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RATIONAL DESIGN AND PRE-CLINICAL PHARMACOLOGY OF DRUGS FOR REVERSING MULTIDRUG RESISTANCE

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Abstract—Drugs that interfere with the action of P-glycoprotein (P-gp), the membrane efflux pump responsible for multidrug resistance (MDR), should be valuable in the treatment of patients with drug-resistant cancer. We have used one class of drug, the phenothiazines, to study the structural features required for optimum interference with the function of P-gp. The structure–activity relationships revealed three important components including the hydrophobicity of the tricyclic ring, the length of the alkyl bridge and the charge on the terminal amino group. *Trans*-flupenthixol is a lead compound that conforms to these structural requirements and demonstrates significant activity as a sensitizer of MDR cell lines to drugs affected by the MDR phenotype. Based on these data, we have proposed a model for the binding of modulators to P-gp and have speculated on the structure of the drug-binding domain. We have developed pre-clinical models of MDR that may help predict clinical activity of chemo-modulators. L1210/VMDRC.06 is a murine lymphocytic leukemia line transformed by a retroviral expression vector containing a full-length cDNA for the human *mdr*1 gene. K562/VBL1–3 are clones of human myeloid blast cells that were transformed with the same vector. Resistance in these lines is not complicated by changes in the cellular content of glutathione or alterations in topoisomerase II. The transformed L1210 line grows in mice as a slowly proliferating non-metastatic peritoneal implant. Both MDR lines are restored to sensitivity by cyclosporin A or *trans*-flupenthixol, and the K562 clones are induced to differentiate by hemin. These lines should provide simple, sensitive screens for new drugs for use against cancers expressing P-gp. We have proposed a model to explain how the pumping activity of P-gp is activated in response to toxic drugs. In this schema, basal activity of P-gp is modulated through phosphorylation/dephosphorylation reactions mediated by protein kinase C (PKC) and calcium sensitive phosphatases. In response to the activation of phospholipase C by toxic drugs and the local production of 1,2-diacylglycerol, PKC is translocated to the cell membrane where it phosphorylates P-gp. Following the extrusion of drug from the cell membrane, phospholipase C activity returns to baseline, diacylglycerol is metabolized, PKC returns to the cytosol and serine/threonine phosphatases dephosphorylate P-gp returning it to the basal state.

Resistance of cancer cells to certain chemotherapeutic drugs is caused by the over-expression of P-glycoprotein (P-gp†), the *mdr*1 gene product [1–3]. P-gp confers drug resistance through its action as a drug efflux pump [4]. Drugs that block the action of P-gp should be useful in treating patients whose tumors are resistant to chemotherapy by virtue of this mechanism.

In this paper we will review our recent studies in three areas: first, structure–activity relationships of phenothiazines as modulators of MDR, including the implications of these studies for the definition of the drug binding site on the molecule; second, the development of two new pre-clinical models of MDR that may be useful for predicting activity of new drugs or drug combinations in the clinic; and third, our working hypothesis on how the efflux activity of P-gp is modulated in response to toxic substances.

Structure–activity relationships of phenothiazines and thioxanthenes

We studied the ability of phenothiazines to increase the sensitivity of the MCF-7/AdrR human breast cancer cell line to doxorubicin. Using this assay system, critical structure–activity relationships were identified including the hydrophobicity of the tricyclic ring, the charge on the terminal amino group and the length of the alkyl bridge connecting the two [5]. Specifically, substitutions on the tricyclic ring which increased hydrophobicity, such as halogens at position 2, increased activity whereas substitutions which decreased hydrophobicity, such as hydroxyl moieties at position 2, decreased activity. Side chains containing positively charged, tertiary amino groups were significantly more potent than side chains containing secondary or primary amino groups. In addition, the incorporation of the charged amino moiety into cyclic structures such as piperazines or piperidines resulted in even greater activity. Finally, a four carbon alkyl group connecting the hydrophobic tricyclic ring to the positively charged terminal amino group gave the best results. Based on these observations, we formulated an idealized structure for modulators of this class (Fig. 1).

We next searched for compounds conforming to this model and tested the thioxanthenes as likely candidates since they differed only by a carbon substitution for the nitrogen in the tricyclic ring, and by having an exocyclic double bond to the side chain.

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† Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance; PKC, protein kinase C; DAG, 1,2-diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; BSO, buthionine sulfoximine; X, any amino acid; TM, transmembrane.

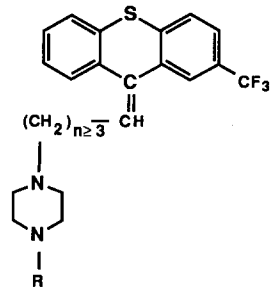

Fig. 1. Idealized structure of antipsychotic drugs capable of reversing MDR based on structure–activity relationships.

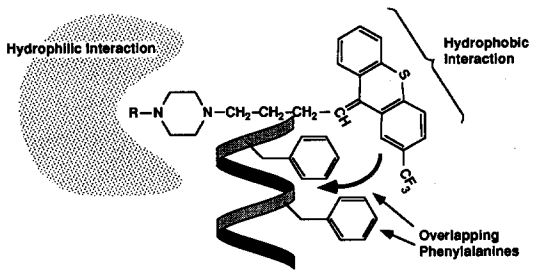

Fig. 2. Hypothetical drug binding site on P-gp based on analogies with the binding of antipsychotic drugs to calmodulin. The phenylalanine side chains are depicted extending outward from the α-helical backbone. The two aromatic rings of the phenylalanines are oriented in such a way by the α-helix to "sandwich" the tricyclic ring of the thioxanthene through π orbital electron interactions. The hydrophobic region is separated from a hydrophilic region of the protein which interacts with the basic side chain of the drug. Adapted from *J Theor Biol* **105**: 63–67, 1983 [12] with permission.

These studies demonstrated that *trans*-flupenthixol, the thioxanthene homolog of the phenothiazine fluphenazine, was at least 5-fold more potent than any of the phenothiazines [6, 7] and at least 3-fold more potent than verapamil. We also found that the *cis*-stereoisomers were usually less potent than the *trans*. This observation has important clinical implications since the more active compound, *trans*-flupenthixol, does not bind with great affinity to dopamine receptors and was relatively devoid of extrapyramidal side effects in clinical trials [8]. We found that the extrapyramidal side effects of trifluoperazine were dose-limiting in Phase I studies of trifluoperazine in combination with bleomycin [9]. Miller and colleagues found similar results using trifluoperazine in combination with doxorubicin [10].

Implications of the structural analysis for the binding site on P-gp

The structure–activity relationship for phenothiazines and thioxanthenes should help define the drug-binding site on P-gp. Based on the similarities between our data with MDR and the data obtained by others for the binding of phenothiazines to calmodulin [11], we propose the model shown in Fig. 2. In this formulation, the drug binding site is contained within a transmembrane α-helical segment containing a hydrophobic region separated by one half turn of the helix from a hydrophilic region [12]. The hydrophobic region is produced by two aromatic phenylalanines which are separated by two or three amino acids, and are oriented by the α-helix in such a way as to overlap the π orbitals of the aromatic groups in the phenothiazines and thioxanthenes. The hydrophilic region is produced by acidic residues present in a proximal domain which interacts with the positively charged amino side chain of the drugs. This phenylalanine repeat motif exists in several TM regions of human and murine P-gp including TM3, TM7, TM8 and TM11 [13, 14]. In support of this proposal, Bruggeman *et al.* [15] demonstrated that peptides containing these TM regions are labeled by azidopine.

In the Phe-X-X-Phe configuration, the two phenylalanines are separated by a 60° turn of the helix. This allows the two phenylalanines to overlap the π orbitals of the tricyclic ring. In the Phe-X-X-X-Phe configuration, the phenylalanines are separated by a 40° turn of the helix. This may not provide optimal overlap of the tricyclic ring but remains an important prospect for a drug binding motif because of its prevalence in the transmembrane domains of P-gp and because of its conservation in human, and in murine forms of the protein. In addition, a Phe-X-X-X-Phe sequence is located immediately adjacent to Ser^{943} in TM11. This residue is homologous to Ser^{941} of the murine *mdr*1 sequence which Gros *et al.* [16] recently determined to be important for conferring resistance to colchicine and adriamycin. In fact, the sequence Phe^{938}-Gly-Ile-Thr-Phe-Ser^{943} is conserved between human and murine *mdr*1 genes but is altered by a single amino acid change ($Phe^{938}\rightarrow Tyr^{938}$) in the *mdr*2 gene of both species [17, 18], thus, destroying the putative binding motif. The fact that P-gps encoded by both *mdr*2 genes are non-functional as multidrug transporters makes this sequence an attractive candidate for a drug binding site.

Unlike the amino acid sequences found in the α-helical domains of calmodulin, there are no acidic amino acids within the α-helical TM domains of P-gp. Since a positively charged amino group appears important for the activity of chemosensitizers [6, 7, 19], this implies the existence of a hydrophilic region of P-gp which folds near the hydrophobic region and interacts with the basic side-chain of the drug (Fig. 2). We have also found that the quaternary ammonium salt of chlorpromazine does not alter MDR (Ford and Hait, unpublished observations) which suggests that the drug must pass through the membrane and perhaps interact with P-gp near a cytoplasmic domain.

Pre-clinical MDR models may help predict clinical activity

Although many drugs are known to sensitize MDR cells to chemotherapeutic drugs, few completely restore the sensitivity of the resistant line to that of the parental line and even fewer have activity *in vivo* [7]. The failure to fully restore sensitivity of MDR lines to chemotherapeutic drugs may be due to the presence of additional mechanisms of drug

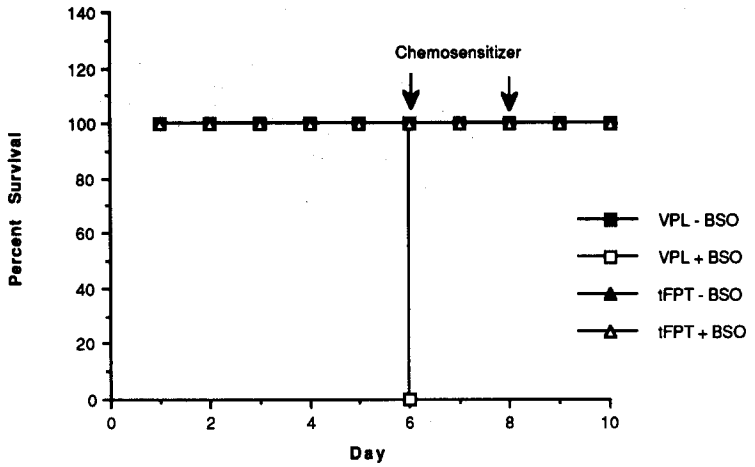

Fig. 3. Effect of BSO on the toxicity of verapamil and *trans*-flupenthixol in mice. Eight-week-old female Swiss Webster mice weighing 20–23 g received normal drinking water (10 mice) or water containing 30 mM BSO (10 mice) *ad lib.* throughout the course of the experiment. On days 6 and 8, mice were injected i.p. with 75 mg/kg verapamil or 30 mg/kg *trans*-flupenthixol, or their vehicles.

resistance produced by prolonged exposure of cells to a cytotoxic selecting agent. Therefore, the blockade of P-gp function, which is only one of several mechanisms of resistance, would be insufficient to fully restore sensitivity to these cell lines. To address this problem, we developed two new clones of MDR cells in collaboration with Drs Michael Gottesman and Ira Pastan. L1210/VMDRC.06 is a murine leukemia transformed by a retroviral expression vector containing a full-length cDNA of the human *mdr*1 gene [20]. It is 5–10-fold resistant to doxorubicin and is cross-resistant to other drugs affected by P-gp [21]. Unlike other MDR lines, L1210/VMDRC.06 does not contain elevated concentrations of glutathione and does not have increased activity or altered structure of topoisomerase II. It is restored to complete sensitivity to doxorubicin and vinblastine by *trans*-flupenthixol or cyclosporin A. Furthermore, it is transplantable into inexpensive laboratory mice where it grows as a solid, non-metastatic tumor within the peritoneal cavity.

K562/VBL cells were transfected in a similar manner to that of L1210 cells and are also multidrug-resistant by virtue of increased expression of P-gp [22]. The K562 clones may be useful pre-clinical models of the blast crisis of chronic myelogenous leukemia which is resistant to chemotherapy and frequently overexpresses the *mdr*1 gene [23]. This line is also fully restored to sensitivity by *trans*-flupenthixol or cyclosporin A. Furthermore, the availability of the transformed K562 clones allowed us to test the influence of P-gp on differentiation therapy. We found that the resistant clones are as susceptible as the sensitive ones to differentiation by hemin.

The failure of modulators to completely sensitize cell lines which contain alternative mechanisms of MDR has important clinical implications. For example, tumors which harbor numerous means to detoxify drugs, such as carcinomas of the colon and kidney, would be unlikely to be fully restored to sensitivity by a drug that inhibited P-gp alone. For this reason, the use of several modulators of resistance is attractive and has been evaluated [24]. However, this approach may be fraught with unforeseen hazards. For example, when we combined BSO (a drug which depletes cellular glutathione) with verapamil (a drug which interacts with P-gp), we failed to demonstrate significant enhancement of doxorubicin cytotoxicity but rather we demonstrated that the tumor cells became supersensitive to the toxic effects of verapamil [25]. Furthermore, when animals were allowed to drink water containing BSO, the injection of verapamil was uniformly lethal (Fig. 3). The identical experiment done with BSO and *trans*-flupenthixol demonstrated that there was no *in vivo* interaction between the two drugs (Fig. 3). Therefore, P-gp antagonists of the thioxanthene class might be better candidates for multiple drug modulation therapy than the calcium channel blockers.

Regulation of P-gp by protein kinase C

Figure 4 depicts a working hypothesis for how the activity of P-gp is regulated by chemotherapeutic agents. This model predicts that exposure of cells to amphipathic substances, such as the anthracyclines, activates phospholipase C which catalyses the breakdown of phosphatidylinositol to IP_3 and DAG. The increase in the plasma membrane concentration of DAG results in the translocation of PKC to the plasma membrane where it phosphorylates P-gp. The phosphorylation of P-gp increases its pumping activity and leads to the removal of doxorubicin from the membrane, thereby returning the activity of phospholipase C to the basal state. Following the metabolism of DAG, P-gp is dephosphorylated through the action of serine/threonine-specific phosphatases which returns the pump to its basal

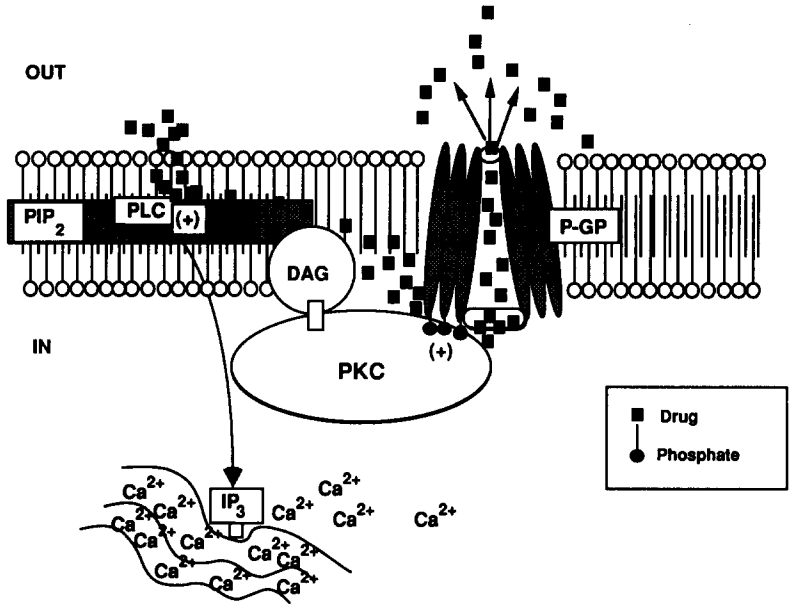

Fig. 4. Model to explain the activation of the regulation of the activity of P-gp by PKC. In this model phospholipase C is activated by cancer drugs (■) to catalyse the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and DAG. DAG production leads to the translocation of PKC to the cell membrane where it phosphorylates P-gp and increases its activity. The extrusion of chemotherapy drug from the cell inactivates phospholipase C and diacylglycerol is metabolized. P-gp is then dephosphorylated by the action of serine/threonine phosphatases which were activated by calcium released from the endoplasmic reticulum by IP_3.

state. The activity of the phosphatases could be increased by the release of intracellular calcium from the endoplasmic reticulum by IP_3.

There is support for this hypothesis. For example, Tritton [26] has shown that doxorubicin increases plasma membrane diacylglycerol, and Chambers *et al.* [27] have found that P-gp is phosphorylated by PKC. In fact, activation of PKC by phorbol esters induces cellular resistance to chemotherapeutic drugs [28, 29] and transfection of multidrug-resistant cells with PKC elevates resistance significantly [30]. In a series of experiments controlled for P-gp content and PKC activity, we have found that phorbol esters increase phosphorylation of P-gp and decrease drug accumulation in a dose-dependent manner. The actions of phorbol esters do not require new protein synthesis and are not mimicked by the inactive 4α-stereoisomers (Aftab and Hait, manuscript in preparation). However, the involvement of PKC in this process may or may not be essential. For example, several kinases in addition to PKC appear capable of phosphorylating P-gp in broken cell preparations [31, 32]. Also, while certain MDR cell lines have been shown to contain increased activity of PKC compared to the parental lines [29, 33, 34], we have shown that this correlation does not universally hold [35].

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