteur sur la P-gp pourrait être un agent reversant. En effet, il diminue l'expression du gène MDR-1 ainsi que le taux de la P-gp. Dans le futur, outre l'optimisation des structures et la recherche d'informations nouvelles sur les liaisons avec le(s) site(s), nous envisageons d'autres investigations qui devraient nous permettre de déterminer si la régulation du gène MDR-1 est transcriptionelle et/ou traductionelle.

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Effect of New Thioacridine Derivatives on P-gp Function and on mdr1 Gene Expression

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Abstract. *We studied the effect of thioacridine derivatives on the function of P-glycoprotein in MDR mouse T-lymphoma cell line L5178 and in MDR human leukemia cell line K562/ADR by rhodamine 123 uptake assay. The effect of some selected thioacridines was also investigated on the expression of the mdr1 gene. Expression was analysed by RT-PCR. Two compounds: 3-amino-9-thio-(4'-nitrobenzyl)acridinone and 2,7-dimethoxy-9-thio-(2'-diethylaminoethyl) acridinone were able to block the function of the P-gp, and also to decrease significantly mdr1 gene expression. Because these two derivatives exert their positive effects as reversing agents they could be potential candidate anticancer agents for further investigation. The thioacridines, which do not affect P-gp function, do not affect or increase the expression of mdr1 gene. Our results showed the structure-activity relationships of these compounds, providing a direction for the development of new, more active compounds.*

The multidrug resistance (MDR) of tumor cells is one of the major problems in cancer chemotherapy. This phenomenon is often associated with the overexpression of a 170 kDa membrane protein, known as P-glycoprotein (P-gp), encoded by the human mdr1 gene, which acts as an efflux pump, transporting the antitumor agents outside from the cells (1, 2, 3).

Many investigators have focused on the reversal of MDR (4, 5). The first success was obtained by Tsuruo *et al* (6), who found that verapamil and trifluoperazine are effective "chemosensitizers". Since that time a broad range of chemical agents have been investigated for their capability to reverse MDR. The "resistance modifiers" were found to belong to a quite different chemical series, sharing only a few common characteristics, like hydrophobicity, a basic nitrogen atom and two or more aromatic rings (5). The proposed mechanism of action of the chemosensitizers is *via* binding to P-gp, by antagonizing the binding of the anticancer drugs and thus inhibiting their efflux from the cells (7, 8).

Another possibility to circumvent the MDR is the down-regulation of the mdr1 gene expression. Muller et al (9,10) demonstrated that veranamil inhibits P-gp, and simultaneously decreases mdr1 gene expression. The decreased gene expression could be the consequence of the decreased transcriptional rate, which could be due to the reduced activity of the mdr1 proximal promoter. Other authors reported that treatment of human colon cell lines with P-gp antagonists like verapamil, nifedipine, nicardipine, diltiazem and cyclosporin (11) or reserpine and yohimbine analogs (12), increase mdr1 gene expression. This increase has been described as regulated both transcriptionally (10) and posttranscriptionally (11). All these studies emphasized the necessity to investigate the effect of various compounds at the protein and at the gene levels.

Based on some earlier studies (5) when some acridines (*e.g.* acridine, acridine orange and quinacrine) were found to be effective modulators of MDR, the MDR reversal effect of some thioacridine derivatives was investigated in our studies.

Materials and Methods

Chemistry. Thioacridinic ethers 1 (Table I) were prepared from the corresponding thioacridinones. Depending on the nature of the alkylating agent, the following methods were used: a) alkylation under phase transfer catalysis conditions with or without catalyst, b) alkylation in dimethyl-formamid (DMF) under reflux in the presence of dipotassium carbonate, c) alkylation in butanone under reflux in the presence of 20% aqueous sodium hydroxide. Oxidation of thioethers with oxygen peroxide leads to sulfoxides or sulfones.

Bis-derivatives 2 and 3 (Table I) were prepared by alkylation of selected thioacridines with 2',2'-bromoacetamido biphenyl in a two step procedure from 2,2'-nitro biphenyl.

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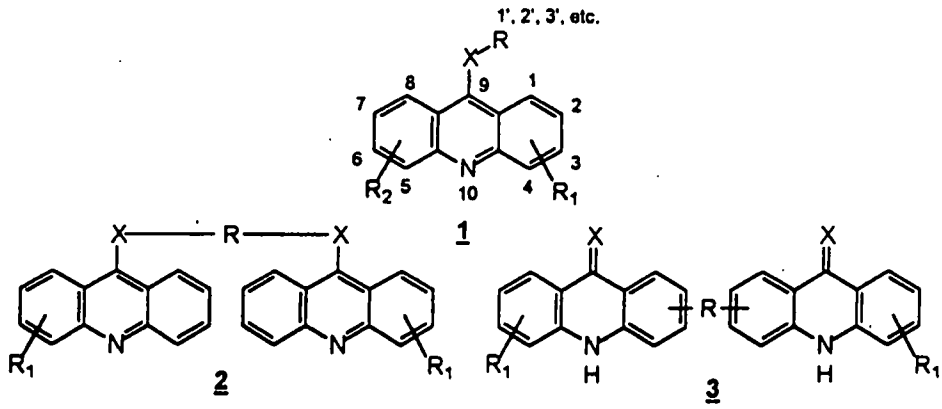
Key Words: P-glycoprotein, mdr1 gene, thioacridines.

Table I. *Structure of the thioacridine derivatives.*



CpdsX	R_1	R_2	R	
1a	S	1,4-dimethoxy	-	ethyl
1b	SO_2	2-methoxy	7-methoxy	ethyl
1c	S	-	-	2'-chloroethyl
1d	S	3-chloro	-	2'-chloroethyl
1e	S	2-methoxy	7-methoxy	2'-chloroethyl
1f	SO	2-methoxy	7-methoxy	2'-chloroethyl
1g	S	2-methoxy	7-methoxy	2'-hydroxyethyl
1 h	SO_2	2-methoxy	7-methoxy	ethenyl
1i	S	3-chloro	-	2',3'-epoxypropyl
1j	S	2-methoxy	7-methoxy	'",3'-epoxypropyl
1k	S	-	-	4'-aminophenyl
1l	S	2-methoxy	6-chloro	4'-aminophenyl
1m	SO	2-methoxy	7-methoxy	benzyl
1n	S	3-amino	-	4'-nitrobenzyl
1p	S	2-methoxy	7-methoxy	4'-nitrobenzyl
1q	S	3-amino	-	2'(diethylamino)ethyl
1r	S	2-methoxy	7-methoxy	2'(diethylamino)ethyl
1s	SO	2-methoxy	7-methoxy	2'(diethylamino)ethyl
2a	S	-	-	3-3'(bis-α,α'-acetamidobiphenyl)
2b	S	3-amino	-	3-3'(bis-α,α'-acetamidobiphenyl)
3a	S	-	-	3-3'(bis-α,α'-aminacetamidobiphenyl)

Cells. a) The L5178Y mouse T-lymphoma parent cell line was infected with the pHa MDR1/A retrovirus as previously described by Pastan *et al* (13). The L5178 MDR cell line and the L5178 Y parent cell line were grown in McCoy's 5A medium supplemented with 10% heat-inactivated horse serum, L-glutamine and antibiotics (and 60 ng/ml colchicine for the MDR cell line).

b) The K562/ADR multidrug resistant cell line was isolated (14) by adaptation to adriamycin from the K562 human chronic myelogenous leukemia parental cell line (15). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine and antibiotics (and 100 nM adriamycin for the K562/ADR cell line).

Rhodamine 123 (R123) uptake assay. The L5178 and K562 cells (2 x 10^6 cells/ml) were resuspended in serum-free medium and distributed (0.5 ml aliquots) to Eppendorf tubes. Compounds to be tested were added at different concentrations and the samples were incubated for 10 minutes

at room temperature. Then the indicator R123 was added to the samples at a final concentration of 5.2 µM and the cells were incubated for 20 minutes at 37°C; washed twice and resuspended in 0.5 ml phosphate-buffer saline (PBS) for analysis. The fluorescence of the cell populations was measured by flow cytometry using a Beckton Dickinson FACScan instrument. Since R123 is a substrate of Pgp, there was a significant difference in fluorescence between MDR and parental cells. Untreated MDR cells accumulate only a low level of R123. Verapamil was used as a reference drug (16). The fluorescence mean intensities (FL) were determined for the treated cells and were compared to these of untreated cells. The percentage of the multidrug resistance reversion (% MDR Rev.) was calculated as follows:

$$\% \text{ MDR Rev.} = \frac{\text{FL(MDR treated)} - \text{FL(MDR untreated)}}{\text{FL(Parent untreated)} - \text{FL(MDR untreated)}} \times 100$$

Cytotoxicity study. K562/ADR cells (1 x 10^5 cells / ml) were grown for 24 hours in the continuous presence of increasing concentrations of selected compounds. Cell viability was determined by flow cytometric analysis using propidium iodide.

Mdr1 gene expression study by RT-PCR. K562/ADR cells (1 x 10^5 cells / ml) were treated with non toxic doses of selected compounds for 24 hours at 37°C. Total cellular RNA was then extracted by the Chomczincki and Sacchi method (17) using the RNAXEL Kit (Eurobio). About 1 µg of total RNA was used for reverse transcription reaction with random primers. Mdr1 and the internal control ß2 microglobuline (ß2m) were amplified with Taq polymerase (Appligen). The sequences of the primers used were:
MDR1 (Sense): 5' GCCTGGCAGCTGGAAGACAAATACACAAAAT 3'
MDR1 (Antisense): 5' GAAGATAGTATCTTTGCCCAGACAGCAGC 3'
ß2m (Sense): 5' CCGACATTGAAGTTGACTTAC 3'
ß2m (Antisense): 5' ATCTTCAAACCTCCATGATG 3' PCR was carried out in a Perkin Elmer system 2400. The reaction conditions included an initial cycle of denaturation at 93°C for 2 minutes, followed by 20 cycles of denaturation for mdr1 and 23 cycles for ß2m at 92°C for 10 second annealing at 52°C for 30 seconds and extension at 72°C for 45 seconds with increments of 20 seconds each cycle and one final cycle of extension at 72°C for 7 minutes at the end. The amplified products were separated by electrophoresis on a 2% agarose gel. The DNA bands were visualized by ethidium bromide staining, and the image was digitalized. Mdr1 expression was normalized to ß2m transcript and was noted as Relative Expression Level (REL):

$$\text{REL} = \frac{\text{Densitometric value of mdr 1}}{\text{Densitometric value of ß2m}}$$

Table II. *Effect of thioacridine derivatives on P-gp function.*

Cpds	L5178 cells (1)		K562. ADR cells (2)	
	% Reversion at			
	10 µM	20 µM	40 µM	80 µM
1a	4	16	3	4
1b	0	0	0	0
1c	2	26	1	1
1d	4	7	13	13
1e	0	2	0	0
1f	3	23	4	9
1g	0	0	0	0
1h	1	4	18	20
1i	6	73	4	25
1j	1	5	6	28
1k	49	75	0	4
1l	24	38	2	24
1m	2	5	1	14
1 n	59	67	54	67
1p	2	4	0	22
1q	27	93	24	21
1r	25	58	35	82
1s	7	23	0	29
2a	87	93	0	2
2b	58	70	1	3
3a	46	74	5	16

Cells were treated with thioacridine derivatives for 10 minutes and with R123 for 20 minutes. Values represent the percent of the multidrug resistance reversion calculated as described in Material and Methods.
(1) For L5178 cells 10 % reversion was obtained with 40 µM verapamil.
(2) For K562/ADR cells 10 % reversion was obtained with 160 µM verapamil.

Results

Screening for possible modulators of MDR among new thioacridines derivatives in L5178 and K562/ADR cells. 21 new thioacridine derivatives were first screened for their ability to reverse the Pgp function in the L5178 resistant cells at two concentrations (10 and 20 µM). Of the 21 compounds tested, 18 were found to be active in reversing the MDR by inhibition of the Rhodamine 123 efflux (Table II). The reversion varied from 1% (lh or lj) to 87% (2a) for 10 µM and from 2% (1m) to 93% (1q and 2a) for 20 µM. Two drugs (1b and 1g) were completely inactive even at the higher concentration.

The efficacy of the same drugs were also investigated on K562/ADR cells (Table II). We did not find any activity at 10 and 20 µM (data not shown). At 40 µM and 80 µM the highest reversion was obtained with compound 1n (54%) and compound lr (82%). Six drugs (1b, 1e, 1g, 1k, 1p, 2a) were inactive at 40 µM and three (1b, 1e, 1g) at 80 µM.

Evaluation of mdr1 RNA expression after exposure of K562/ADR cells to some selected thioacridines. Six representative of the previously tested compounds were analyzed for their possible effect on mdr1 gene expression. Initial experiments attempted to establish the highest non toxic dose for a 24 h treatment (Table III). With 1a, 1c, 1g, 1n, 1r and 3a this dose was µ5 M, 1 µM, 12 µM, 6 µM, 3 µM and 6 µM respectively.

It can be seen in Figure 1., that depending on the drug, treatment produced a 30% decrease (1n and 1r), a 30% increase (1g) or 40% increase (1a) in mdr1 gene expression in

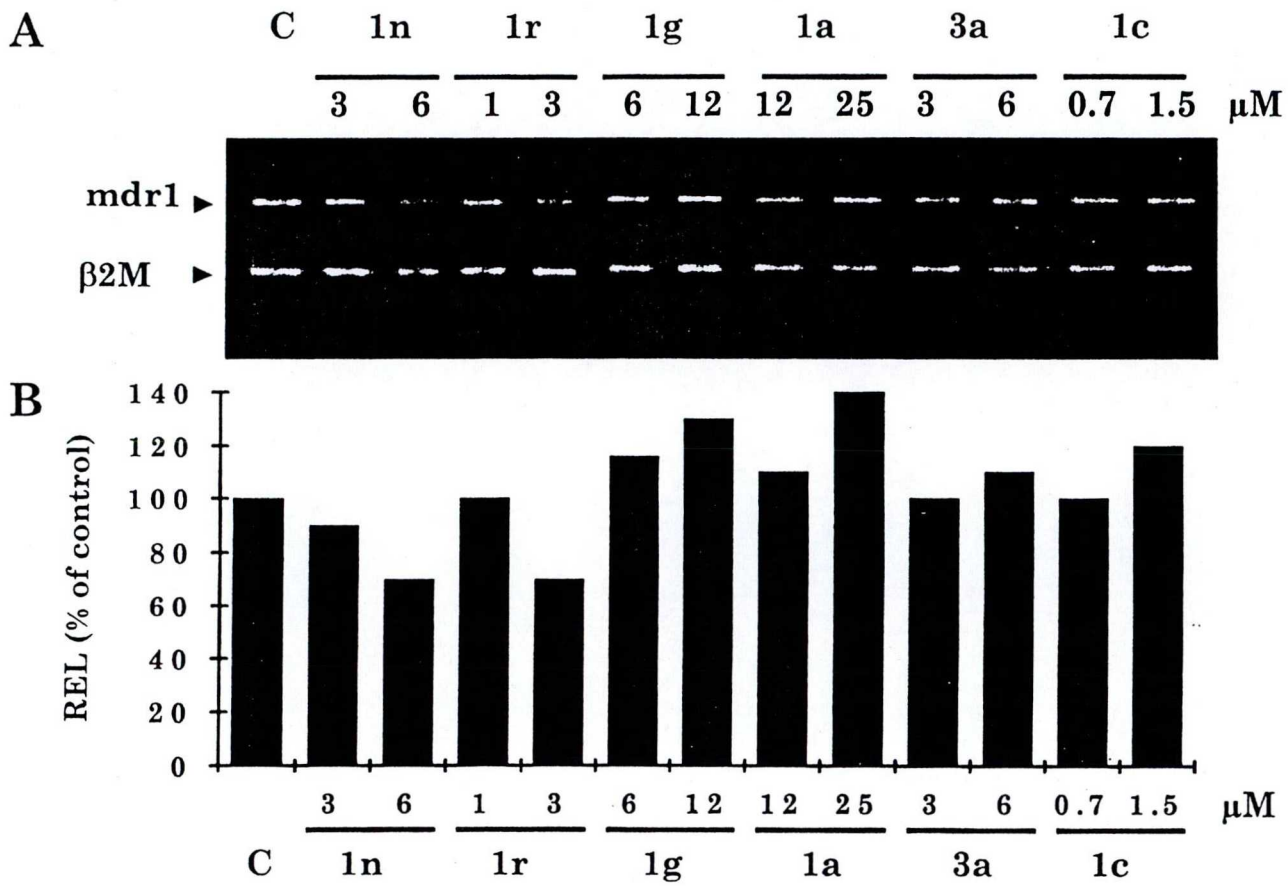


Figure 1. *Effect of thioacridine derivatives on mdr1 gene expression in K562/ADR treatment. Cells were incubated with non toxic doses of drugs and RNA was extracted after 24 hours A: Representative RT-PCR analysis of mdr1 gene expression. B: Representation of the results obtained after quantification of digitalized gel image. REL quantitates the level of mdr1 mRNA normalized to β2M (mean of 10 RT-PCR realized after 3 independent treatment)*

Table III. *Cytotoxicity of thioacridine derivatives in K562/ADR cells.*

	% of dead cells at							
Cpds (control)	0 μM	1 μM	3 μM	6 μM	12 μM	25 μM	50 μM	100 μM
1n	15	8	9	13	40	90	100	100
1r	12	9	11	34	36	99	99	100
1g	16	4	6	8	8	30	32	44
1a	15	10	10	10	12	10	17	24
3a	15	8	10	17	20	100	100	100
1c	15	7	23	29	47	52	74	80

K562/ADR cells were treated for 24 hours with different concentrations of some thioacridine derivatives. Values represent the percentage of dead cells evaluated by a flow cytometer using propidium iodide.

K562/ADR cells. In some cases (1c and 3a) no modification was found in the gene expression level.

Discussion

In this paper we have shown that among a new series of thioacridines, there are compounds which inhibit the function of the P-glycoprotein. We observed this effect in both L5178 mouse T-lymphoma cell line and in K562/ADR human chronic myelogenous leukemia cell line. However, in the case of human cells, the reversing activity was observed at higher concentrations than in mouse cells (40-80 μM instead of 10-20 μM). This phenomenon can be linked to our observation that

the cell surface is two times larger and the quantity of the P-glycoprotein in the membrane three times higher in the K562/ADR cells compared-to L5178 cells (data not shown). The highest inhibition of the efflux pump was shown with compounds 1n and 1r, in both cell lines. These derivatives are in L5178 cells more effective P-gp inhibitors than verapamil, since at 10 μM they inhibit the P-gp function by 70% (1n) or 25% (1r) while only 10 % was acheived with 40 μM verapamil. Moreover, in K567/ADR cells this inhibitory effect was about 70% (1n) and 80% (1r) at 80 μM, while we have to increase the concentation of verapamil to 160μM to achieve only 10% of inhibition. Compound 2a, 2b and 3a are also very active, but this activity strongly decreases from L5178 to K562/ADR cell line. At the present time, there is no explanation for this observation and thus, concerning of these bis-derivatives, further investigation is needed. Finally, there are also compounds (1b and 1g) which are completly inactive in L5 178 and in K562/ADR cells, as well.

Thus, compounds can be ranked into three groups, which are almost the same in both cell lines investigated: a) inactive or almost inactive compounds (*e.g.* 1b, 1e, 1g), b) moderately active compounds (*e.g.* 1a, 1d, 1h, 1m) and c) very active compounds (*e.g.* 1n, 1r).

We were able to show for the first time an apparent structure-activity relationship in the ability of some new thioacridines to block the function of the P- glycoprotein. As a rule, sulfoxides and sulfones are poorly active derivatives. Where the parent sulfide is active, derivatives oxygenated on the sulfur atom are notably less active (1s<1r). As regards the side chain, protonatable nitrogen is required for a good activity, insofar as only compounds 1l, 1n, 1r, 1q are really active. This is clearly shown in the following sequences: 1g~1e<1i<<1r and 1b<1m~1h<1s. Furthermore, a substituent directly branched onto the heterocyclic moiety usually decreased the activity, apart from the protonatable cases (1n). However, no definitive conclusions can be drawn, because of the restricted number of substituents tested; although it is a proved fact that 2,7-dimethoxy substitution is of interest.

Another approach to reverse the MDR phenotype is to down regulate mdr1 gene expression. To achieve this we selected some thioacridines, both active (1n, 1r) and non active (1a, 1b, 1c, 1g, 3a) on Pgp function, and investigated their influence on mdr1 gene expression. It was previously described by Muller *et al* (10) that some P-gp inhibitors can modify mdr1 gene expression by acting on the mdr1 promoter. The L5178 cells, resistant by transfection with pHa-mdr1 were then not appropriate for our study, since the mdr1 gene is not under the control of its own promoter. Thus, for the mdr1 gene expression studies we selected the multidrug resistant K562/ADR cells, isolated by their adaptation to adriamycin.

Compounds In and Ir (P-gp inhibitors) decreased the expression level of the mdr1 gene by 30%, at the highest non toxic dose. This result is in agreement with those previously obtained for verapamil in the same cell line (10). However, Herzog *et al*, described an increase in the mdr1 mRNA level after treatment of colon carcinoma cells by verapamil (11). For this reason, it should be useful to study the concentration dependent effect of 1n and 1r in cell lines other than K562/ADR.

Verapamil, which was found to be less toxic than 1n or 1r can be used at higher concentrations leading to a better decrease in mdr1 expression: 50% with 30 μM. Verapamil (data not shown) compared to 30% with 6 μM 1n or 3 μM 1r. This does not mean that these two thioacridines are less effective than verapamil, but their therapeutic index is more limited. Further studies merit consideration to determine whether our compounds downregulate the mdr1 gene by action on the mdr1 promoter as described for verapamil (10), or if different mechanisms are involved.

Compounds 1g and 1a increase the expression level of the mdr1 gene by 30-40%. A similar phenomenon has been described with some other compounds (*e.g.* reserpine and yohimbine analogs) (12), but these compounds are P-gp inhibitors, while 1g and 1a are not. Thus, the explanation given by Bhat *et al* (17) that the increase in mdr1 gene expression can be the consequence of a functional blockade of P-gp with a positive feed-back, is not acceptable; the more so since it has already been reported that cytotoxic drugs which are not Pgp substrates are able to enhance mdr-1 and Pgp induction (18). Additional investigation is needed to understand the mechanism of this increase. 1g and 1a cannot be taken in the consideration as chemosenzitisers as they do not block the efflux pump, and in addition they increase mdr1 gene expression. Compounds such as 1c, which did not have significant influence neither at the protein or at the gene level, are of no interest in MDR studies.

In conclusion, this paper presents evidence that some thioacridines (*e.g.* In: 3-amino-9-thio-(4'-nitrobenzyl) acridinone and 1r: 2,7-dimethoxy-9-thio-(2-diethylaminoethyl) acridinone) which are able to inhibit the P-gp activity and also to downregulate mdr1 gene expression, could be potential candidates as MDR reversing agents. Three pharmacophoric groups can be identified in the thioacridines investigated: a) an extracyclic non oxygenated sulfur atom, b) a protonatable side chain and finally c) the tricyclic moiety. These selected pharmacophores will be used for portraying the mechanism of action of these compounds on the function of the P-glycoprotein by computer simulation.

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