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Pharmacology of Drugs That Alter Multidrug Resistance in Cancer*

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I. Introduction

A. Definition and Characteristics of the Multidrug Resistant Phenotype

Clinical resistance to chemotherapeutic drugs is a major problem in the treatment of cancer. One form of drug resistance, termed 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 due to enhanced outward transport of drugs mediated by a membrane glycoprotein "drug transport pump" (Biedler and Riehm, 1970; Juliano and Ling, 1976). Most experimental models of MDR have been obtained by growing human, mouse, or hamster cell lines in progressively greater concentrations of cytotoxic drugs in culture, although MDR cell lines have been developed in vivo (Dano, 1972). Cells selected for resistance with one drug display significant cross-resistance to the other drugs, which include natural products such as the anthracyclines, *Vinca* alkaloids, epipodophyllotoxins, colchicine, and actinomycin D, but not drugs such as bleomycin, methotrexate, or alkylating agents. This fairly consistent pattern of cross-resistance is termed the MDR phenotype.

The degree of cross-resistance displayed by MDR cells to individual drugs varies among cell lines. However, the similarity in the pattern of resistance to this set of chemotherapeutic agents suggests a single underlying mechanism responsible for MDR. Because many of the drugs affected by MDR are believed to possess different mechanisms of cellular toxicity, investigations into the determinants of this form of drug resistance have focused on the identification of a unifying cellular defense mechanism against toxic agents rather than on individual alterations in target enzymes. The most consistent alteration found in MDR cell lines is an increased expression of a high molecular weight cell surface glycoprotein (P-gp) and the concomitant decrease in accumulation and retention of cytotoxic drugs (Riordan and Ling, 1985).

Several excellent reviews of MDR have been published recently (Beck, 1987; Pastan and Gottesman, 1987; Bradley et al., 1988; Moscow and Cowan, 1988; Endicott and Ling, 1989). Thus, only a brief summary of the current understanding of the cellular pharmacology and molecular biology of this form of drug resistance will be presented. Following this, we present a detailed discussion of the biochemistry and clinical pharmacology involved in the reversal of MDR by drugs, which as a group we will refer to as chemosensitizing agents.

B. Cellular Pharmacology of Multidrug Resistance

The early studies demonstrating that MDR cells were associated with a relative decrease in intracellular cytotoxic drug accumulation when compared to sensitive cells were performed independently by Riehm and Biedler (1971) and Ling and Thompson (1974) using Chinese hamster cells resistant to daunomycin and colchicine, respectively. Subsequently, well-characterized MDR cell lines that express P-gp have all been shown to display decreased drug accumulation, generally ascribed to enhanced outward rather than diminished inward transport (Inaba and Johnson, 1978; Inaba et al., 1979). However, a careful analysis of membrane transport in at least one highly resistant MDR cell line (DC-3F/VCRd-5L) revealed marked alterations in both inward and outward drug transport and found significant differences between the transport kinetics for different chemotherapeutic agents (Sirotnak et al., 1986). A number of investigators have explored the basis for decreased drug retention in many other MDR cell lines and have generally found an energy-dependent mechanism responsible for the outward transport of all drugs to which the cells display resistance. Accordingly, experiments in which MDR cells were depleted of ATP by removing glucose or adding metabolic inhibitors resulted in a reversal of the accumulation defect, whereas replacing glucose restored MDR (Dano, 1973). Furthermore, influx of anthracyclines appears to occur by simple Fickian diffusion (Dalmark and Storm, 1981), in a similar manner in both sensitive and MDR cells (Kessel and Wilberding, 1985a; Willingham et al., 1986), although recent evidence suggests that the rate of diffusion for doxorubicin uptake is less in MDR P388 cells than in sensitive cells (Ramu et al., 1989). These and other observations led to an acceptance of the hypothesis that an outward transport pump of broad specificity is responsible for the transport defect in MDR cells that results in altered sensitivity to multiple drugs (Skovsgaard, 1978).

Alternative explanations may explain decreased drug retention in certain cells. For example, alterations in the binding of drugs to cellular proteins or organelles have been shown in MDR cell lines with vincristine (Sirotnak et al., 1986) and colchicine (Beck, 1987). Clearly, the kinetics of drug transport in MDR cells are complex and remain to be fully defined for many lines. The relationship among drug influx, whether passive or facilitated, intracellular drug binding and metabolism, and drug efflux, both passive and active, all contribute to the resulting cellular accumulation for each drug affected by the MDR phenotype and between each MDR cell line. Nevertheless, the outward transporter or drug pump

§ Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; DNA, deoxyribonucleic acid; cDNA, complementary DNA; RNA, ribonucleic acid; mRNA, messenger RNA; PKC, protein kinase C; VAD, vinblastine, doxorubicin, and dexamethasone; GST, glutathione-*S*-transferase; GSH, glutathione; CaM, calmodulin; DMDP, N-(3,4-dimethoxyphenethyl)-N-methyl-2-(2-naphthyl-*m*-dithane-2-propylamine; SDB, N-solanesyl-N,N′-bis(3,4-dimethoxybenzyl); NASV, N-(*p*-azidosalicyl)-N′-(β-aminoethyl)vindesine; NASAV, N-(*p*-azidosalicyl-aminomethyl verapamil; CsA, cyclosporin A; ILS, increase in the mean or median life span; TPA, 12-O-tetradecanoylphorbol-13-acetate; W7, N-(4-aminohexyl)-5-chloronaphthalensulfonamide; W-12, N-(6-aminobutyl)-2-naphthalensulfonamide; W-13, N-(6-aminobutyl)-5-chloronaphthalensulfonamide.

hypothesis has remained consistent with recent advances in the understanding of the MDR phenotype.

Finally, it must be remembered that most early studies were done with highly resistant cell lines that are likely to possess several mechanisms of drug resistance in addition to defective drug accumulation. For example, using P388 MDR cells, Ganapathi et al. (1984b) found significant cross-resistance to highly lipophilic anthracyclines such as N-trifluoroacetyladriamycin and aclacinomycin, yet the MDR line accumulated the same concentration of these drugs as the parental line. Kessel and Wilberding (1985a) found that this same MDR line could be induced to accumulate equal concentrations of anthracyclines as that of controls, yet cytotoxicity in the MDR line was significantly less.

C. Molecular Biology of Multidrug Resistance

1. Overexpression of P-glycoprotein. In early studies on drug resistance in Chinese hamster ovary cells, an approximately 170,000-Da plasma membrane P-gp associated with MDR but not detectable in drug-sensitive cells was identified by Ling's group (Juliano and Ling, 1976). The presence of P-gp was found to correlate with both decreased accumulation of drugs and degree of resistance in Chinese hamster ovary cells (Kartner et al., 1983a). Since then, several monoclonal antibodies to P-gp have been developed, and most established MDR cell lines from mouse, hamster, or human origin have been shown to express a 150- to 180-kDa membrane protein immunologically cross-reactive with P-gp (Kartner et al., 1983a,b, 1985; Gerlach et al., 1986b).

It is now nearly certain that P-gp is an active outward transporter of drugs having broad specificity as predicted by earlier studies of the cellular pharmacology of MDR. For example, (*a*) a number of radiolabeled drugs, such as [^{3}H]vinblastine, and photoactivatable drug analogs bind in a specific, saturable and energy-dependent manner to MDR plasma membranes but not to sensitive cell membranes (Cornwell et al., 1986a,b; Naito et al., 1988); (*b*) the major membrane protein labeled by these drugs has a molecular weight of 170 kDa and is immunologically cross-reactive with monoclonal antibodies to P-gp (Safa et al., 1986; Cornwell et al., 1986a, 1987); (*c*) purified, solubilized P-gp has been shown to possess ATPase activity (Hamada and Tsuruo, 1988a,b); (*d*) structural analyses of P-gp accomplished by sequencing cDNA clones from P-gp-encoding genes are particularly provocative (Chen et al., 1986; Gros et al., 1986a). For example, the deduced amino acid sequence of P-gp reveals it to consist of two homologous domains containing 12 transmembrane segments (fig. 1) and bearing striking homology to the ATP-binding domains of periplasmic transport proteins of bacteria such as the hyl B α-hemolysin export pump (Higgins et al., 1982), as well as the yeast *Saccharomyces cerevisiae STE6* gene product, which functions to export hydrophobic lipopeptide pheromones (McGrath and Varshavsky, 1989). Furthermore, this deduced sequence reveals two consensus ATP-binding domains on P-gp (Chen et al., 1986); (*e*) transfection of expression vectors containing cDNAs coding for P-gp (Gros et al., 1986c; Ueda et al., 1987) into sensitive cells confers the MDR phenotype, and these stable transfectants overexpress P-gp and display enhanced outward transport of labeled vinblastine (Hammond et al., 1989; Schurr et al., 1989); (*f*) kinetic studies of drug transport in several different experimental models of MDR have further implicated P-gp. For example, inside-out plasma membrane vesicles prepared from MDR KB cells accumulate labeled vinblastine in an ATP-dependent, saturable manner against a concentration gradient (Horio et al., 1988). Accumulation is inhibited by competitive and noncompetitive inhibitors of ATP. Also, cytotoxic drugs affected by MDR and chemosensitizers that reverse MDR were found to be competitive inhibitors of vinblastine accumulation. Both wild-type and *mdr1*-transfected MDCK kidney epithelial cells express P-gp on their apical membranes (Pastan et al., 1988). When grown as a highly polarized epithelium on porous filters, these cells display transepithelial transport of labeled vinblastine, vincristine, and daunomycin in a basal to apical direction, and this process is inhibited by cytotoxic drugs and chemosensitizers (Horio et al., 1989). Similarly, Arias and colleagues (Kamimoto et al., 1989) recently showed that P-gp is expressed on bile canalicular membranes of rat liver cells and that vesicles made from these membranes, but not rat liver sinusoidal membranes, transport labeled daunomycin in an energy-dependent, osmotically sensitive and saturable manner.

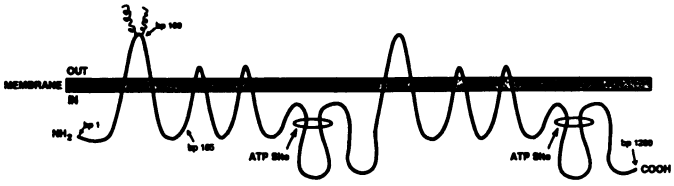

FIG. 1. Model of the structure of the human *mdr1* gene product, P-gp. The protein is 1280 base pairs (bp) in length, with 12 transmembrane domains. Putative N-linked glycosylation sites are shown near base pair 100, and the two ATP-binding sites are circled. A glycine to valine mutation occurs at position 185 in certain colchicine-selected MDR cell lines.

These data suggest that P-gp functions to transport a variety of drugs across cell membranes in a manner analogous to that defined for active transport or ion channel proteins (fig. 2). Well-defined carrier molecules such as the Ca^{++}-Mg^{++}ATPase or the Na^+-K^+ exchange pump are known to bind solutes at specific sites, to transport solutes in an energy- and temperature-dependent, saturable manner, to have characteristic binding constants (K_m), and to be blocked specifically by competitive and noncompetitive inhibitors (Friedman, 1986). Unlike these carrier molecules, however, P-gp is less selective with regard to substrate. It appears to transport certain hydrophobic cationic molecules. It is not known whether this requires an exchange for other organic or

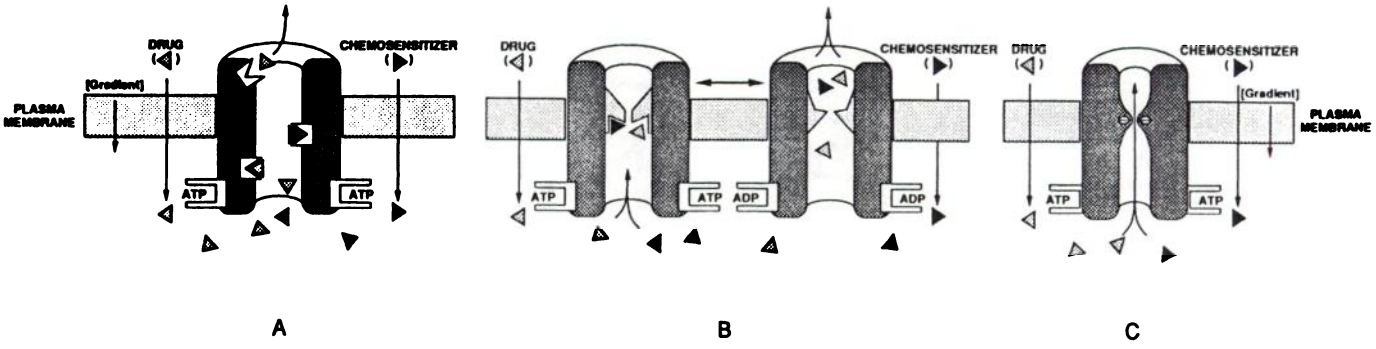

FIG. 2. Functional representations of P-gp. Model A depicts a translocating carrier protein, which utilizes ATP energy to actively transport substrate across the plasma membrane. Chemosensitizer serves as a competitive inhibitor by occupying drug-binding sites. Model B depicts a gated-pore carrier protein. In this model, P-gp undergoes a comformational change, pumping drug and/or chemosensitizer to the *trans* side of the membrane, with each energy-dependent cycle. Model C depicts an ion channel protein, which utilizes ATP energy to actively pump drug (cations) against a concentration gradient. ADP, adenosine diphosphate.

inorganic cations during the transport process. It has been suggested that P-gp may contain multiple drug-binding sites, each having different affinities for different classes of drugs. In fact, Bruggemann et al. (1989) digested photoaffinity-labeled P-gp and used specific antibodies to identify two azidopine-labeled fragments, one from the carboxy terminus and one from the amino terminus of the protein. Because azidopine is a calcium channel blocker that inhibits active transport of vinblastine in MDR KB cells and excess vinblastine inhibits azidopine binding to P-gp, it was inferred from these experiments that there may also be two vinblastine-binding sites on P-gp. However, it is not proven that azidopine and vinblastine bind to the same site on P-gp or whether binding of one drug alters the other's binding site. Yoshimura et al. (1989) used sequence-specific antibodies to synthetic peptides corresponding to two domains of P-gp to demonstrate labeled azidopine binding to both segments but found more efficient binding to the carboxy half of the molecule. Clearly, more work is necessary to define the single or multiple drug-binding sites in each of the duplicate domains of P-gp and to determine the affinity of these sites for various drugs and transport inhibitors.

It is not yet known whether P-gp undergoes a reversible, conformational change during the transport of drugs, analogous to other active transport carrier molecules, such as in membrane translocating or gated-pore models (Friedman, 1986). It is also not known whether the counter- or cotransport of another solute molecule is necessary for cytotoxic drug transport by P-gp.

The structure, function, and biochemical properties of P-gp also bear certain similarities to ion channel proteins (fig. 2). For instance, most ion channels contain multiple hydrophobic membrane-spanning regions, hydrophilic elements that form the aqueous transmembrane pores, and sites for glycosylation on the externally facing segments and phosphorylation on the cytoplasmic segments (Krueger, 1989). P-gp also is composed of hydrophobic membrane-bound regions, hydrophilic N-terminal and nucleotide-binding regions, and sites for glycosylation and phosphorylation (Chen et al., 1986). Ion channels can use energy derived from the hydrolysis of ATP to pump or exchange inorganic cations such as Na^+, K^+, or Ca^{++} across the membrane (Krueger, 1989), although, more commonly, solutes of appropriate size and charge diffuse down a concentration gradient. Similarly, P-gp transports drugs that are hydrophobic, organic cations. Finally, direct phosphorylation of ion channels by PKC can affect long-term changes in its activity (Shearman et al., 1989), and P-gp is known to be phosphorylated by PKC in MDR cells (Center, 1985; Mellado and Horwitz, 1987). Experiments in which treatment of MDR cells with phorbol ester result in enhanced phosphorylation of P-gp along with reduced drug accumulation (increased drug resistance) (Fine et al., 1988; Chambers et al., 1990) suggest that PKC-mediated phosphorylation does modulate P-gp function. The mechanism for competitive inhibitors such as P-gp chemosensitizers, however, is much less clear in this model, although noncompetitive inhibition could occur at ATP-binding sites or at phosphorylation sites. Whether the P-gp transporter functions more like a carrier protein or a channel is unknown, but several lines of evidence suggest the former (Friedman, 1986). For example, carrier transport rates are generally more sensitive to temperature than channel-mediated fluxes. Carrier transport rates (10^2-10^4 substrate molecules/s) are generally less than channel-mediated rates (10^6-10^9/s). Solute flux in carrier-mediated transport is affected more strongly by the concentration of other solutes, both in terms of co- and countertransported solutes, and inhibitors. Finally, the effect of competitive inhibitors such as MDR chemosensitizers on the cytotoxic drug transport kinetics of P-gp strongly supports its similarity to carrier transport proteins.

In summary, the evidence strongly suggests that P-gp functions as an energy-dependent multidrug transporter and its expression forms the genetic basis for MDR.

Furthermore, P-gp appears to function in a manner similar to that of other active transport or ion channel proteins, although additional studies are needed to confirm this suspicion.

2. Overexpression of mdr genes. In certain MDR cell lines, such as the human epidermal KB lines, the amplification of gene(s) coding for P-gp allowed the use of in-gel renaturation (Roninson, 1983) to isolate and ultimately clone *mdr* genes from hamster, mouse, and human cell lines (Gros et al., 1986b; Roninson et al., 1986). The degree of resistance in several MDR cell lines was found to be proportional to the amount of a 4.5-kb mRNA which hybridized to cloned gene segments from human KB cells (*mdr1* gene) (Chen et al., 1986). Gerlach et al. (1986a) used an alternative approach to clone the *mdr1* gene. Monoclonal antibodies to P-gp were used as probes to screen a cDNA expression library for P-gp-encoding sequences from a Chinese hamster ovary MDR cell line. The cDNA clones obtained by each of these two methods were found to be homologous and to hybridize strongly to each other, indicating that P-gp is the product of the *mdr1* gene (Ueda et al., 1986). The sequences of these cDNAs led to the deduced structural characteristics of P-gp discussed above.

Perhaps the most compelling evidence that MDR results from the expression of a single gene came from transfection experiments. Transfer of total genomic DNA from human MDR cells (Shen et al., 1986b) and, more recently, transfection of retroviral and plasmid expression vectors containing full-length cDNAs coding for P-gp (Gros et al., 1986c; Ueda et al., 1987) into sensitive cells conferred the full MDR phenotype in the wild-type cells. These transfectants showed a four-fold decrease in [^{3}H]vinblastine accumulation due to enhanced outward transport of drug (Hammond et al., 1989).

Roninson and coworkers (Choi et al., 1988) found that KB cells transfected with a *mdr1* cDNA derived from a KB cell line selected for MDR with colchicine possess a preferential increase in resistance to colchicine. This *mdr1* protein transcript has a single amino acid mutation at position 185 that appears to be responsible for the altered pattern of MDR.

II. Clinical Relevance of Multidrug Resistance

The rapid advances in understanding the cellular and molecular biology of MDR in vitro have allowed the question to be addressed of whether MDR has a clinical counterpart. Most experimentally induced MDR cell lines have been selected for at least 100-fold drug resistance, although it has been argued that clinically relevant drug resistance is on the order of two- to 10-fold (Young, 1989). It is possible that the pharmacological characteristics displayed by these highly resistant cells are a laboratory phenomenon only, exploiting otherwise functionally unrelated cellular mechanisms that bear no relevance to clinical drug resistance. Whether the MDR phenotype occurs and is responsible for intrinsic or acquired drug resistance through a P-gp-associated mechanism in human tumors is still uncertain. Nevertheless, the common observation that patients who relapse from therapy with certain drugs often become refractory to treatment with other unrelated drugs is reminiscent of the MDR phenotype and therefore demands careful exploration.

A. Expression of P-Glycoprotein in Normal Human Tissues

To understand the clinical importance of MDR, the normal physiological role and tissue distribution of P-gp in a variety of normal human tissues have been studied. Immunohistochemical staining techniques with monoclonal antibodies against P-gp have been used, and high concentrations of the protein have been found in adult adrenal glands, kidney, and placenta (Sugawara et al., 1988a) and, more abundantly, in adrenal cortex than adrenal medulla (Sugawara et al., 1988b). Western blots have also demonstrated the presence of P-gp in liver and low concentrations in small bowel (Hitchins et al., 1987). At a cellular level, P-gp has been localized to the apical biliary surface of hepatocytes, the columnar epithelial cells of colon and jejunum (Thiebaut et al., 1987), and the apical brush border of epithelial cells from renal proximal tubule cells (Lieberman et al., 1989). The polarized expression of P-gp in these tissues suggests a secretory role for the membrane glycoprotein. Another possible function for P-gp was raised by the observation that endothelial cells of human capillary blood vessels at the blood-brain barrier and blood-testis barrier also express P-gp, but cells from larger blood vessels and other tissue capillaries do not. This suggests that P-gp may serve to exclude toxic compounds from the central nervous system and other pharmacological sanctuaries (Cordon-Cardo et al., 1989; Thiebaut et al., 1989).

P-gp expression in nonneoplastic tissues has also been studied. An initial survey found low levels of *mdr1* mRNA in most tissues, with higher levels in adrenal, kidney, liver, and colon (Fojo et al., 1987), although significant variability existed. Recently, Kioka et al. (1989) isolated a full-length *mdr1* cDNA from normal human adrenal tissue. The deduced amino acid sequence of P-gp expressed by this cDNA, as well as the pattern of drug resistance expressed by NIH 3T3 cells transfected with this adrenal cell *mdr1* cDNA, was found to be the same as that in vinblastine-selected KB-V1 cells, as opposed to colchicine-selected KB-C1 cells containing the position 185 amino acid substitution (Choi et al., 1988).

Therefore, P-gp almost certainly has a normal physiological function in human tissues related to secretion and/or protection of tissues from various naturally occurring toxins or commonly encountered xenobiotics. It is likely that continued analysis will eventually yield a natural substrate or substrates for P-gp that may bear

resemblance to the chemotherapeutic drugs that induce its overexpression.

B. Expression of P-Glycoprotein in Human Tumors

P-gp and *mdr1* mRNA expression have also been analyzed in human tumors. Most data reported to date were from tumors in patients not involved in prospective trials analyzing the relationship among P-gp expression, drug treatment, and clinical drug resistance. Because many patients with tumors refractory to drug treatment have received multiple courses of combination chemotherapy, these findings must be interpreted cautiously. However, these published results do provide support for a possible role for P-gp in clinical drug resistance.

Increased P-gp expression is frequently found in tumors derived from tissues known to normally overexpress P-gp. For example, *mdr1* mRNA is overexpressed in pheochromocytomas (from adrenal medulla) and adrenocortical tumors (from adrenal cortex) (Fojo et al., 1987). Similarly, *mdr1* mRNA expression in untreated renal cell carcinomas was higher than in other urogenital tumors derived from tissues that do not normally express P-gp (Kakehi et al., 1988). The levels of mRNA from tumors in this study were shown to inversely correlate with the sensitivity of the tumors to vinblastine, as determined by an in vitro cellular toxicity assay, although this association could not be shown for doxorubicin.

Goldstein et al. (1989) analyzed more than 400 tumor samples taken from patients both treated and untreated with chemotherapeutic drugs. Expression in untreated solid tumors was found to be high for colon cancer, renal cell carcinoma, hepatoma, adrenocortical carcinoma, and pheochromocytoma, whereas untreated breast cancer, non-small cell lung cancer, and many other tumors from tissues that do not normally overexpress P-gp had low or undetectable levels of *mdr1* mRNA. These findings suggest that the *mdr1* gene continues to be expressed in certain tissues after malignant transformation occurs. Whether P-gp plays a role in the failure of initial chemotherapy in these intrinsically resistant tumors is not known. It is important to remember that renal cell, adrenal, and colon carcinoma are also clinically resistant to drugs that are not known to be affected by P-gp, such as alkylating agents, antimetabolites, and bleomycin. Thus, mechanisms other than P-gp-associated MDR must also contribute to intrinsic clinical drug resistance.

Expression of P-gp appears to be an adverse prognostic factor for tumor response to chemotherapy in children with soft tissue sarcoma, a usually highly drug-sensitive tumor. P-gp was detected in nine patients with childhood sarcoma, all of whom relapsed after an initial clinical response to vincristine, dactinomycin, and cyclosphosphomide, whereas only one of 20 patients with P-gp-negative tumors relapsed after a clinical response (Chan et al., 1990). Significant P-gp expression has also been found in untreated tumors from 10 of 12 skin biopsies in patients with Kaposi's sarcoma (Schwartsmann et al., 1989), a stubbornly recurring malignancy.

Expression of P-gp has been found in untreated hematological malignancies in which the cell of origin does not normally express P-gp, such as in myelodysplastic syndromes (Holmes et al., 1989) and in chronic myelogenous leukemia in blast crisis, but not in chronic phase (Goldstein et al., 1989). Expression of *mdr1* mRNA has been shown to be elevated in some lymphomas, with seven of 12 samples from patients with indolent cancers possessing moderate P-gp mRNA levels but only one of 11 more aggressive lymphomas having comparable levels of expression (Moscow et al., 1989a). However, several investigators have demonstrated a significant increase in P-gp or *mdr1* mRNA expression in hematological malignancies following initial chemotherapy or during clinical relapse. Ma et al. (1987) used immunoblots to show P-gp overexpression in sequential biopsies from two patients with acute nonlymphoblastic leukemia progressing while receiving therapy. Similar findings of a relationship between P-gp expression and clinical resistance to anticancer therapy was reported for two patients with chronic myeloid leukemia in blast crisis and one patient with acute nonlymphoblastic leukemia (Carulli et al., 1988). Comparison of treated vs. untreated hematological malignancies also revealed increased P-gp expression, as demonstrated in nine of 13 cases of refractory or secondary acute myeloblastic leukemia vs. two of eight untreated cases (Holmes et al., 1989), six of 10 treated and clinically resistant cases of acute myelocytic leukemia vs. one of six untreated cases (Nooter et al., 1990), 11 of 17 treated vs. two of 15 untreated cases of acute lymphoblastic leukemia (J. M. Berry, N. A. Brophy, and B. I. Sikic, personal communication), and three of 15 cases of acute lymphoblastic leukemia relapsed on salvage chemotherapy vs. one of nine untreated, initially chemoresponsive cases of acute lymphoblastic leukemia (Rothenberg et al., 1989). Ito et al. (1989), however, reported that Southern blots and immunofluorescence studies failed to detect increased *mdr1* mRNA or P-gp expression, respectively, either initially or after relapse in a sequential study of 14 patients with acute myeloblastic leukemia and five with acute lymphoblastic leukemia.

Multiple myeloma has become a focus for studies of P-gp expression. This disease initially responds to chemotherapy with the VAD regimen of vinblastine, doxorubicin, and dexamethasone in 70% of patients, but nearly all relapse. It has been shown that myeloma cells from most patients who have relapsed after VAD treatment express P-gp (Dalton et al., 1989b; Salmon et al., 1989). The percentage of cells expressing P-gp in these treated patients was significantly greater than found in untreated patients (Epstein and Barlogie, 1989; Epstein et al., 1989). In support of a functional role for P-gp in the drug resistance seen in myeloma, cells from patients

refractory to chemotherapy that also expressed P-gp displayed significant resistance to doxorubicin in vitro (Salmon et al., 1989). Finally, the incubation of myeloma cells from two patients with verapamil in vitro resulted in a modest increase in cellular accumulation of labeled doxorubicin and vincristine (Dalton et al., 1989a).

Increased expression of P-gp has also been observed in some solid tumors following initial chemotherapy, although the relationship between expression and clinical resistance is still uncertain. For instance, Goldstein et al. (1990) reported high levels of *mdr1* mRNA expression in five of 18 pediatric patients with neuroblastoma treated with doxorubicin, vincristine and VM-26 but only three of 31 untreated patients with this disease. Ovarian and breast cancer express low levels of P-gp before treatment. Advanced, nonresponsive ovarian cancer was found to overexpress P-gp in two of five cases (Bell et al., 1985). Bourhis et al. (1989) reported that three of 15 tumors from patients who failed to respond to chemotherapy (of whom 10 had received prior doxorubicin) overexpressed *mdr1* mRNA as opposed to none of 35 untreated ovarian carcinomas. Similarly, Sikic's group found 11 of 33 treated vs. 8 of 50 untreated ovarian tumors, as well as five of 15 previously treated breast cancers, expressed high levels of *mdr1* mRNA (J. M. Berry, N. A. Brophy, and B. I. Sikic, personal communication).

Significant P-gp expression has been detected by immunohistochemical staining in three samples from patients with breast cancer who had received either an anthracycline (epirubicin) or a *Vinca* alkaloid (vindesine), whereas 20 other breast cancers in this study from patients who had received no chemotherapy or treatment with alkylating agents, antimetabolites, or hormonal agents had little or no P-gp expression (Schneider et al., 1989). Significant *mdr1* mRNA expression was also detected in 25 of 49 primary breast cancer biopsies from untreated patients using Northern and dot blot hybridization (Kieth et al., 1990). Kacinski et al. (1989a) showed significant expression of *mdr1* mRNA in cells from nine of 16 untreated breast cancers using in situ hybridization and found a strong negative correlation between *mdr1* cDNA hybrids and expression of progesterone receptors levels (Kacinski et al., 1989b). This study also raises the possibility that in situ hybridization may be a more sensitive technique for detecting *mdr1* expression in heterogeneous solid tumors.

Clearly, P-gp overexpression has been observed in untreated patients with a number of intrinsically resistant tumors and with increased frequency in patients with several tumor types with acquired resistance following treatment. However, to prove an association between intrinsic or acquired drug resistance and P-gp expression, careful analyses of the possible correlations between tumor P-gp levels and treatment responses to specific drugs must be performed, and prospective sampling of pre- and posttreatment P-gp levels from individual patients must be determined. Finally, controlled trials designed to detect increased clinical response of tumors with intrinsic or acquired resistance to chemotherapeutic regimens containing inhibitors of P-gp function will be critical to understanding the therapeutic importance of clinical MDR.

III. Alternative Mechanisms Involved in Multiple Drug Resistance

Although the study of MDR has focused on P-gp, a rapidly increasing number of biochemical and molecular alterations have been described in cell lines selected for the expression of resistance to multiple cytotoxic drugs. In fact, these findings have encouraged investigators to thoroughly characterize and define MDR cell lines as "classic" P-gp-associated cells with drug accumulation defects or "atypical" MDR cells which appear to possess other mechanisms for resistance to multiple chemotherapeutic drugs. This should be discouraged because what is truly typical is yet to be defined, and the term lacks sufficient descriptive qualities to be meaningful.

Many of these more recently described molecular alterations associated with resistance to multiple drugs have been found both in cells lacking P-gp expression and in cells that overexpress P-gp. Therefore, it is difficult and impractical to link descriptive terminologies to specific drug resistance phenotypes, because their genotypes may be overlapping. Indeed, clinical drug resistance in human tumors is most likely made up of various combinations of cellular mechanisms for resistance.

In the context of this review, the term "chemosensitizer" refers only to drugs that possess modulatory activity in P-gp-expressing MDR cells. Alternative or additional molecular mechanisms for MDR may present excellent targets for new drug development. The two best characterized additional mechanisms observed in cells that are resistant to multiple drugs are changes in the expression or activity of enzymes involved in the glutathione detoxification pathway and alterations in the nuclear enzyme topoisomerase II.

GST is a multifunctional phase II detoxification enzyme that catalyzes the conjugation of electrophilic substances and endogenous xenobiotics to the tripeptide thiol, GSH, forming stable, excretable metabolites (Habig et al., 1974; Jakoby, 1978; Chasseaud, 1979), and prevents oxidative damage through intrinsic, organic peroxidase activity (Batist et al., 1986). Alterations in the activity of GST and other GSH-related enzymes have been identified in several cell lines associated with resistance to alkylating agents (Tew and Clapper, 1986; Buller et al., 1987; Evans et al., 1987; Lewis et al., 1988; Saburi et al., 1989). In addition, the doxorubicin-selected MDR human breast cancer cell line, MCF-7 Adr^R, which possesses an amplified and overexpressed *mdr1* gene (Fairchild et al., 1987), also shows a seven- to 12-fold increased selenium-dependent GSH peroxidase activity

and a 45-fold increased GST activity, the latter resulting from the increased expression of a single, anionic (π) isozyme of GST (Batist et al., 1986; Mimnaugh et al., 1989). This suggested that GSH peroxidase or GST may have independent activity in conferring cellular resistance to doxorubicin. Transfection and expression of cDNA clones of the GST-π isozyme in drug-sensitive MCF-7 cells that do not overexpress P-gp conferred low level (1. 5- to 2-fold) resistance to alkylating agents and carcinogens but no increase in resistance to natural product drugs such as doxorubicin (Moscow et al., 1989b). Thus, the role of GST in MDR involving non-alkylating agents has not been clearly shown.

Other evidence, however, does suggest that the GSH system may have a role in addition to that of P-gp in conferring drug resistance to certain agents within MDR cells. For instance, depletion of GSH, the substrate for all GST enzymes, in MCF-7 Adr^R cells with the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine (Meister, 1983) resulted in a five- to seven-fold increase in cellular sensitivity to doxorubicin for one group (Dusre et al., 1989), although we have found only a 1.5-fold increase in the sensitivity of these 200-fold doxorubicin-resistant cells after similar treatments with buthionine sulfoximine (Ford and Hait, 1989).

The topoisomerases are enzymes that catalyze the breaking and rejoining of DNA required for genomic unwinding and are necessary for DNA replication (Liu, 1983). Attention has focused on the role of topoisomerase II in drug resistance, because it is believed to be a target for many DNA-intercalating and -nonintercalating drugs such as doxorubicin, mitoxantrone, etoposide, tenoposide, and 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (Chen et al., 1984; Nelson et al., 1984; Tewey et al., 1984). Alteration in the quantity or function of topoisomerase II has been suggested as a possible mechanism for cellular resistance to this group of drugs (Ross et al., 1988), and in fact, several MDR cell lines are characterized by decreased levels of topoisomerase II activity in the absence of P-gp expression or changes in drug accumulation (Pommier et al., 1986; Beck et al., 1987; Charcosset et al., 1988; Deffie et al., 1989; Zwelling et al., 1989; de Jong et al., 1990).

Multifactorial drug resistance has also been associated with alterations in the activity of topoisomerase II and overexpression of P-gp in MDR cells. For example, Ganapathi et al. (1989) characterized a series of L1210 cells selected for progressively increasing resistance to doxorubicin. These lines displayed the MDR phenotype and showed increased expression of P-gp and reduced topoisomerase II-mediated DNA cleavage of DNA.

Many other changes have been described in cells that are resistant to several drugs. Certain of these changes appear to occur only in conjunction with overexpression of the *mdr1* gene, suggesting that they may be the product of genes passively transcribed along with *mdr1* amplification. For instance, sorcin, a 22-kDa anionic calcium-binding protein, has been shown to be overproduced in vincristine-resistant mouse and Chinese hamster lung and ovary cells (Meyers et al., 1985, 1987) and in L1210 cells selected for MDR with etoposide, tenoposide, doxorubicin, dactinomycin, or vincristine (Roberts et al., 1989). The gene for sorcin has been shown to be coamplified in MDR cells along with *mdr1* (Van der Bliek et al., 1986), and thus it is unclear whether sorcin itself plays a role in the establishment, maintenance, or pattern of MDR.

Other low molecular weight cytosolic proteins are uniquely expressed in MDR, such as a 21-kDa protein found in colchicine-resistant KB cell lines (Shen et al., 1986a) and a 20-kDa protein phosphorylated to a greater degree in MCF-7 Adr^R cells exposed to phorbol esters (Fine et al., 1988). The significance or function of these proteins in MDR is completely unknown. Similarly, several membrane proteins unique to MDR cells have been observed in addition to P-gp, such as an 85-kDa glycoprotein in K562/ADM cells (Hamada et al., 1988) and a 150-kDa protein immunologically distinct from P-gp (McGrath and Center, 1988).

Numerous biochemical changes have been noted in MDR cells, particularly in MCF-7 Adr^R cells, including changes in phase I cytochrome P450 enzymes such as aryl hydrocarbon anhydroxylase (Ivy et al., 1988), alterations in the regulation of the hexose monophosphate shunt (Yeh et al., 1987), and increased activity of the drug-metabolizing enzymes DT-diaphorase and glucuronyl transferase (Cowan et al., 1986). One would suspect that these alterations in MDR cells have a significant regulatory or functional role rather than being simply a coincidental result of the selecting procedure.

It appears that cells have a vast capacity to protect themselves from toxic substances, and the study of MDR has uncovered many of these methods. A clinically important question is the order in which these cellular defense mechanisms are expressed, and whether this order differs from that of normal tissues, because early changes would be the first targets for drugs designed to prevent the emergence of resistance. Thus, numerous opportunities exist to further define the regulatory mechanisms for MDR and for exploring the molecular and biochemical basis for alternative forms of MDR.

IV. Pharmacological Reversal of Multidrug Resistance by Chemosensitizers

A major goal in experimental as well as clinical investigations of drug resistance is to discover unique methods by which to reverse or circumvent it. Therefore, many investigators have focused on the pharmacological reversal of MDR. Through the identification of drugs that reverse or antagonize MDR, we hope to gain a better understanding of the various biochemical mechanisms involved in this form of cellular drug resistance and to provide possible agents for use in the clinic.

The first report of the pharmacological reversal of MDR came from Tsuruo and his colleagues (1981), who showed that the calcium channel blocker, verapamil, and the CaM antagonist, trifluoperazine, greatly potentiated the antiproliferative activity of vincristine and produced an increased cellular accumulation of vincristine in an MDR murine leukemia cell line in vitro and in vivo. Since this original observation, many compounds have been shown to antagonize MDR in a variety of cell lines and in vivo tumor models when coadministered with chemotherapeutic agents to which the cells are resistant. The pharmacological agents, MDR cells lines, and cytotoxic drugs used to date, both in vitro and in vivo, for the reversal of MDR are given in tables 1–6. A variety of assays have been used by various investigators to measure the antiproliferative or cytotoxic effects of drugs in MDR cell lines, resulting in a broad range of reported values for similar agents. In general, agents used to antagonize MDR, termed "chemosensitizers" or "resistance modifiers," affect the drug accumulation defect present in MDR cells, usually without completely reversing it, and cause little or no potentiation of drug cytotoxicity in sensitive cells. Investigators have generally determined the magnitude of chemosensitizers' effects by comparing the IC_{50} values for a cytotoxic drug in the absence and presence of a relatively nontoxic, fixed concentration of chemosensitizer. The ratio of these two values has been referred to as the "fold-sensitization," "dose-modifying factor," "degree of potentiation," or "MDR ratio." This approach has been used to calculate the fold reversal of cellular drug resistance for each chemosensitizer listed in tables 1–5.

The biochemical mechanism(s) or the molecular targets for any of the structurally diverse group of known chemosensitizers are still uncertain, partly because many of these compounds have profoundly different effects on cellular physiology and are often cytotoxic on their own. Nevertheless, there is considerable interest in devising clinical protocols utilizing relatively nontoxic chemosensitizers to circumvent clinical drug resistance in man (Skovsgaard et al., 1984; Ozols et al., 1987; Miller et al., 1988; Dalton et al., 1989a; Gottesman and Pastan, 1989).

The chemosensitizers described to date may be grouped into six broad categories: (*a*) calcium channel blockers, (*b*) CaM antagonists, (*c*) noncytotoxic anthracycline and *Vinca* alkaloid analogs, (*d*) steroids and hormonal analogs, (*e*) miscellaneous hydrophobic, cationic compounds, and (*f*) cyclosporins. Although these compounds share only broad structural similarities, most are extremely lipophilic, and those in the first five groups are all heterocyclic, amphipathic substances (see figs. 3–7 for representative structures from each anti-MDR group). This suggests that there may be one or more specific receptor sites for anti-MDR drugs, which have unique, although as yet poorly defined, structural requirements for efficient binding.

In the following sections we review the literature concerning the effects of chemosensitizers in vitro, in vivo, and in human trials and will focus on the need to carefully define the structural requirements for antagonism of MDR by chemosensitizers and to elucidate their mechanism of action to develop more specific and effective agents for clinical use.

A. Effects of Chemosensitizing Agents on Cultured Cells

1. Verapamil. Based on the knowledge that certain MDR cell lines displayed an increase in outward transport of drugs due to alterations in drug transport at the plasma membrane level (Inaba and Johnson, 1978), Tsuruo's group examined the anti-MDR effect of the calcium channel blocker verapamil, presumably because of its known action on a membrane target (Fleckenstein, 1977) and its documented inhibition of hormone secretion (Eto et al., 1974). Measuring cellular proliferation, Tsuruo et al. (1981) showed that 2.2–6.6 μM verapamil reversed the approximately 30-fold resistance to vincristine and seven-fold resistance to vinblastine displayed by a MDR murine leukemia cell line, P388/VCR. Incubation of P388/VCR cells with 6.6 μM verapamil for 5 h also caused a 10-fold increase in the accumulation of [^{3}H]vincristine. In addition, verapamil caused a three-fold increase in the antiproliferative effect of vincristine and vinblastine and a two-fold increase in [^{3}H]vincristine accumulation in sensitive P388 cells (Tsuruo et al., 1981). Concentrations of verapamil >6.6 μM produced significant cytotoxicity when used alone. Because verapamil was shown not to alter vincristine binding to tubulin, the apparent cytotoxic target of *Vinca* alkaloids (Owellen et al., 1974), it was concluded that verapamil's pharmacological chemosensitizing effect was due to alterations in drug accumulation (Tsuruo et al., 1981).

In subsequent reports, Tsuruo's group demonstrated that 6.6 μM verapamil fully reversed the 20-fold vincristine resistance and the three-fold doxorubicin resistance in a MDR human acute myelogenous leukemia line K562/VCR (Tsuruo et al., 1983a) and partially reversed the 40-fold doxorubicin resistance in P388/ADM cells (Tsuruo et al., 1982). Again, this chemosensitizing effect was associated with increased drug accumulation, shown to be associated with the inhibition of an energy-dependent outward transport mechanism (Tsuruo et al., 1982).

Since these early observations, many investigators have demonstrated the chemosensitizing activity of verapamil in various cell lines (table 1) measuring both inhibition of cell growth and cytotoxicity. For example, resistance to vinblastine was partially reversed by verapamil in vinblastine-resistant human leukemic lymphoma CCRF-CEM cells (Beck, 1984) and in doxorubicin-resistant B16 murine melanoma cells (Formelli et al., 1988), as measured by short-term cell growth inhibition. Clonogenic assays demonstrated modest increases in cytotoxicity when verapamil was given with daunomycin in MDR Chinese hamster ovary cells (Cano-Gauci

TABLE 1

*Summary of studies using verapamil to reverse multidrug resistance**

Verapamil (μM)	Cell line	Cytotoxic drug (-fold resistance)	-Fold reversal	Reference
6.6	P388/VCR	VCR (31)	122	Tsuruo et al., 1981
		VLB (7)	7	
2.2		VCR (31)	34	
		VLB (7)	7	
3.3	P388/VCR	VCR (20)	26	Tsuruo et al., 1982
6.6			84	
10	P388/VCR	VCR (20)	40	Tsuruo et al., 1985
		VLB (12)	7	
3.3	P388/ADM	DOX (43)	12	Tsuruo et al., 1982
6.6			13	
10	P388/ADR	DOX (27)	9	Tsuruo et al., 1985
		DAU (19)	9	
30	P388/ADR	DOX (40)	28	Ramu et al., 1984d
10	P388/ADR	DOX (51)	11	Klohs et al., 1986
		DAU (38)	9	
10	P388/DOX	DOX (37)	8	Kramer et al., 1988
6	P388/ADR	DOX (100)		Radel et al., 1988
5	P388/DOX	DOX (100)	6	Ford et al., 1990
2	P388/ADR	DOX (69)	9	Yoshinari et al., 1989
40	EA/DAU	DAU (5)	4	Slater et al., 1982
6.6	K562/VCR	VCR (17)	61	Tsuruo et al., 1982
		DOX (3)	4	
6	C6/DOX	DOX (20)	10	Huet and Robert, 1988
1	CHO/B30	DAU (100)	8	Cano-Gauci and Riordan, 1987
10	8226/DOX40	DOX (50)	40	Bellamy et al., 1988a
10	CEM/VLB$_{100}$	VLB (420)	22	Zamora et al., 1988
10	CEM/VLB$_{1K}$	VLB (930)	21	Beck et al., 1988
6	MES-SA/Dx5	DOX (100)	7	Harker et al., 1986
		DAU (100)	14	
		MITO (60)	8	
		ActD (1200)	9	
		VLB (100)	1	
		VCR (240)	1	
10	MCF-7 Adr^R	DOX (100)	1	Fine et al., 1988
		VCR (100)	10	
20	MCF-7 Adr^R	DOX (100)	7	Kramer et al., 1988
5	MCF-7 Adr^R	DOX (200)	13	Ford et al., 1990
		VLB (100)	33	
		VCR (200)	15	
		COLCH (400)	4	
20	KB-Ch^R 8-5-11-24	COLCH (220)	63	Fojo et al., 1985
		DOX (21)	23	
		VLB (21)	38	
		VCR (73)	183	
5	KB-V1	VLB (500)	15	Ford et al., 1990
		VCR (2000)	18	
		DOX (200)	30	
		COLCH (166)	20	
2	2780^{AD}	DOX (100)	4	Schuurhuis et al., 1987
1	2780^{AD}	DOX (170)	6	Rogan et al., 1984
1	1847^{AD}	DOX (5)	6	
22	DC-3F/AD	DOX (100)	20	Delaporte et al., 1988
		ActD (4200)	85	
6.6	NCI-H69/Cx4	DOX (85)	19	Twentyman et al., 1986
		VCR (750)	72	
	MOR/DOX	DOX (12)	5	
	COR-L23/DOX	DOX (12)	4	
10	HCT-8	DOX (NR)	4	Klohs and Steinkampf, 1988
	Colon 26	DOX (NR)	4	
	LoVo	DOX (NR)	3	

* Abbreviations: ActD, actinomycin D; COLCH, colchicine; DOX, ADR, ADM, doxorubicin; DAU, daunomycin; MITOX, mitoxantrone; VCR, vincristine; VLB, vinblastine; NR, not reported.

and Riordan, 1987) and with doxorubicin in doxorubicin-resistant C6 rat glioblastoma cells (Huet and Robert, 1988). Slater et al. (1982) showed that 2–6 μM verapamil caused significant increases in the inhibition of RNA and DNA synthesis by daunomycin in daunomycin-resistant Ehrlich ascites carcinoma cells, as measured by [^{3}H]uridine and [^{3}H]thymidine incorporation into RNA and DNA, respectively. Fresh tumor cells from patients who relapsed while receiving doxorubicin were more sensitive to doxorubicin in the presence of verapamil when assayed by the human tumor clonogenic assay (Goodman et al., 1987). The responsiveness to drugs of most sensitive cells from which the MDR lines were derived was not significantly affected by verapamil at noncytotoxic doses.

The effect of verapamil on cross-resistance to chemotherapeutic drugs other than those used for initial selection is of great interest, with respect to both the mechanism and the pharmacology of the chemosensitizing activity of verapamil and in defining the mechanisms by which P-gp transports structurally distinct molecules. In certain studies, verapamil produced as great an effect on cross-resistance to drugs as on primary resistance to the selecting agent. For example, using the MES-SA human uterine sarcoma cell line selected for 100-fold resistance to doxorubicin and shown to overexpress the *mdr1* gene product (Sikic et al., 1989), Sikic's group showed that 6 μM verapamil caused a seven-fold sensitization to doxorubicin, associated with increased [^{14}C]doxorubicin accumulation and retention, and a similar magnitude of reversal of cross-resistance to daunomycin, actinomycin D, and mitoxantrone (Harker et al., 1986). Verapamil had no effect on resistance to melphalan, a drug not included in the phenotypic definition of MDR. Of note, verapamil increased accumulation of [^{3}H]vinblastine in these cells without altering MES-SA/Dx5 resistance to vinblastine. Similarly, using an NIH 3T3 cell line transfected with and expressing an *mdr1* gene derived from a colchicine-resistant KB cell line, we found 12 μM verapamil completely reversed primary resistance to colchicine (40-fold) and cross-resistance to doxorubicin (20-fold) (Ford et al., 1990).

In certain studies, verapamil was more effective in reversing resistance to the selecting agents than to the cross-resistant drugs. For example, Beck's group (1986) reported that 10 μM verapamil produced a 75- and 87-fold decrease in the 244- and 1163-fold resistance to vinblastine and vincristine in the vinblastine-selected CEM/VLB100 cell line but only a two- to five-fold potentiation of doxorubicin and daunomycin, to which the cells were 100-fold cross-resistant. Similarly, although 5 μM verapamil caused a 13-fold reversal of 200-fold doxorubicin resistance and 33-fold reversal of 100-fold vinblastine resistance in MCF-7 Adr^R cells, it resulted in only a four-fold change in the 400-fold cross-resistance to colchicine (Ford et al., 1990).

Yet, in other studies, verapamil appeared to have a greater effect on cross-resistance than on primary resistance to selecting agents. Fojo et al. (1985) demonstrated that 20 μM verapamil completely reversed the 20- to 70-fold cross-resistance to doxorubicin, vinblastine, and vincristine in colchicine-selected KB human carcinoma cells, but caused only a 60-fold reduction in the 220-fold resistance to colchicine. Our data with vinblastine-selected KB cells also showed that verapamil caused a relatively greater reversal of cross-resistance to doxorubicin and colchicine than to the primary *Vinca* alkaloid resistance (Ford et al., 1990). Finally, in doxorubicin-selected P388/ADR cells, the degree of potentiation by verapamil of a series of five anthrapyrazoles was linearly related to the level of cross-resistance to these drugs (Klohs et al., 1986). Thus, verapamil affects resistance to selecting agents and cross-resistance to other drugs equally in some but not all MDR cell lines. In addition, certain data suggest that colchicine resistance responds differently to verapamil than that of anthracyclines or *Vinca* alkaloids, although no consistent pattern has yet emerged.

Why MDR cell lines display different degrees of resistance and cross-resistance to drugs, and why some cross-resistance is less sensitive to modulation by verapamil, is unclear. An intriguing possibility is that alterations in the *mdr1* gene at the genomic level (Choi et al., 1988) or posttranslational modifications in P-gp may cause changes in the affinity of cytotoxic drugs or verapamil for the putative drug-binding site(s). Alternatively, multiple isoforms of P-gp, as well as multiple mechanisms of drug resistance to various chemotherapeutic agents operating within a single cell line, could explain these phenomena.

The effect of verapamil on the well-characterized changes in drug accumulation observed in MDR cells has been carefully documented. Kessel and Wilberding (1985a) probed the effect of verapamil on anthracycline cellular kinetics in P388/ADR cells. Drug influx was not altered by verapamil or by the metabolic inhibitor sodium azide, consistent with a diffusional model of anthracycline inward transport. Drug efflux from [^{3}H]daunomycin-loaded cells was also not affected by either modifying agent in P388-sensitive cells, whereas outward transport of daunomycin from MDR cells was inhibited by varying degrees in MDR cells by 2–20 μM verapamil (Inaba et al., 1979). Similar studies were performed with the human myeloma MDR cell line 8226/DOX40 (Bellamy et al., 1988). In these studies, 10 μM verapamil increased net accumulation of [^{14}C]doxorubicin in resistant, but not sensitive, cells due to decreased outward drug transport. Verapamil did not alter the initial rate of anthracycline influx. Furthermore, Bellamy and colleagues (1988) used DNA alkaline elution techniques to determine the number of single-stranded, double-stranded, and protein-associated DNA breaks in both sensitive and

resistant cells after a 1-h exposure to doxorubicin and found an approximately five-fold decrease in the formation of DNA lesions in MDR cells compared to sensitive cells. However, in the presence of 10 μM verapamil, sensitive and resistant cells sustained equivalent amounts of single-stranded, double-stranded, and protein-associated DNA damage by doxorubicin. A major proposed mechanism for anthracycline cytotoxicity is through topoisomerase II-mediated DNA strand breaks (Ross et al., 1978). The work of Bellamy et al. is, therefore, consistent with a model in which verapamil inhibits P-gp-mediated transport of doxorubicin, leading to increased intracellular doxorubicin concentrations and increased cellular toxicity.

Therefore, it has been clearly shown that verapamil inhibits the P-gp-associated, energy-dependent outward drug transport common to MDR cells. In addition, it is likely that at least some of the sensitization produced by verapamil is secondary to the resultant increase in intracellular accumulation of chemotherapeutic agents. As will be reviewed below, a number of investigators have shown that photoactivated verapamil analogs bind to P-gp and that verapamil inhibits the binding of many chemotherapeutic drugs as well as other chemosensitizers to P-gp (Cornwell et al., 1987; Safa et al., 1987; Akiyama et al., 1988; Beck et al., 1988; Safa, 1988a,b), suggesting that verapamil's mechanism of action is through antagonizing P-gp drug binding and transport.

Although verapamil has been widely studied and is a potent and effective antagonist of resistance to a number of drugs in most MDR cell lines in vitro, it possesses potentially life-threatening cardiovascular effects in humans at plasma concentrations in the 2- to 6-μM range needed for antagonism of MDR (DeFaire and Lundman, 1977; Candell et al., 1979) and is cytotoxic at higher doses to normal (Lampidis et al., 1986) and tumor tissue (Beck et al., 1986). Therefore, more potent and less toxic chemosensitizing agents than verapamil have been investigated.

2. *Verapamil analogs.* The activity of structural analogs of verapamil is shown in table 2. Kessel and Wilberding (1985b) examined 14 analogs and found that tiapamil, which contains a dithiane tetraoxide substituent on verapamil's carbon backbone (fig. 3), was 50-fold less potent than verapamil in causing a partial (15-fold) reversal of the 100-fold doxorubicin-resistant P388/ADR line, whereas the analog DMDP was only seven-fold less potent, and the dithiane-substituted analog Ro 11–2933 was 10-fold more potent than verapamil in mediating this effect. The activity of the four most active tiapamil analogs correlated with their effect on drug accumulation (Kessel and Wilberding, 1985b).

Another group examined the activity of DMDP in the same cell line and found that nontoxic doses (3 μM) caused similar effects on doxorubicin cytotoxicity and accumulation to that of 6 μM verapamil and that DMDP was six-fold more toxic to cells than verapamil when used alone (IC_{50} 14 μM) (Radel et al., 1988).

Yamaguchi and coworkers reported that SDB-ethylenediamine, a synthetic isoprenoid with a structurally similar carbon backbone to verapamil containing a 9 carbon isoprene side group (fig. 1), produced a two- to five-fold increase in doxorubicin cytotoxicity in the P388/ADR cell line, as well as partial reversal of cross-resistance to vincristine, vinblastine, and daunomycin (Yamaguchi et al., 1986). This compound was also effective in P388/VCR cells and in colchicine-selected MDR human epidermal carcinoma KB-Ch^R-24 (Nakagawa et al., 1986; Yamaguchi et al., 1986). SDB-ethylenediamine has far less activity as a calcium channel blocker than verapamil (Yamaguchi et al., 1986). Furthermore, a radiolabeled, photoactivatable SDB analog labels P-gp in MDR human KB-C2 cells, and a number of *Vinca* alkaloids, anthracyclines, and chemosensitizers, including verapamil, inhibit this binding (Akiyama et al., 1989).

All of the previously discussed studies of the effect of verapamil on MDR have used a racemic mixture. Because the *S*-enantiomer of verapamil selectively binds to calcium channels (Gilman et al., 1985) recent studies have compared the effects of the *S*- and *R*-enantiomers of verapamil and its analogs desmethoxyverapamil and emopamil on MDR (Keilhauer et al., 1989; Qian and Beck, 1990; Pirker et al., 1990) and found them to be equally active chemosensitizers in drug-resistant CEM/Vlb and KB-C1 cells. The closely related analogs gallopamil and davapamil were more potent calcium channel blockers but less potent chemosensitizers in MDR cells (Pirker et al., 1990). Therefore, the use of less cardiotoxic enantiomers of verapamil and its analogs may provide a means for reaching clinically effective anti-MDR levels in patients.

Thus, certain compounds structurally related to verapamil partially reverse MDR but lack other physiological effects of verapamil on cell membranes. This also suggests that through structure-activity relationships it may be possible to identify and exploit those structural features necessary for anti-MDR activity, while diminishing those important for blocking calcium channels.

3. *Other calcium channel blockers.* A number of calcium channel blockers structurally dissimilar to verapamil have been studied for chemosensitizing activity, and many have been found to be quite active (table 2) (Helson, 1984; Tsuruo, 1989). Indeed, Tsuruo et al. (1982, 1983a) found that prenylamine and caroverine, agents known to block calcium channels (Braasch and Fleck, 1961; Ishida et al., 1980), were as active, although two- to three-fold less potent, than verapamil for altering the sensitivity of P388/VCR and K562/VCR cells to vincristine and P388/ADM cells to doxorubicin.

Additional investigators found that two other classes of structurally dissimilar calcium channel blockers, diltiazem and the nifedipine analogs (fig. 3), had significant

TABLE 2

*Summary of studies using verapamil analogs and other calcium channel blockers to reverse multidrug resistance**

Chemosensitizer	Dose	Cell line	Cytotoxic drug (-fold resistance)	-Fold reversal	Reference
Tiapamil	500 μM	P388/ADR	DOX (100)	15	Kessel and Wilberding, 1985b
DMDP (Ro 11-3651)	20 μM	P388/ADR	DOX (100)	15	Kessel and Wilberding, 1985b
	3 μM	P388/DOX	DOX (100)	19	Radel et al., 1988
Ro11-2933	1 μM	P388/ADR	DOX (100)	15	Kessel and Wilberding, 1985b
	2 μM	A2780-DX2	DOX (30)	4	Jamali et al., 1989
		A2780-DX3	DOX (50)	10	
		A2780-DX6	DOX (500)	71	
SDB-ethylenediamine	34 μg/ml	KB-Ch^R-24	DOX (28)	35	Nakagawa et al., 1986
			VCR (89)	55	
	100 μg/ml	P388/ADM	DOX (37)	3	Yamaguchi et al., 1986
			DAU (19)	2	
			VCR (17)	3	
			Act-D (10)	4	
		P388/VCR	VCR (8)	33	
			DOX (10)	10	
Diltiazem	20 μM	P388/ADR	DOX (51)	3	Klohs et al., 1986
	35 μM	K562/VCR	VCR (21)	9	Tsuruo et al., 1983a
		P388/VCR	VCR (20)	29	Tsuruo et al., 1985
			VLB (12)	7	
		P388/ADR	DOX (27)	9	Tsuruo et al., 1985
			DAU (19)	5	
	100 μM	P388/VCR	VCR (15)	29	Tsuruo et al., 1983b
		P388/ADM	DOX (49)	16	Tsuruo et al., 1983b
Nifedipine	3 μM	P388/ADR	DOX (50)	1	Ramu et al., 1984d
	100 μM	P388/VCR	VCR (15)	12	Tsuruo et al., 1983b
		P388/ADM	DOX (49)	4	Tsuruo et al., 1983b
Niludapine	10 μM	K562/VCR	VCR (21)	48	Tsuruo et al., 1983a
		P388/VCR	VCR (15)	26	Tsuruo et al., 1983b
		P388/VCR	VCR (20)	25	Tsuruo et al., 1985
			VLB (12)	7	
		P388/ADM	DOX (49)	10	Tsuruo et al., 1983b
		P388/ADM	DOX (27)	10	Tsuruo et al., 1985
			DAU (12)	3	
	2 μM	P388/ADR	DOX (69)	6	Yoshinari et al., 1989
Nimodipine	35 μM	K562/VCR	VCR (21)	54	Tsuruo et al., 1983a
		P388/VCR	VCR (15)	26	Tsuruo et al., 1983b
		P388/ADM	DOX (49)	15	Tsuruo et al., 1983b
Nicardapine	3 μM	K562/VCR	VCR (21)	25	Tsuruo et al., 1983a
		P388/VCR	VCR (15)	33	Tsuruo et al., 1983b
		P388/ADM	DOX (49)	4	Tsuruo et al., 1983b
		P388/ADR	DOX (50)	4	Ramu et al., 1984d
	10 μM	K562/VCR	VCR (21)	52	Tsuruo et al., 1983a
		P388/VCR	VCR (15)	70	Tsuruo et al., 1983b
		P388/VCR	VCR (20)	70	Tsuruo et al., 1985
			VLB (12)	7	
		P388/ADM	DOX (49)	27	Tsuruo et al., 1983b
		P388/ADR	DOX (27)	27	Tsuruo et al., 1985
			DAU (19)	9	
Dihydropyridine analogs					
NK-138	10 μg/ml	KB-C1	DAU (13)	10	Nogai et al., 1989
			VCR (150)	107	
			VLB (52)	35	
NK-194	50 μg/ml	KB-C1	DAU (13)	11	Nogai et al., 1989
			VCR (150)	250	
			VLB (52)	66	
BS 300	2 μM	P388/ADR	DOX (69)	15	Yoshinari et al., 1989
BS 304	2 μM	P388/ADR	DOX (36)	17	Yoshinari et al., 1989
Caroverine	6.6 μM	P388/VCR	VCR (20)	19	Tsuruo et al., 1982
		P388/ADM	DOX (40)	8	Tsuruo et al., 1982
		K562/VCR	VCR (17)	11	Tsuruo et al., 1983a
Prenylamine	6.6 μM	P388/VCR	VCR (20)	22	Tsuruo et al., 1982
		P388/ADM	DOX (40)	11	Tsuruo et al., 1982
		K562/VCR	VCR (17)	40	Tsuruo et al., 1983a
Bepridil	4 μM	CH^R C5	DOX (195)	74	Schuurhuis et al., 1987
		2780^{AD}	DOX (140)	10	Schuurhuis et. al., 1987
AHC-52	1 μg/ml	P388/VCR	VCR (20)	22	Shinoda et al., 1989
		P388/ADR	DOX (200)	20	

* Abbreviations: ActD, actinomycin D; DOX, doxorubicin; DAU, daunomycin; VCR, vincristine; VLB, vinblastine.

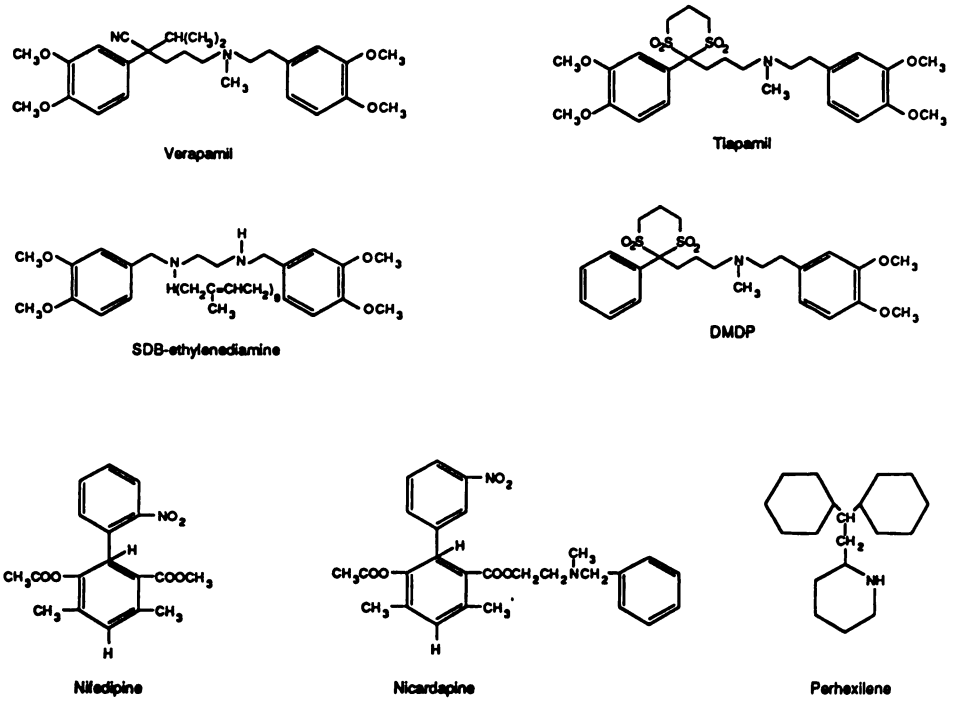

FIG. 3. Structures of calcium channel blockers with chemosensitizing activity.

anti-MDR activity (Tsuruo et al., 1983b; Ramu et al., 1984d; Tsuruo et al., 1985). Although nifedipine is known to be a potent calcium channel blocker (Fleckenstein et al., 1979), it was a poor antagonist of MDR in P388/VCR and K562/VCR cells (Tsuruo et al., 1983b) and had no effect in potentiating doxorubicin in P388/ADR cells (Ramu et al., 1984d). However, the dihydropyridine analogs, niludipine, nimodipine, and nicardapine, were found to be potent antagonists of MDR, with 3.5–10 μM nicardapine fully reversing vincristine resistance in P388/VCR and K562/VCR cells (Tsuruo et al., 1983b; Tsuruo et al., 1985) and partially reversing doxorubicin resistance in P388/ADR cells (Tsuruo et al., 1983b; Ramu et al., 1984d). These studies stressed the lack of correlation between these drugs' effects on calcium channels and on MDR.

In an extensive analysis of the chemosensitizing activity of dihydropyridine analogs, a series of 24 derivatives were screened for their ability to reverse MDR (Nogai et al., 1989). Several lead compounds were identified that partially reversed KB-C1 cell resistance to daunomycin, vincristine, vinblastine, and actinomycin-D (table 2). However, no specific structural features were identified that appeared to correlate with chemosensitizing activity.

Several groups have shown that perhexiline maleate, an active calcium channel blocker structurally unrelated to dihydropyridines or verapamil (fig. 1) (Fleckenstein, 1977) can also antagonize MDR. Noncytotoxic concentrations (10 μM) increased the sensitivity of a MDR human breast cancer cell line MCF-7^{Ad} to doxorubicin and vinblastine (Ramu et al., 1984a) and substantially decreased resistance of P388/ADR cells to these drugs (Foster et al., 1988). This effect was associated with increased drug accumulation. Like verapamil, however, perhexiline maleate is a suboptimal candidate for the modulation of tumor resistance in humans, because of its dose-limiting hemodynamic side effects. Bepridil, a calcium channel blocker with fewer cardiovascular effects, was found to be a potent inhibitor of resistance to doxorubicin in a colchicine-selected Chinese hamster ovary cell line (Schuurhuis et al., 1987) at concentrations that are clinically achievable (2–4 μM) (Benet, 1985).

Thus, a number of calcium channel blockers from different structural classes have been identified that are active antagonists of MDR in cultured cells. Unfortunately, because of the cardiovascular effects of these drugs, most will probably not be useful in man. However, the several dihydropyridine chemosensitizers that lack calcium channel-blocking activity hold promise as relatively specific agents. Of those calcium channel blockers already in clinical use, bepridil appears to be the most promising for continued pre-clinical development for the in vivo reversal of drug resistance.

4. Calmodulin antagonists. The second group of anti-MDR agents are drugs that possess the ability to inhibit CaM-mediated processes, such as the Ca^{++}/CaM-dependent form of cyclic nucleotide phosphodiesterase (Levin and Weiss, 1976). The activity of CaM antagonists against MDR was described by Tsuruo et al. (1982), who studied the effect on MDR of several drugs known to perturb intracellular calcium homeostasis (table 3), based on the finding that verapamil functioned as a chemosensitizer in MDR cells. Nontoxic concentrations of the phenothiazine antipsychotic, trifluoperazine,

TABLE 3

*Summary of studies using calmodulin antagonists to reverse multidrug resistance**

Chemosensitizer	Dose (μM)	Cell line	Cytotoxic drug (-fold resistance)	-Fold reversal	Reference
Trifluoperazine	3.3	P388/VCR	VCR (20)	10	Tsuruo et al., 1982
		P388/ADM	DOX (43)	5	
	2	P388/ADR	DOX (51)	2	Klohs et al., 1986
	4	P388/DOX	DOX (100)	5	Ganapathi and Grabowski, 1983
	4	P388/DOX	DOX (100)	10	Ganapathi et al., 1984b
	5	P388/DOX	DOX (100)	10	Ganapathi et al., 1984a
			DAU (100)	15	
	2	P388/DOX	VLB (20)	5	Ganapathi et al., 1986a
			VCR (100)	7	
	3.3	P388/ADM	DOX (27)	5	Tsuruo, 1983
		P388/VCR	VCR (26)	10	
		K562/VCR	VCR (17)	25	
	6.6	K562/VCR	VCR (17)	25	Tsuruo et al., 1983a
	5	KB-Ch^R-24	COLCH (115)	58	Akiyama et al., 1986
			DOX (50)	19	
			DAU (36)	15	
			VLB (40)	40	
			VCR (68)	12	
	5	L1210/0.025	DOX (5)	3	Ganapathi and Grabowski, 1988
		L1210/0.05	DOX (10)	4	
		L1210/0.1	DOX (20)	7	
		L1210/0.25	DOX (40)	8	
	4	MCF-7 Adr^R	DOX (200)	3	Ford et al., 1989
Thioridazine	1	P388/ADR	DOX (50)	3	Ramu et al., 1984d
	4	KB-Ch^R-24	COLCH (115)	14	Akiyama et al., 1986
			DOX (50)	30	
			DAU (36)	28	
			VLB (40)	44	
			VCR (68)	15	
Chlorpromazine	4	P388/DOX	DOX (100)	3	Ganapathi et al., 1984b
	10	CEM/VLB$_{100}$	VLB (42)	11	Zamora et al., 1988
	5	KB-Ch^R-24	COLCH (115)	4	Akiyama et al., 1986
			DOX (50)	9	
			DAU (36)	7	
			VLB (40)	4	
			VCR (68)	2	
	3	MCF-7 Adr^R	DOX (200)	2	Ford et al., 1989
Prochlorperazine	4	P388/DOX	DOX (100)	5	Ganapathi et al., 1984b
	4	MCF-7 Adr^R	DOX (200)	3	Ford et al., 1989
Trifluopromazine	4	MCF-7 Adr^R	DOX (200)	3	Ford et al., 1989
Perphenazine	4	MCF-7 Adr^R	DOX (200)	3	Ford et al., 1989
Clomipramine	10	P388/VCR	VCR (20)	22	Tsuruo et al., 1982
		P388/ADM	DOX (43)	4	
	6.6	K562/VCR	VCR (17)	14	Tsuruo et al., 1983a
	6.6	P388/VCR	VCR (26)	12	Tsuruo et al., 1983
		P388/ADM	DOX (27)	4	
		K562/VCR	VCR (17)	14	
Fluphenazine	3	MCF-7 Adr^R	DOX (200)	3	Ford et al., 1990
			VLB (100)	20	
			VCR (200)	12	
			COLCH (400)	1	
		KB-V1	VLB (500)	10	Ford et al., 1990
			VCR (2000)	6	
			DOX (200)	3	
			COLCH (166)	4	
		P388/DOX	DOX (100)	2	Ford et al., 1990
			VLB (20)	3	
cis-Flupenthixol	3	MCF-7 Adr^R	DOX (200)	5	Ford et al., 1989
trans-Flupenthixol	5	MCF-7 Adr^R	DOX (200)	15	Ford et al., 1990
			VLB (100)	36	
			VCR (200)	42	
			COLCH (400)	8	
	5	KB-V1	VLB (500)	40	Ford et al., 1990
			VCR (2000)	57	
			DOX (200)	35	
			COLCH (166)	20	
	12	NIH 3T3/MDR1	COLCH (40)	40	Ford et al., 1990
			DOX (20)	20	
cis-Chlorprothixene	5	MCF-7 Adr^R	DOX (200)	2	Ford et al., 1990
trans-Chlorprothixene	5			7	
cis-Clopenthixol	5	MCF-7 Adr^R	DOX (200)	3	Ford et al., 1990
trans-Clopenthixol	5			15	
W-12	20	P388/DOX	DOX (100)	1	Ganapathi et al., 1984b
W-13	20	P388/DOX	DOX (100)	2	Ganapathi et al., 1984b

* Abbreviations: COLCH, colchicine; DOX, ADR, ADM, doxorubicin; DAU, daunomycin; VCR, vincristine; VLB, vinblastine.

caused a five- to 10-fold increase in vincristine and doxorubicin sensitivity in 20- and 40-fold resistant P388/VCR and P388/ADM cells, respectively (Tsuruo et al., 1982) and fully reversed 17-fold K562/VCR resistance to vincristine (Tsuruo et al., 1983a). Like verapamil, trifluoperazine also caused a four- to five-fold increase in [^{3}H]vincristine and [^{3}H]doxorubicin accumulation and did not significantly alter drug cytotoxicity or accumulation in sensitive cell lines (Tsuruo et al., 1982, 1983a). In these experiments, verapamil appeared to be more effective than trifluoperazine for antagonizing MDR, however, greater concentrations of verapamil (6.6 μM) than trifluoperazine (2–3 μM) were used because of the greater intrinsic cytotoxicity of trifluoperazine (Tsuruo et al., 1982). Tsuruo et al. (1982, 1983a) did report that equimolar concentrations of trifluoperazine and verapamil (6.6 μM) produced equivalent increases in ^{3}H-drug accumulation in all three cell lines. We also found that verapamil caused a two- to three-fold greater reversal of primary and cross-resistance to anthracyclines, *Vinca* alkaloids, and colchicine than equimolar concentrations of trifluoperazine in a variety of human and mouse MDR cell lines and resulted in greater intracellular doxorubicin accumulation in MCF-7 Adr^R cells (Ford et al., 1989, 1990).

Ganapathi et al. carefully studied the phenothiazines and focused on the effect of trifluoperazine on cellular drug resistance, cross-resistance, and drug accumulation (table 3). In 100-fold doxorubicin-resistant P388/DOX cells, 4–5 μM trifluoperazine caused a five- to 10-fold increased sensitivity to doxorubicin in MDR but not in sensitive cells (Ganapathi and Grabowski, 1983; Ganapathi et al., 1984a). Trifluoperazine (5 μM) also partially antagonized MDR in a series of L1210 murine leukemia cell lines of variable resistance to doxorubicin (five- to 40-fold). In these studies, the magnitude of the effect of trifluoperazine was related to the degree of resistance (Ganapathi and Grabowski, 1988) (table 3).

The effect of trifluoperazine on cross-resistance (i.e., to drugs other than the selecting agent) is less clear. Trifluoperazine enhanced the cellular accumulation and cytotoxicity of daunomycin in the 100-fold daunomycin-cross-resistant P388/DOX cells to the same extent as to doxorubicin but not in sensitive P388 cells (Ganapathi et al., 1984a). Trifluoperazine had significant effects on the cytotoxicity of *Vinca* alkaloids in both MDR and sensitive P388 cells, causing two- to 10-fold increases in the toxicity of vinblastine and vincristine in the 20- and 100-fold cross-resistant P388/DOX cells, respectively, as well as in sensitive P388 cells (Ganapathi et al., 1986a). Conversely, the effect of trifluoperazine on the accumulation of [^{3}H]vinblastine was not similar in the P388/DOX and P388 sensitive cells, producing an eight-fold increase in the resistant and only a two-fold increase in the sensitive line (Ganapathi et al., 1986a). Thus, trifluoperazine's enhancement of *Vinca* alkaloid cytotoxicity does not clearly parallel its effect on cellular drug levels.

Studies by Ganapathi and colleagues have questioned the primary role of the drug accumulation defect in resistance to anthracyclines as opposed to other chemotherapeutics. For instance, this group compared the cellular toxicity when similar intracellular concentrations of doxorubicin or daunomycin were achieved by either exposing cells to 5 μM trifluoperazine or by increasing extracellular drug concentrations and found that greater cytotoxicity occurred with trifluoperazine treatment (Ganapathi et al., 1986b). The cellular accumulation of doxorubicin was also examined in a series of L1210 cell lines which display 5-, 10-, 20-, and 40-fold resistance to doxorubicin (Ganapathi and Grabowski, 1988) and are associated with progressively increasing expression of P-gp (Ganapathi et al., 1989). In these cells, cellular concentrations of doxorubicin were inversely proportioned to resistance. Exposure to 5 μM trifluoperazine caused a constant, 1.5-fold increase in doxorubicin accumulation in each resistant subline, independently of the magnitude of resistance. Therefore, there was a relative decrease in the total change in doxorubicin accumulation conferred by trifluoperazine in increasingly resistant cell lines. Yet, the effect of trifluoperazine on the cytotoxicity of doxorubicin increased in proportion to the level of resistance (table 3) (i.e., the magnitude of chemosensitizing activity increased with increasing resistance), supporting a lack of correlation between trifluoperazine's effect on drug accumulation and toxicity in MDR cells.

The relationship of drug accumulation to cytotoxicity is difficult to assess because drug accumulation and retention may represent only one of many factors influencing the cytotoxicity of chemotherapeutic agents. Nevertheless, the disparity between modulation of accumulation and cytotoxicity in the studies of Ganapathi et al. suggests that trifluoperazine, a drug known to effect many cellular enzymes and receptors (Pang and Briggs, 1976; Creese and Sibley, 1980; Ruben and Rasmussen, 1981; Hait and Lazo, 1986), may alter cell sensitivity to drugs in additional ways unrelated to changes in accumulation. Indeed, several investigators have shown that CaM antagonists such as trifluoperazine modulate cell sensitivity to bleomycin through increased DNA damage or through inhibition of DNA repair mechanisms (Chafouleas et al., 1984; Lazo et al., 1985). Because certain MDR cells may also have multiple mechanisms of resistance in addition to P-gp-mediated defects in drug accumulation, reversing the accumulation defect alone may not, and usually does not, fully reverse cellular drug resistance.

In an effort to identify CaM antagonists with greater activity and specificity for reversing MDR, we examined the structure-activity relationships for a series of 22 phenothiazines for potentiation of doxorubicin activity in 200-fold resistant MCF-7 Adr^R cells (Ford et al., 1989).

Hydrophobicity of the tricyclic ring and specific structural features of the amino side chain were independently important for anti-MDR activity (fig. 4). For instance, phenothiazines with tertiary, cationic amino groups incorporated into a cyclic piperazinyl structure, at a distance of at least 4 carbons from a tricyclic ring containing a halogen substitution at position C_2, possessed the greatest chemosensitizing activity. Phenothiazines that fulfilled these criteria, such as prochlorperazine, fluphenazine, and trifluoperazine, caused at most a three-fold reversal of doxorubicin resistance in this study (table 4).

By searching for related drugs that shared these structural features, we identified a novel class of chemosensitizers that possessed significantly greater activity. Specifically, the thioxanthene class of antipsychotics differs in structure from the phenothiazines by a carbon instead of a nitrogen in the tricyclic ring and an exocyclic double bond, conferring stereoisomerism to the drugs (fig. 2). The *trans*-isomer of each in a series of 16 thioxanthenes showed greater activity than the *cis*-isomer and the respective phenothiazine homolog for reversing doxorubicin resistance in MCF-7 Adr^R cells. This effect was not related to differential uptake of the stereoisomers (Ford et al., 1990). Similar to the phenothiazines, the most potent thioxanthene chemosensitizers possessed halogenated tricyclic rings connected to piperazinyl or piperadinyl side groups. The lead compound, *trans*-flupenthixol, reversed MDR in a number of human and murine MDR cell lines and in sensitive cells transfected with the *mdr1* gene and increased doxorubicin accumulation in MCF-7 Adr^R cells to a greater extent than either its stereoisomer *cis*-flupenthixol, its phenothiazine structural homolog fluphenazine, or the calcium channel blocker verapamil (table 3) (Ford et al., 1990).

trans-Flupenthixol was not more potent than *cis*-flupenthixol at antagonizing MDR in an NIH 3T3 line transfected with an *mdr1* expression vector (Ford et al., 1990). The *mdr1* cDNA used in this expression vector was derived from a colchicine-selected KB MDR cell line (Ueda et al., 1987). As mentioned above, this latter cell line contains a P-gp with a single amino acid change at position 185 and this genetic event is associated with a preferential increase in resistance to colchicine, possibly due to alterations in the affinity of P-gp drug-binding sites (Choi et al., 1988). Therefore, the apparent loss of flupenthixol's stereospecificity for antagonism of MDR in 3T3/MDR1 cells may also be related to this mutation, by changing the affinity of a drug-binding domain affecting both chemosensitizers and cytotoxic drugs. However, we also found that *cis*- and *trans*-flupenthixol were equally effective inhibitors of a labeled, photoactivatable verapamil analog ([^{3}H]azidopine) binding to P-gp containing this mutation at position 185 (Ford et al., 1990) and that the same lack of stereospecificity was seen in a nonmutated P-gp.

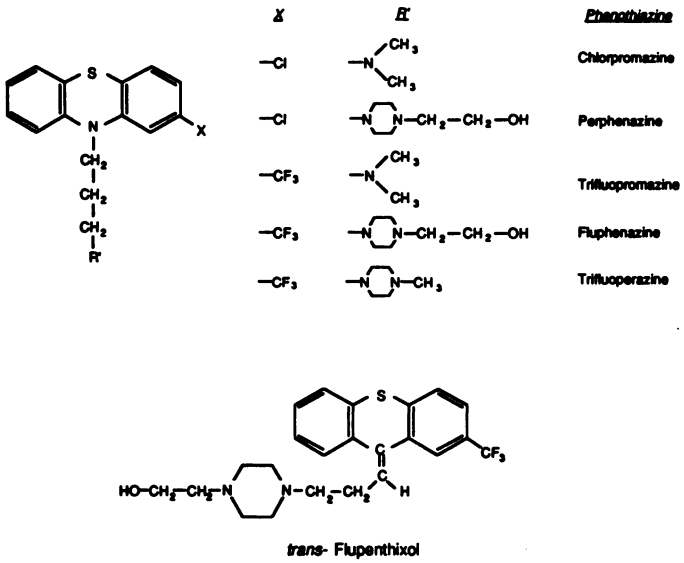

FIG. 4. Structures of phenothiazines and thioxanthenes with chemosensitizing activity.

The clinical pharmacology and toxicology of *trans*-flupenthixol (further reviewed in section IV, C, 3) suggest that it may be suitable for in vivo use. Clinical trials of the antipsychotic effects of thioxanthenes in humans showed that *cis*-flupenthixol was far more effective than *trans*-flupenthixol and that the latter drug was far less toxic (Johnstone et al., 1978). This observation may be explained by biochemical and crystallographic evidence that *cis*-flupenthixol is a potent antagonist of dopamine receptors (Post et al., 1975; Huff and Molinoff, 1984), whereas *trans*-flupenthixol has virtually no activity as a dopamine antagonist, resulting in its apparent lack of extrapyramidal side effects (Nielsen et al., 1973). Because extrapyramidal side effects have proven to be dose limiting in phase I trials that combined trifluoperazine with bleomycin (Hait et al., 1989a) or doxorubicin (Miller et al., 1988), further preclinical development is warranted for this potentially useful clinical chemosensitizer.

In summary, certain CaM antagonists, such as the phenothiazines and thioxanthenes, are effective chemosensitizers, enhance cytotoxic drug accumulation and retention in MDR cells, and may have other effects on DNA damage and repair. Furthermore, specific structural features and spatial relationships appear to be critical for chemosensitizing activity for these classes of drugs. This suggests that application of these principles may enable the rational design of far more potent and specific chemosensitizers. Indeed, if the previously discussed hypothesis regarding alterations in chemosensitizer-binding affinities is valid, it may be possible to target individual MDR subtypes, defined by their particular pattern of cross-resistance and *mdr1* sequence, with chemosensitizers that preferentially bind to that P-gp molecule and/or compete more effectively with specific classes of chemotherapeutic agents.

5. Anthracycline and Vinca alkaloid analogs. Soon after the discovery that MDR cells accumulate less drug be-

TABLE 4

Summary of studies using anthracycline and Vinca *alkaloid analogs, lysosomotropics, steroids, and other drugs to reverse multidrug resistance**

Chemosensitizer	Dose	Cell line	Cytotoxic drug (-fold resistance)	-Fold reversal	Reference
ID-8279	2 μM	P388/VCR	VCR (50)	50	Inaba et al., 1984
			DAU (10)	5	
Vindoline	10 μg/ml	P388/VCR	VCR (50)	50	Inaba and Nagashima, 1986
	20 μM		VLB (20)	50	
			DOX (10)	10	
			DAU (10)	10	
		P388/DOX	DOX (200)	100	
			DAU (200)	100	
	50 μM	CEM/VLB$_{100}$	VLB (420)	>42	Zamora et al., 1988
	10 μM	CEM/VLB$_{1K}$	VLB (930)	13	Beck et al., 1988
Chloroquine	10 μM	P388/ADR	DOX (50)	2	Ramu et al., 1984d
	2 μg/ml	KB-Ch^{R}-24	DOX (38)	4	Shiraishi et al., 1986
	10 μM		DAU (18)	3	
			VCR (139)	3	
			VLB (24)	2	
			ActD (24)	4	
	50 μM	CEM/VLB$_{100}$	VLB (157)	13	Zamora and Beck, 1986
			VCR (600)	10	
			DOX (112)	3	
			DAU (124)	4	
	50 μM	CEM/VLB$_{100}$	VLB (420)	10	Zamora et al., 1988
	50 μM	CEM/VLB$_{1K}$	VLB (930)	10	Beck et al., 1988
Quinacrine	0.5 μg/ml	P388/VCR	VCR (15)	15	Inaba and Maruyama, 1988
	1 μM	P388/ADR	DOX (240)	5	
	5 μM	CEM/VLB$_{100}$	VLB (420)	12	Zamora et al., 1988
	50 μM	CEM/VLB$_{1K}$	VLB (930)	10	Beck et al., 1988
	1 μM	MCF-7/DOX	DOX (200)	1	Ford et al., 1989
Quinidine	10 μM	P388/VCR	VCR (16)	82	Tsuruo et al., 1984
		K562/VCR	VCR (65)	50	
		P388/ADM	DOX (41)	8	
Quinine	100 μM	CEM/VLB$_{100}$	VLB (420)	>32	Zamora et al., 1988
Perhexiline maleate	10 μM	P388/ADR	DOX (20)	12	Ramu et al., 1984a
			VLB (25)	14	
	10 μM	MCF-7^{Ad}	DOX (12)	3	Foster et al., 1988
			VLB (500)	9	
Progesterone	2 μM	J7.V1-1	VLB (1000)	2	Yang et al., 1989
	10 μM		VLB (1000)	9	
Deoxycorticosterone	2 μM	J7.V1-1	VLB (1000)	2	Yang et al., 1989
Tamoxifen	3 μM	P388/ADR	DOX (32)	12	Ramu et al., 1984b
	10 μM	MCF-7^{Ad}	DOX (12)	3	Foster et al., 1988
			VLB (500)	8	
	10 μM	MCF-7 Adr^{R}	DOX (200)	12	DeGregorio et al., 1989
Toremifene	10 μM	MCF-7 Adr^{R}	DOX (200)	15	DeGregorio et al., 1989
Triparanol	3 μM	P388/ADR	DOX (32)	10	Ramu et al., 1984b
Reserpine	5 μM	CEM/VLB$_{100}$	VLB (420)	75	Pearce et al., 1989
			VCR (NR)	30	
			DOX (NR)	10	
	5 μM	CEM/VLB^{1K}	VLB (930)	120	Beck et al., 1988
Yohimbine	5 μM	CEM/VLB$_{100}$	VLB (420)	25	Pearce et al., 1989
Rescinnamine	5 μM	CEM/VLB$_{100}$	VLB (420)	100	Pearce et al., 1989
			VCR (NR)	60	
			DOX (NR)	10	
Trimethoxyohimbine	5 μM	CEM/VLB$_{100}$	VLB (420)	95	Pearce et al., 1989
			VCR (NR)	25	
			DOX (NR)	10	
Cepharanthine	2 μg/ml	KB-Ch^{R}-24	DOX (117)	25	Shiraishi et al., 1987
	3 μM		DAU (17)	16	
			VCR (105)	87	
			ActD (43)	39	
Cefoperazone	1000 μM	MES-SA/Dx5	DOX (100)	14	Gosland et al., 1989
			VP16 (NR)	29	
			VLB (100)	16	
Ceftriaxone	1000 μM	MES-SA/Dx5	DOX (100)	8	Gosland et al., 1989
			VP16 (NR)	10	
			VLB (100)	2	
Erythromycin	650 μM	WEHI 164/ActD	ActD (100)	10	Hofsli and Nissen-Meyer, 1989a
			DOX (100)	20	
Amiodarone	4 μM	DHD/K12/TR	DOX (NR)	32	Chauffert et al., 1986
Dipyridamole	10 μM	P388/ADR	DOX (50)	15	Ramu et al., 1984d
Aclacinomycin A	5 μg/ml	Friend leukemia	DOX (1000)	2	Tapiero et al., 1988
	10 μM		DAU (1000)	3	

* Abbreviations: ActD, actinomycin D; Ch, colchicine; DOX, ADR, ADM, doxorubicin; DAU, daunomycin; VCR, vincristine; VLB, vinblastine; NR, not reported.

cause of an active transport mechanism (Skovsgaard, 1978), Skovsgaard (1980) tested the hypothesis that a specific drug transport pump would be competitively inhibited by an excess of a nontoxic substrate. The ideal anthracycline structural analog for this purpose was N-acetyl-daunorubicin, which lacks the free amino group of daunomycin essential for DNA intercalation and, thus, has a lower affinity for DNA, resulting in less nuclear accumulation, less cytotoxicity, and higher cytoplasmic concentrations (Zunino et al., 1972). Skovsgaard (1980) found that a 30-fold excess of N-acetyl-daunorubicin caused marked inhibition of active daunomycin transport from MDR but not from sensitive Ehrlich ascites cells, leading to increased net daunomycin accumulation. In addition, N-acetyl-daunorubicin had a seven-fold lower affinity for both sensitive and resistant cell nuclei than daunomycin and did not compete with [^{3}H]daunomycin binding to DNA. The effect of N-acetyl-daunorubicin on cellular resistance to daunomycin was unfortunately not examined in cultured cells, although a 1:20 combination of daunomycin and its N-acetyl analog increased life span in mice with MDR Ehrlich ascites cells inoculated intraperitoneally compared to no effect with either drug alone (Skovsgaard, 1980).

Inaba's group (1984) analyzed the chemosensitizing effects of three additional anthracycline analogs on MDR cells and found significant enhancement of vincristine toxicity in P388/VCR cells in vitro when used in 100-fold excess. One of these analogs also partially reversed the cross-resistance of these cells to daunomycin, implying that either a similar drug transport mechanism effects both anthracyclines and *Vinca* alkaloids or the daunomycin analogs compete with both anthracyclines and *Vinca* alkaloids for separate binding sites on P-gp. Similarly, a number of relatively nontoxic *Vinca* alkaloid analogs, such as vindoline, effectively antagonized both the primary resistance and cross-resistance of MDR P388/ADM and P388/VCR cells (Inaba and Nagashima, 1986) when used in 1000-fold excess (table 4). These analogs were five- to 10-fold less potent than the anthracycline analogs as chemosensitizers and as enhancers of cytotoxic drug accumulation. It would be important to determine whether these nontoxic analogs competitively inhibit the photoaffinity labeling of the photoactive vinblastine analog ^{125}I-NASV (Akiyama et al., 1988) or the photoactive verapamil analog ^{125}I-NASAV (Safa, 1988b) to P-gp.

This class of modifier is of particular interest because the modifiers themselves possess cytotoxic activity. The combination with other chemotherapeutics, both at subtoxic doses, may produce a combination with increased therapeutic index.

6. Steroids and hormonal analogs. Horwitz's group recently studied the effect of steroids on MDR (Yang et al., 1989), prompted by the discovery of high levels of *mdr1* mRNA in the pregnant murine uterus (Arceci et al., 1988). They found that progesterone and deoxycorticosterone, but not estradiol, caused an increase in labeled vinblastine accumulation and a modest reversal of vinblastine resistance in MDR J7.V1–1 murine macrophages, similar in degree to equivalent doses of verapamil (table 4, fig. 5). Furthermore, progesterone was the most potent in a series of steroids tested for inhibition of azidopine photoaffinity labeling of endometrial P-gp, as well as inhibition of labeled vinblastine or vincristine binding to MDR cell membrane (Naito et al., 1989; Yang et al., 1989). These observations suggest the possibility that certain steroid hormones may be natural substrates for P-gp, and that less physiologically active steroid analogs may also be able to reverse MDR with greater specificity. In fact, the antiestrogens tamoxifen, toremifene, and other structurally related triparanol analogs can partially overcome resistance in P388/DOX cells and MCF-7 Adr^R cells independently of their effect on estrogen receptors (fig. 5) (Ramu et al., 1984b; Foster et al., 1988; DeGregorio et al., 1989).

7. Miscellaneous hydrophobic cationic compounds. The search for agents to circumvent MDR has led to the identification of numerous compounds that are not known to be calcium channel blockers or CaM antagonists and are not otherwise pharmacologically related. Most of these compounds are amphipathic and lipophilic in nature and share a broad structural similarity that includes a heterocyclic ring nucleus separated at a distance from a cationic, amino group (fig. 6). Chemosensitizing agents as diverse as the antiarrhythmics amiodarone (Chauffert et al., 1986) and quinidine (Tsuruo et al., 1984), the alkaloid derivative cepharanthine (Shiraishi et al., 1987), the lysosomotropic amines chloroquine and propranolol (Shiraishi et al., 1986; Zamora and Beck, 1986; Zamora et al., 1988), the antimalarial quinacrine (Inaba and Maruyama, 1988; Zamora et al., 1988), the indole alkaloids reserpine and yohimbine (Beck et al., 1988; Pearce et al., 1989), the platelet anticoagulant dipyridamole (Howell et al., 1989; Ramu and Ramu, 1989; Kusumoto et al., 1988), and the antibiotics erythromycin (Hofsli and Nissen-Meyer, 1989a,b), cefoperazone, and ceftriaxone (Gosland et al., 1989) have been reported to partially overcome resistance and cross-resistance to cytotoxic drugs and to increase drug accumulation and retention in various MDR cell

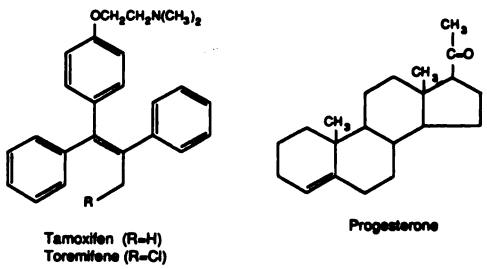

FIG. 5. Structures of steroid hormones and antiestrogen analogs with chemosensitizing activity.

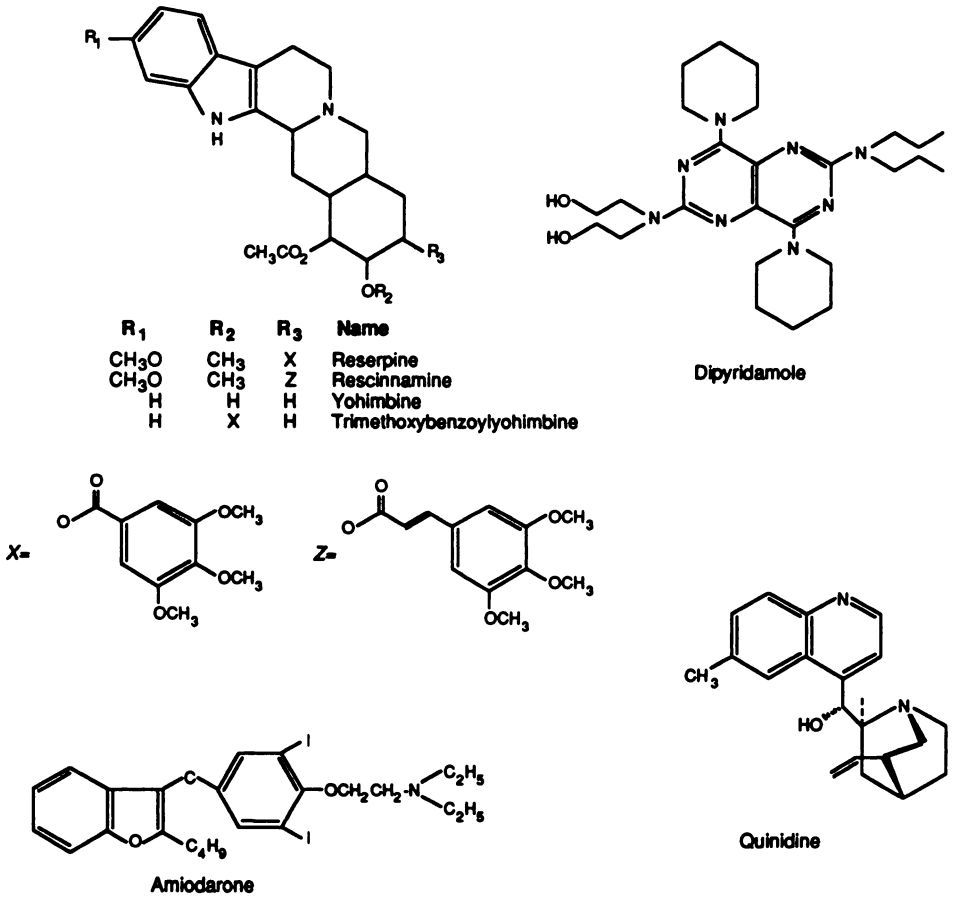

FIG. 6. Structures of other hydrophobic cationic compounds with chemosensitizing activity.

lines (table 4). Whether these many types of pharmacological agents are acting through a common mechanism to antagonize MDR, whether numerous drug-target interactions are capable of mediating this effect, or whether as a result of their degree of hydrophobicity these drugs simply cause nonspecific membrane perturbations leading to increased drug accumulation is presently unclear.

It is important to note that not all hydrophobic cationic drugs alter MDR. In fact, most of the chemotherapeutics affected by MDR share these features, suggesting that they may serve as partial or full agonists or antagonists of the process. For example, certain mitochondrial dyes such as rhodamine 123 or the bisquinaldinium, dequalinium, structurally resemble drugs that antagonize CaM and, in fact, inhibit its activity (Bodden et al., 1986). However, MDR cells are cross-resistant to these types of drugs, by virtue of decreased accumulation (Lampidis et al., 1985; Hait and Pierson, 1990), and other CaM antagonists like trifluoperazine can completely reverse this phenomenon (Hait and Pierson, 1990). Trifluoperazine, on the other hand, accumulated to the same extent in sensitive and MDR P388 cells. Therefore, it is possible that some of these compounds serve as substrates for the P-gp multidrug transporter but possess varying affinities resulting in either pure or mixed agonist vs. antagonist activity, whereas others such as phenothiazines may have distinct mechanisms of action.

8. Cyclosporins. In addition to well-known immunosuppressive properties, cyclosporins have chemosensitizing activity in sensitive and MDR tumor cell lines (Twentyman, 1988b). Structurally and pharmacologically quite different from other known chemosensitizers, CsA is a hydrophobic cyclic peptide of 11 amino acids (fig. 7) (Wenger, 1989). The primary immunosuppressive activity of CsA is through specific inhibition of an early stage of T lymphocyte activation, apparently by interaction with the cytosolic receptor protein, cyclophilin (Handschumacher et al., 1984), and/or with nuclear proteins important for transcriptional activation of lymphokine-encoding genes (Emmel et al., 1989). Interest in CsA's potential anti-MDR activity was in part due to reports of its CaM-binding properties (Colombani et al., 1985), although it was later shown that CsA does not specifically inhibit CaM-mediated processes (Hait et al., 1986, Legrue et al., 1986).

Although there were several earlier reports that CsA could potentiate various cytotoxic drugs in nonresistant tumor cells (Kloke and Osieka, 1985; Osieka et al., 1986), Slater et al. (1986b) first studied its effect in MDR cells. It was found that CsA caused a three- to four-fold poten-

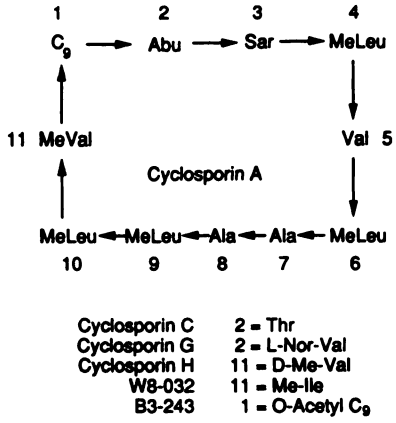

FIG. 7. Amino acid sequence of CsA and some other cyclosporin analogs with chemosensitizing activity [modified from Twentyman (1988a)]. C9 is a new amino acid (2*S*, 3*R*, 4*r*, 6*E*)-3-hydroxy-4-methyl-amino-6-octenoic acid; MeVal, N-methyl-L-valine; MeLeu, N-methyl-L-leucine; MeIle, N-methyl-L-isoleucine.

tiation of daunomycin toxicity in Ehrlich ascites cells that had a very low level of resistance (two-fold) to daunomycin. It is difficult to know whether CsA antagonized the MDR process per se or simply potentiated anthracycline activity in general, because a two-fold potentiation of daunomycin cytotoxicity was also noted in sensitive Ehrlich ascites cells (Slater et al., 1986b). Since then, CsA was found to reverse resistance and cross-resistance in MDR, but not sensitive cells (table 5) (Twentyman et al., 1987; Twentyman, 1988b; Hait et al., 1989b), and also to produce drug potentiation in certain sensitive as well as resistant cells (Chambers et al., 1989; Gaveriaux et al., 1989). For example, CsA caused an 11-fold enhancement of doxorubicin toxicity in sensitive Chinese hamster ovary Aux B1 cells and a 62-fold enhancement in colchicine-selected MDR CH^RC5 cells (Chambers et al., 1989).

The mechanism(s) by which CsA affects MDR appears to be more complex than other chemosensitizers studied to date. Unlike calcium channel blockers or CaM antagonists, as discussed above, cyclosporins appear to increase the effect of chemotherapeutics on certain sensitive as well as MDR lines. In addition, CsA has variable effects on drug accumulation. Slater et al. (1986a) found that CsA reversed resistance to vincristine but had no effect on drug accumulation in a human acute lymphoblastic leukemia cell line selected for vincristine resistance with low level cross-resistance to daunomycin. However, daunomycin accumulation rather than vincristine accumulation was measured in this experiment, and because these cells do not display an accumulation defect to daunomycin, these data are difficult to interpret. Hait et al. (1989b) found that treatment with CsA enhanced the sensitivity to doxorubicin in MDR P388/DOX cells but did not increase the sensitivity of P388 cells. CsA produced a small increase in doxorubicin accumulation in both lines. Chambers et al. (1989) reported that CsA increased doxorubicin accumulation in sensitive Aux B1 Chinese hamster ovary cells, in parallel with an up to 10-fold increase in doxorubicin cytotoxicity and doxorubicin-associated DNA single-strand breaks measured by alkaline elution. However, CsA had no effect on doxorubicin accumulation or DNA single-strand breaks in the MDR derivative, CH^RC5 but did alter drug sensitivity (Chambers et al., 1989). In contrast, Nooter's group found that 0.5–3 μM CsA partially reversed the accumulation defect to daunomycin in another Chinese hamster ovary MDR cell line, CH^RB3 but did not affect daunomycin accumulation in Aux B1 cells, as determined by flow cytometry (Silbermann et al., 1989). Similarly, Nooter et al. (1989) found CsA to increase daunomycin accumulation and toxicity in P388/DAU cell. Thus, it appears that the mechanism by which CsA antagonizes MDR may not be solely due to modification of drug transport.

These mixed results from studies of drug accumulation are paralleled by equally disparate results of the interaction of CsA with P-gp. It has recently been reported that CsA itself is accumulated less in the P-gp-associated CH^RC5 cells than the parent Aux B1 line and that this difference could be reversed with 100 μM verapamil (Goldberg et al., 1988). This suggests that CsA may serve as a P-gp substrate and competitively modify MDR in a manner similar to the nontoxic anthracycline analogs, perhaps in addition to its other drug-potentiating effects found in both sensitive and MDR cells. However, Hait et al. (1989b) found no difference in radiolabeled CsA accumulation in P388 sensitive vs. P388/DOX cells. Furthermore, the 100 μM concentrations of verapamil used by Goldberg's group (10- to 20-fold greater than standard concentrations of verapamil used to affect resistance and accumulation of chemotherapeutics) argues against a specific interaction between CsA and P-gp. Studies by Foxwell et al. (1989) showed photolabeling of CH^RC5 P-gp by a radiolabeled CsA derivative, which was inhibited by 20-fold excess cold CsA, as well as verapamil and diltiazem. The specificity of this binding is difficult to prove, because the extremely hydrophobic CsA molecules also bind to many other cellular proteins and excess cold drug also inhibits those interactions. However, the authors suggest that CsA specifically binds to P-gp because cold CsA inhibits [^{3}H]azidopine photolabeling of the 170-kDa protein band more than the 20- to 120-kDa labeled proteins on an autoradiographic gel.

Future experiments probing the effects of CsA on both primary and cross-resistance to a wide range of drugs in both sensitive and well-defined MDR cells lines as well as in normal cells are clearly necessary to determine whether the chemosensitizing effects of CsA are unique to cells of the MDR phenotype or whether CsA independently enhances the effect of certain drugs. Animal studies and early clinical reports strongly suggest that CsA sensitizes normal tissues to chemotherapeutic drugs (see sections C, 1 and C, 2).

TABLE 5

*Summary of studies using cyclosporines to reverse multidrug resistance**

Chemosensitizer	Dose	Cell line	Cytotoxic drug (-fold resistance)	-Fold reversal	Reference
Cyclosporin A	13 μg/ml	EA/DAU	DAU (2)	3	Slater et al., 1986b
	13 μg/ml	GM3639/L_{100}	VCR (60)	50	Slater et al., 1986a
			DAU (5)	4	
	10 μM	Hepatoma 129	DAU (NR)	2	Meador et al., 1987
	12 μg/ml				
	5 μg/ml	NCI-H69/LX4	DOX (60)	25	Twentyman et al., 1987
			VCR (1200)	200	
	2 μg/ml	NCI-H69/LX4	DOX (100)	9	Twentyman, 1988a
			VCR (150)	32	
	1 μg/ml	CH^R C5	DOX (72)	62	Chambers et al., 1989
		Aux B1 (S)	DOX (1)	11	
	1 μg/ml	CH^RC5	CH (60)	9	Gaveriaux et al., 1989
			DAU (100)	32	
			VCR (25)	38	
		Aux B1 (S)	CH (1)	11	
			DAU (1)	7	
			VCR (1)	16	
	1 μg/ml	P388/DOX	DOX (100)	8	Hait et al., 1989b
	3 μM	P388/DAU	DAU (13)	9	Nooter et al., 1989
	3.6 μg/ml				
Cyclosporin C (2-threonine)	5 μg/ml	NCI-H69/LX4	DOX (60)	8	Twentyman et al., 1987
Cyclosporin G (11-L-Nor-Val)	5 μg/ml	NCI-H69/LX4	DOX (60)	90	Twentyman et al., 1987
	1 μg/ml	CH^RC5	CH (60)	73	Gaveriaux et al., 1989
			DAU (100)	76	
			VCR (25)	59	
		Aux B1 (S)	CH (1)	8	
			DAU (1)	6	
			VCR (1)	16	
Cyclosporin H (11-D-Me-Val)	5 μg/ml	NCI-H69/LX4	DOX (60)	2	Twentyman et al., 1987
	1 μg/ml	CH^RC5	CH (60)	1	Gaveriaux et al., 1989
			DAU (100)	1	
			VCR (25)	1	
		Aux B1 (S)	CH (1)	7	
			DAU (1)	5	
			VCR (1)	7	
11-Me-Ile (W8-032)	2 μg/ml	NCI-H69/LX4	DOX (100)	9	Twentyman, 1988a
			VCR (150)	167	
	1 μg/ml	CH^RC5	CH (60)	35	Gaveriaux et al., 1989
			DAU (100)	93	
			VCR (25)	35	
		Aux B1 (S)	CH (1)	11	
			DAU (1)	5	
			VCR (1)	13	
O-Acetyl C_9 (B3-243)	2 μg/ml	NCI-H69/LX4	DOX (100)	55	Twentyman, 1988a
			VCR (150)	50	
	1 μg/ml	CH^RC5	CH (60)	32	Gaveriaux et al., 1989
			DAU (100)	95	
			VCR (25)	32	
		Aux B1 (S)	CH (1)	7	
			DAU (1)	5	
			VCR (1)	11	
11-Me-Leu	1 μg/ml	CH^RC5	DOX (72)	8	Chambers et al., 1989
		Aux B1 (S)	DOX (1)	12	
	1 μg/ml	P388/DOX	DOX (100)	8	Hait et al., 1989b
6-Me-Ala	1 μg/ml	CH^RC5	DOX (72)	16	Chambers et al., 1989
		Aux B1 (S)	DOX (1)	8	
	1 μg/ml	CH^RC5	CH (60)	41	Gaveriaux et al., 1989
			DAU (100)	72	
			VCR (25)	32	
		Aux B1 (S)	CH (1)	8	
			DAU (1)	5	
			VCR (1)	13	

* Abbreviations: CH, colchicine; DOX, doxorubicin; DAU, daunomycin; S, sensitive; VCR, vincristine; NR, not reported.

The activity of several nonimmunosuppressive cyclosporin analogs on MDR is of great interest with regard to their mechanism of action as well as clinical potential. Hait et al. (1987) described the modulation of MDR by a nonimmunosuppressive analog, 11-methyl-leucine cyclosporin, in P388/DOX cells. Work by Twentyman initially demonstrated a correlation between the chemosensitizing activity of three additional analogs, cyclosporin C (2-threonine), G (2-L-nor-valine), and H (11-D-methyl-valine) (fig. 7) and their immunosuppressive effects in a human small cell lung cancer line (Twentyman et al., 1987). However, this group later found that the nonimmunosuppressive cyclosporins W8–032 (11-Me-Ile) and B3–243 (O-acetyl-C_9) were actually more effective chemosensitizers than identical concentrations of CsA (table 5) (Twentyman, 1988a). Similarly, the nonimmunosuppressive 11-methyl-leucine analog was shown to be less potent but equally effective as CsA in sensitizing P388/DOX cells to doxorubicin (Hait et al., 1989b). 11-Methyl-leucine cyclosporin, as well as a 6-methyl-alanine analog, also increased the sensitivity of Chinese hamster ovary-sensitive and MDR cells to doxorubicin and epirubicin (Chambers et al., 1989). In another study of the effect of 15 cyclosporin analogs on colchicine, daunomycin, and vincristine sensitivity in MDR CH^RC5 and sensitive Aux B1 cells, no correlation was found between the immunosuppressive and anti-MDR properties of the derivatives (Gaveriaux et al., 1989). Furthermore, 14 of these compounds caused a five- to 10-fold increase in the sensitivity of the parental Aux B1 cell line to chemotherapeutic drug cytotoxicity. Because nonimmunosuppressive analogs do not bind to cyclophilin, these results rule out the possibility that reversal of drug resistance is related to this cytosolic protein.

In summary, although the cyclosporins clearly sensitize MDR cells to a variety of chemotherapeutic drugs, it is uncertain whether the mechanism is through a direct effect on P-gp, through an indirect effect on cellular drug metabolism, through potentiation of chemotherapeutic drug toxicity, or a combination of these. Indeed, CsA has been shown to potentiate a number of drugs in vitro and in vivo in both tumor cells and normal tissues (Twentyman, 1988b). If cyclosporin does have additional, specific chemosensitizing activity against MDR cells, it is likely that it is mediated through different or additional mechanisms than most other chemosensitizers, because it does not consistently alter drug accumulation. This leads to the important possibility that CsA, in combination with other presumably P-gp-specific chemosensitizing agents, such as verapamil or *trans*-flupenthixol, may act synergistically. The nonimmunosuppressive analogs that are believed to be less nephrotoxic may be particularly useful.

B. Structure-Activity Relationships among Chemosensitizers

The various classes of drugs with activity against MDR possess very different cellular effects but must share structural features important for anti-MDR activity. It may be possible to define cellular targets and structural features that enhance the interaction with these target(s) by studying the anti-MDR activity of drugs within a single pharmacological class. If specific structural features determine anti-MDR activity, rather than nonspecific properties, it is likely that their mechanism of action is mediated by classical receptor-ligand interactions, implying that the rational design of agents may result in increased specificity and potency. It seems likely that this is true and that drugs can be classified as either complete or partial antagonists or agonists of the receptor governing MDR activity.

To this end, several groups have performed systematic and statistically significant structure-activity relationships of the chemosensitizing effects of individual classes of pharmacological agents. For example, we have studied a series of phenothiazines with individual molecular alterations for their ability to sensitize the human MDR cell line MCF-7 Adr^R to doxorubicin (Ford et al., 1989). Our results suggested that a hydrophobic ring and a tertiary amino group were both important structural components for effective anti-MDR activity (fig. 4). Specifically, substitutions on the phenothiazine tricyclic ring that increased hydrophobicity of the ring, such as halogens at position C_2, increased anti-MDR activity, whereas those that decreased hydrophobicity, such as hydroxyl groups, decreased activity. A highly significant correlation was found between lipophilicity and chemosensitizing activity for phenothiazine drugs with alterations in ring structure. However, specific structural features of the amino side chain were also important. For example, tertiary amines (chlorpromazine) were more potent chemosensitizers than primary or secondary amines, and piperazinyl amines, particularly those with *para*-methyl (trifluoperazine) or ethyl (fluphenazine) substitutions, were more effective than noncyclic, aliphatic amines (trifluopromazine, perphenazine) (fig. 4). Finally, the distance between the amino group and the phenothiazine ring proved important, with a four-carbon alkyl bridge being more effective than shorter chains.

The structural principles derived from this study of the phenothiazine class of pharmaceuticals allowed us to identify a structurally similar class of chemosensitizers, the thioxanthenes (fig. 4). Similar to the phenothiazines, thioxanthenes with halogenated tricyclic rings and piperazinyl amino side groups were particularly effective chemosensitizers (Ford et al., 1990). The distance between the amino group and the thioxanthene ring remained an important determinant for activity, and moreover, the fixed spatial relationship between the two groups proved highly significant. Because of an exocyclic double bond connecting the side chain to the thioxanthene ring, stereoisomers of each thioxanthene analog exist. We found that the *trans*-isomers of each thioxanthene pair were more effective chemosensitizers than the

cis-isomers. Therefore, our studies of structure-activity relationships with phenothiazines and thioxanthenes suggest that, for these classes of drugs, compounds with tertiary, cationic amino groups incorporated into a cyclic ring structure in a particular spatial orientation and at a distance of at least three carbons from a hydrophobic conjugated ring were optimal for reversing MDR.

Ramu (1989) has come to similar conclusions regarding the importance of a substituted amino group located at a precise distance from a ring structure, through the study of three different classes of anti-MDR agents. For example, the chemosensitizer perhexilene maleate possesses two cyclohexyl rings separated by a three-carbon chain from a piperidine amino group (Ramu et al., 1984a) (fig. 3). Replacement of the two-ring structures by a noncyclic group (guanethidine) resulted in a complete loss of anti-MDR activity (Ramu et al., 1984d), whereas replacement with phenyl rings (proadifen or prenylamine) enhanced activity (Ramu, 1989). Furthermore, Ramu's results with several related compounds, chlorocyclizine, cinnarizine, and meclizene, further support the importance of an N-methyl or N-ethyl piperazine structure for anti-MDR activity (Ramu, 1989).

Results from the structure-activity relationships of 43 dipyridamole analogs demonstrated that (*a*) the spatial relationships of the piperidine and diethanolamine substituents of dipyridamole (fig. 6) were important for chemosensitizing activity, (*b*) tertiary amino groups were more effective than primary amino groups, and (*c*) replacement or loss of the piperidine, but not the diethanolamine, groups resulted in a loss of anti-MDR activity (Ramu and Ramu, 1989). Similar to results with other classes of chemosensitizers, one of the most effective dipyridamole chemosensitizers (RA-168) contained an N-methyl piperidine substituent.

Triparanol, an effective chemosensitizer analog of the triphenylethylene class of drugs, contains three substituted phenyl rings connected by a two-carbon chain to a tertiary amino group (fig. 3). Related triphenylethylenes lacking the amino side chain lose activity for reversing MDR in P388/DOX cells (Ramu et al., 1984b). Similar to our findings with the phenothiazines chlorproethazine and chlorpromazine, a diethyl substituted amino group (triparanol) conferred greater anti-MDR activity than a dimethyl group (tamoxifen) (Ramu, 1989). Furthermore, incorporation of the tertiary amine into a cyclic pyridinyl structure enhanced activity (nitromifene, nafoxidine).

An important analysis of structural characteristics important for chemosensitizing activity comes from studies with indole alkaloids and other aromatic amines. Similar to the previously discussed structure-activity relationships, Beck and colleagues (Zamora et al., 1988) have reported that quinoline derivatives such as primaquine and quinacrine, which possess conjugated ring structures attached to substituted amino side groups, display significant anti-MDR activity. Acridine, which completely lacks an amino side group, retains only partial activity at a 10-fold higher concentration. In a series of related studies of reserpine and yohimbine analogs, the presence of a pendant benzoyl moiety appeared to enhance activity (Pearce et al., 1989). The addition of this group resulted in compounds such as reserpine, trimethoxybenzoylyohimbine, and rescinnamine, with aromatic ring domains separated by a distance from a tertiary, cationic nitrogen (fig. 6). Compounds that lacked the former ring structure, such as reserpic acid and yohimbine, also lacked significant anti-MDR activity. Whereas these alkaloids are quite different in their basic structure from those compounds discussed by Ford et al. (1989, 1990), Ramu et al. (1984a,b,d, 1989), and Ramu and Ramu (1989), three-dimensional configurational analyses show a similar relationship between hydrophobic, planar ring groups and cationic, tertiary amines (Pearce et al., 1989).

Studies with several other groups of chemosensitizers agree with the principles emerging from these structure-activity relationships. For example, a number of dihydropyridine analogs were demonstrated to possess anti-MDR activity, and many of the more active of these possess one or more tertiary amine side groups attached to the dihydropyridine ring (Yoshinari et al., 1989). Also, Sikic and colleagues (Gosland et al., 1989) identified several cephalosporin antibiotics with chemosensitizing activity. The most active of the five drugs studied, cefoperazone, was the only one to contain an N-ethyl piperazine amino group, connected to the cephalosporin ring structure.

In summary, systematic structure-activity relationships point out the importance of two particular structural features common to most active anti-MDR pharmaceuticals, a hydrophobic, conjugated planar ring and a substituted, preferably cyclic, tertiary amino group. The apparent importance of the spatial orientation between the two moieties, as illustrated by studies with stereoisomers, is intriguing because it suggests that binding site(s) exist with specific structural requirements for effective antagonist or partial agonist activity. These structural requirements are reminiscent of those found to be important for interactions between phenothiazines and CaM (Prozialeck and Weiss, 1982). Drug-binding studies with synthetic peptides and molecular modeling provided a rationale for the importance of both hydrophobicity and molecular structure for the phenothiazine-CaM interaction. The induction of an α-helix formation by the binding of Ca^{++} to CaM results in two distinct regions, a hydrophobic pocket containing two aromatic phenylalanine residues (Phe 89 and 92) oriented to form a charge transfer complex with the aromatic, tricyclic nucleus of the phenothiazines and a hydrophilic region at a distance of one-half helical turn formed by glutamic acid residues (Glu 83, 84, and 87), which interact with the positively charged nitrogen atom of the phenothi-

azine side chain (Reid, 1983). The relationship between structure and hydrophobicity for the phenothiazine's anti-MDR activities suggests that in these systems, similar to CaM, chemosensitizers interact in both a hydrophobic and electrostatic manner with a protein target. Like CaM, it is likely that this target possesses a hydrophobic domain in close proximity to a negatively charged amino acid. Therefore, use of structure-activity relationships to better define determinants important for chemosensitizing activity may not only result in the discovery of more potent, less toxic agents for the reversal of MDR but may also lead to a precise definition of the drug-binding site.

C. In Vivo Effects of Chemosensitizers

The goals in studying the in vitro effects of chemosensitizers on MDR are two-fold: to further elucidate the biochemical and molecular mechanism(s) involved in MDR and to identify and characterize compounds that may act as effective, nontoxic modulators of intrinsic or acquired drug resistance in patients. A number of agents identified as having anti-MDR activity in vitro have been tested in mouse tumor models with varying success, and there is intense interest in the results from current and proposed clinical trials of these agents in man.

Ideally, chemosensitizers should produce selective enhancement of cytotoxicity. The potential utility of any chemosensitizing agent in vivo depends both on its ability to potentiate the cytotoxicity of anticancer drugs at clinically achievable concentrations and on its associated toxicity and untoward side effects. Therefore, it is crucial that the enhanced toxicity against cancer cells is not accompanied by a similar enhancement of drug toxicity to normal cells. Because this ideal chemosensitizer has not been identified, it is necessary to carefully test the effect of potential drugs for clinical use in appropriate animal models before human trials begin.

1. Effects of chemosensitizers in murine tumor models. The role of murine tumor models in identifying effective anticancer drugs is well documented (Martin et al., 1986). The standard in vivo model for the study of pharmacological reversal of MDR has been measurement of the effect on life span of combinations of cytotoxic and chemosensitizing drugs, injected intraperitoneally daily for 8–10 days into mice inoculated intraperitoneally with either 1×10^5–1×10^6 sensitive or MDR ascites tumor cells (Skovsgaard et al., 1984). A number of the chemosensitizing agents discussed in the preceding sections have been shown to cause an ILS in this model (table 6). For instance, daily intraperitoneal administration of verapamil at doses of 50–100 mg/kg caused approximately 30–50% ILS in P388/VCR-bearing mice treated with vincristine or vinblastine (Tsuruo et al., 1981, 1983b; Yamaguchi et al., 1986), 25–40% ILS in P388/ADR-bearing mice treated with doxorubicin (Tsuruo et al., 1983b; Radel et al., 1988), and 120% ILS in daunorubicin-resistant Ehrlich ascites-bearing mice treated with daunorubicin (Slater et al., 1982). Doses of verapamil >125 mg/kg proved toxic when given to mice, particularly in combination with doxorubicin or vincristine (Tsuruo et al., 1981).

Other calcium channel blockers have shown similar activity in vivo (table 6). Accordingly, daily treatment with 125 mg/kg diltiazem, 75 mg/kg nicardipine, or 75 mg/kg niludipine in combination with doxorubicin or vincristine caused 30–50% ILS in P388/DOX- and P388/VCR-bearing mice (Tsuruo, 1983; Tsuruo et al., 1983b).

Two structural analogs of verapamil that possessed in vitro activity have also been tested in vivo. The synthetic isoprenoid, SDB-ethylenediamine, caused ≤48% ILS in P388/VCR-bearing mice in combination with vincristine (Yamaguchi et al., 1986). Initial studies of the tiapamil analog DMDP showed that 15- to 60-mg/kg doses were not effective in potentiating doxorubicin activity in P388/ADR tumor-bearing mice (Radel et al., 1988). In fact, DMDP caused a decrease in the mean survival time, presumably due to enhanced toxicity in combination with doxorubicin, because measurement of intracellular drug levels in ascites tumor cells revealed adequate anti-MDR concentrations of the chemosensitizer. More recent findings also show that 30–60 mg/kg DMDP could restore in vivo intracellular concentrations of doxorubicin in P388/DOX cells to that of their sensitive counterparts and that 9 μM plasma concentrations of DMDP could be achieved with the maximum tolerated dose of 100 mg/kg (Yin et al., 1989). However, whether these doses of DMDP caused increased toxicity in combination with doxorubicin was not addressed in this study.

The chemosensitizers quinidine and quinacrine also possess noteworthy activity in vivo (Tsuruo et al., 1984; Inaba and Maruyama, 1988), the latter causing an 85% ILS in the P388/VCR model.

The anthracycline analog, N-acetyl-daunorubicin, which possessed no demonstrable toxicity when given in a ratio of 20:1 with daunorubicin, caused a 53% ILS in mice bearing Ehrlich ascites tumors resistant to daunorubicin (Skovsgaard, 1980).

There has only been one report of the activity of CaM antagonists in reversing MDR in mouse tumor models. Tsuruo et al. (1983b) found that 20–30 mg/kg clomipramine, in combination with vincristine or doxorubicin, caused a 31–33% increase in mean survival time of mice bearing P388/VCR or P388/ADM tumors. There have been no published results of the effect of phenothiazine CaM antagonists on MDR tumors in mice, in contrast to the extensive work done in cultured cells with these compounds. However, preliminary results with the thioxanthene CaM antagonist *trans*-flupenthixol show that treatment of P388/DOX-tumor bearing mice with *trans*-flupenthixol, 50 mg/kg, i.p., a dose that alone caused no toxicity, together with doxorubicin, 1–3 mg/kg, caused a 28–62% increase in intracellular doxorubicin accumulation in the MDR cells in vivo. Similar treatment of mice

TABLE 6

*Summary of in vivo studies to reverse multidrug resistance**

Chemosensitizer	Tumor	Dose (μg/ml)			Schedule	ILS (%)	Reference
		CS	Drug				
Verapamil	P388/VCR	0	VCR	0.03	QD × 10	0	Tsuruo et al., 1981
		0		0.2		0	
		50		0.1		34	
		50		0.2		29	
		100		0.03		29	
		100		0.1		45	
Verapamil	EA/DR	0	DAU	0.4	QD × 5	8	Slater et al., 1982
		25		0.4		120	
		50		0.4		106	
Verapamil	P388/DOX	0	DOX	0	QD × 8	2	Radel et al., 1988
		0		1		7	
		75		1		25	
Verapamil	Sarcoma 180	0	BOU	0.6	QD × 4	0	Chitnis et al., 1985
		10		0.0		5	
		10		0.6		45	
Verapamil	P388/VCR	100	VCR	0.1	QD × 10	45	Tsuruo, 1983
	P388/ADM	125	ADM	1.0		37	
SDB-ethylene diamine	P388/VCR	0	VCR	0.03	QD × 8	10	Yamaguchi et al., 1986
		0		0.1		22	
		40		0.0		12	
		40		0.03		48	
		40		0.1		44	
DMDP	P388/DOX	0	DOX	10	QH × 4 (DMDP)	16	Radel et al., 1988
		60		0	QD × 1 (DOX)	4	
		60		10		−36	
Diltiazem	P388/ADM	100	DOX	1	QD × 10	27	Tsuruo et al., 1983b
		125		1		40	
Diltiazem	P388/VCR	125	VCR	0.1	QD × 10	32	Tsuruo, 1983
	P388/ADM	125	ADM	1.0		40	
Nicardapine	P388/ADM	75	DOX	1	OD × 10	43	Tsuruo et al., 1983b
Nicardapine	P388/VCR	100	VCR	0.1	QD × 10	51	Tsuruo, 1983
	P388/ADM	125	ADM	1.0		43	
Niludapine	P388/ADM	75	DOX	1	QD × 10	33	Tsuruo et al., 1983b
Niludapine	P388/VCR	100	VCR	0.1	QD × 10	50	Tsuruo, 1983
	P388/ADM	125	ADM	1.0		33	
AHC-52	P388/VCR	0	VCR	0.1	QD × 5 (V)	27	Shinoda et al., 1989
		25		0.1	BID × 5 (CS)	45	
		50		0.1		74	
		75		0.1		97	
Clomipramine	P388/VCR	100	VCR	0.1	QD × 10	31	Tsuruo, 1983
	P388/ADM	125	ADM	1.0		8	
N-Acetyl-daunorubicin	EA/DR	0	DAU	1.5	QD × 4	0	Skovsgaard, 1980
		30		0.0		0	
		30		1.5		53	
Quinidine	P388/VCR	0	VCR	0.0	QD × 10	0	Tsuruo et al., 1984
		0		0.2		19	
		125		0.0		2	
		125		0.2		41	
Quinacrine	P388/VCR	0	VCR	0.0	QD × 5	0	Inaba and Maruyama, 1988
		0		0.1		30	
		40		0.0		29	
		40		0.1		85	
Cyclosporin A	EA/DR	0	DAU	0.0	QD × 5	0	Slater et al., 1986b
		0		0.3		11	
		80		0.3		>215	
		12.5		0.3		183	
		5		0.3		169	
Cyclosporin A	Hep 129	0	DAU	0.0	QD × 2	0	Meador et al., 1987
		0		0.4		26	
		10		0.0		10	
		10		0.4		69	

* Abbreviations: CS, chemosensitizer; BOU, bouvadin; DOX, ADM, doxorubicin; DAU, daunomycin; EA/DR, Ehrlich ascites/daunomycin resistant; VCR, vincristine; QD, 1 time/day; BID, 2 times/day; QH, every hour.

bearing P388-sensitive cells, which were shown to accumulate eight-fold more doxorubicin in vivo than the MDR cell, had no effect on intracellular doxorubicin accumulation. However, nontoxic doses of *trans*-flupenthixol, 10–50 mg/kg, i.p., administered with doxorubicin, 1–3 mg/kg, for 5–7 days in P388 tumor-bearing mice resulted in increased toxicity in combination, causing a 0–30% decrease in life span compared to no drug or either drug alone (J. M. Ford and W. N. Hait, unpublished observations).

By far the most successful in vivo murine trial of a chemosensitizer, to date, has been with CsA. Slater et al. (1986b) found that up to 80 mg/kg/day for 5 days of CsA could be given to mice without toxicity and that, in combination with 0.3 mg/kg daunomycin daily for 5 days, caused an ILS of >200% in daunorubicin-resistant Ehrlich ascites tumor-bearing mice, with 10 of 10 mice treated surviving for >60 days and apparently cured. CsA, 80 mg/kg, or daunomycin, 0.3 mg/kg, when used alone, caused only a 26% ILS and 11% ILS, respectively, in the MDR murine model. When the dose of CsA was reduced, as little as 5 mg/kg CsA together with daunomycin still produced an impressive 169% ILS in the MDR tumor-bearing mice.

We have studied the toxicity of combining CsA with vinblastine and, unlike the studies with doxorubicin, have observed severe toxicity. For example, all mice receiving CsA, 40–80 mg/kg, i.p., with vinblastine, 10 mg/kg, i.v., died within 3 days of a single dose, whereas those receiving either drug alone had no demonstrable toxicity. Animals were found to tolerate 5 mg/kg CsA with 5 mg/kg/vinblastine weekly for 2 weeks without hematological or other demonstrable side effects.

The apparent synergistic (Berenbaum, 1989) toxicity seen in these "dose-finding" studies support the hypothesis that P-gp is critical in protecting normal tissues from cytotoxicity by chemotherapeutics and other toxic agents, and drugs that antagonize P-gp enhance the toxic effects of these agents on the host.

Houghton's group (Horton et al., 1989) has studied this possibility. When verapamil was given at a constant infusion of 6.25 mg/kg/h, clinically relevant anti-MDR plasma concentrations of 10 μM were achieved for ≤96 h in mice. However, the addition of standard doses of vincristine resulted in significant lethality. It was further shown that verapamil markedly increased the accumulation of vincristine in small intestine, liver, and kidney, tissues known to express P-gp (see section II, A). However, verapamil did not alter the uptake or retention of vincristine in sensitive or vincristine-resistant human tumor xenografts. Therefore, these studies raise a cautionary note with regard to the ability to achieve a therapeutic index with chemosensitizers, if antagonism of normal tissue P-gp results in significantly increased toxicity of chemotherapeutic agents to the host.

Although these in vivo results provide an important corollary to the in vitro study of chemosensitizers, the relative simplicity of the experimental design used ignores many critical factors that contribute to clinical effectiveness. For example, because many chemosensitizers are metabolized by the liver, intraperitoneal injection of these drugs leads to extensive first-pass metabolism. Also, the in vivo cellular pharmacokinetics of chemosensitizers have been poorly studied. Measurements of the intracellular drug concentrations achieved in tumors and in tissues are likely to be more meaningful than steady state plasma concentrations, because most lipophilic chemosensitizers have high volumes of distribution and are rapidly sequestered within tissues. The pharmacokinetics and anti-MDR activity of chemosensitizer metabolites also deserve attention, because they may also possess significant activity.

The use of human tumor xenografts in nude mice has proven to be valuable for the identification of effective chemotherapeutic drugs in humans (Giovanella and Fogh, 1985). This system may also apply to the study of the effects of chemosensitizers in vivo, through use of either previously selected, well-characterized MDR human tumors or sensitive human tumors that have been previously studied and propagated in mouse models, into which a cloned human *mdr1* cDNA is transfected. The advantage of the latter is that drug sensitivity and optimal treatment of the parent line would already be known, and drug resistance in the MDR line would presumably be entirely due to expression of the human *mdr1* gene. Inoculation of tumors in extraperitoneal, subcutaneous sites also enables a careful measurement of tumor response to anti-MDR treatment and investigation of intracellular drug accumulation.

In summary, experiments performed using simple mouse tumor models have suggested that clinical reversal of drug resistance is feasible but may be limited by increased toxicity. However, the eventual development of successful chemosensitizer protocols in humans may require additional models and understanding of the effect of drug interactions on metabolism and toxicity.

2. Effects of chemosensitizers in human trials. The clinical experience with chemosensitizers for the modification of acquired or intrinsic drug resistance is limited. The few trials have been complicated by significant toxicity, lack of studies to determine the mechanism(s) of resistance present clinically, and lack of documented reversal of resistance at the cellular level. For example, attempts to combine verapamil with single-agent chemotherapy have, in general, been unsuccessful because of the intolerable cardiac effects sustained at levels of verapamil nearing the effective anti-MDR range (3–6 μM). In a phase I study of escalating doses of verapamil plus 1.5 mg/m^2 of vinblastine given for 5 days in 17 patients with advanced, unresponsive malignancies, no objective responses were noted, and the majority of patients developed electrocardiographic changes including first-de-

gree heart block and junctional rhythms (Benson et al., 1985). Peak plasma concentrations of verapamil achieved during the continuous constant infusion (0.12 mg/kg/h, i.v.) were 0.45 μM, well below the concentrations necessary to produce anti-MDR effects in culture (table 1). Similarly, a pilot study of oral verapamil and doxorubicin (50 mg/m^2 every 3 weeks) in 13 patients refractory to chemotherapy (eight patients to doxorubicin) was limited by nausea, hypotension, and cardiac arrhythmias (Presant et al., 1986). Although one partial response and two minor responses were noted in patients who had received prior doxorubicin, plasma or tissue levels of doxorubicin or verapamil were not measured. However, reports of the clinical pharmacokinetics of verapamil suggest that high plasma levels are unlikely with oral administration (Frishman et al., 1982). In neither of these studies did verapamil appear to potentiate the cardiac toxicity or myelosuppression of chemotherapeutic drug treatment. In fact, studies of the effect of verapamil on doxorubicin, vincristine, and vinblastine toxicity to normal myeloid and macrophage colony formation show no enhancement of toxicity in normal human bone marrow samples or in marrows from patients recovering from cytotoxic chemotherapy (Fine et al., 1987).

A trial of escalating doses of intravenous verapamil plus doxorubicin (50 mg/m^2) in eight patients with refractory ovarian cancer resulted in unacceptable toxicity, including severe hypotension, heart block, and congestive heart failure, without objective responses to therapy (Ozols et al., 1987). However, because none of the patients had received previous doxorubicin or other natural product chemotherapeutic drugs but rather combinations of cyclophosphamide and cisplatin, there was little reason to believe, and no data to document, that the resistance of these tumors involved the MDR mechanism. The verapamil infusion (9 μg/kg/min) led to median and peak plasma verapamil levels of 2 and 5 μM, respectively. Similarly, a trial of continuous infusion of verapamil (5 μg/kg/min, i.v.) for 6 days in combination with a bolus injection of vincristine (2 mg/m^2, i.v.) and 5 days of VP-16 (200 mg/m^2/day) in pediatric patients with cancer also produced varying degrees of heart block and hypotension, with median and peak verapamil levels of 1 and 4 μM, respectively (Cairo et al., 1989). Although these concentrations are within the range of experimentally relevant in vitro anti-MDR concentrations, further verapamil dose escalation is clearly limited by hemodynamic side effects.

A well-designed study of the clinical use of verapamil for modification of tumor resistance was recently reported by Dalton and colleagues (1989a). In this trial, seven patients with multiple myeloma and one patient with non-Hodgkin's lymphoma, all refractory to standard treatment with 4 days of vincristine (0. 4 mg/day), doxorubicin (9 mg/m^2/day), and dexamethasone (40 mg/day), were studied for tumor expression of P-gp, cellular accumulation of doxorubicin and vincristine, and response to treatment with the identical VAD regimen plus verapamil. Tumor cells from six of eight patients were shown to express P-gp by immunoperoxidase staining of bone marrow biopsies or lymph node biopsies, whereas no P-gp was expressed by normal bone marrow cells. Analysis of tumor cells from two P-gp-positive patients showed enhanced accumulation of doxorubicin or vincristine in the presence of verapamil in vitro. Finally, the infusion of verapamil (0.1 mg/kg, i.v., loading dose, followed by a 0. 15 mg/kg/h continuous infusion starting 12 h before initiation of VAD and continuing until 24 h after completion of the 4-day cycle) resulted in transient, objective remissions in three of six P-gp-positive patients. The two P-gp-negative patients did not respond to VAD plus verapamil, nor did three of six P-gp-positive patients. Measured serum verapamil levels were 133–1342 ng/ml (<3 μM), and side effects included asymptomatic hypotension, primary atrioventricular block, and two cases of atrioventricular dissociation with junctional rhythms. No increase in myelosuppresion was noted with the addition of verapamil.

Thus, this trial suggests that clinical modulation by verapamil of known P-gp-expressing tumors to an anthracycline/*Vinca* alkaloid-based chemotherapeutic regimen may be possible. However, a subsequent preliminary report of a similarly designed trial of VAD plus verapamil at comparable doses in 10 patients with multiple myeloma, refractory to prior treatment with VAD, showed only one response (26 weeks), whereas all other patients continued to progress on this regimen (Trumper et al., 1989). Unfortunately, neither tumor P-gp expression nor plasma verapamil levels were measured, making these negative results difficult to interpret.

In another recently reported trial, trifluoperazine was given with doxorubicin to 36 patients with either acquired (previously responsive to doxorubicin) or intrinsic (never responded to doxorubicin) tumor resistance (Miller et al., 1988). Doxorubicin (60 mg/m^2) was given as a constant infusion with a 20-mg/day oral dose of trifluoperazine that was increased to 100 mg/day. One complete response and six partial responses were seen. All responses were in patients who had acquired, rather than intrinsic, resistance to doxorubicin. Dose-limiting side effects of this regimen were the extrapyramidal effects of trifluoperazine, including motor restlessness, akathesia, facial dystonia, and resting tremor. Trifluoperazine plasma levels ranged widely but were all <0. 3 μM and, thus, at least 10-fold less than those found optimal in culture (table 5).

A problem with all of these studies, assuming that plasma concentrations correlate with the more important tissue concentrations, is that the concentrations of chemosensitizers at which maximum activity is found in vitro are clinically unachievable without significant toxicity. One approach that may circumvent this problem is

the use of combinations of chemosensitizers with additive or synergistic anti-MDR activity but with nonoverlapping toxicities. For example, by performing isobologram analyses (Berenbaum, 1989) in cultured MDR cells, we demonstrated that the calcium channel blocker verapamil and the CaM antagonist *trans*-flupenthixol were additive in their effects in antagonizing MDR (Ford et al., 1990). This suggests that clinical protocols may be designed using several chemosensitizing agents, each at less toxic doses, resulting in equivalent antagonism of P-gp-mediated drug resistance. An additional problem in most of the reported studies is that the mechanism of tumor resistance in patients has not been determined. In fact, only in the trial of Dalton et al. (1989a) with VAD plus verapamil has the expression of P-gp been analyzed. With the recent advent of molecular diagnostic techniques, measurement of P-gp expression and *mdr1* mRNA levels using immunological and genetic probes, it will be possible to better define the potential for anti-MDR chemosensitizer treatment in specific patients. As more specific and potent chemosensitizers with less clinical toxicity and side effects enter clinical trials, it will be critical to carefully plan and coordinate prospective therapeutic trials and diagnostic procedures, by measuring MDR markers before, during, and after treatment and analyzing both tumor and plasma drug concentrations.

The selection of appropriate types of cancer for studying MDR and its clinical modulation will also be critical when testing the potential relevance of this form of drug resistance. Criteria for an "ideal model" include a tumor that (*a*) consistently overexpresses P-gp, (*b*) is either intrinsically resistant to drugs included in the MDR phenotype or acquires resistance to such drugs after initial treatment response, (*c*) must be accessible for sequential biopsies, (*d*) includes a reasonable life expectancy despite relatively early relapse, and (*e*) occurs with relative frequency. Malignancies that fit such criteria particularly well include the leukemias and lymphomas, and myeloma, cutaneous T cell lymphoma, and Kaposi's sarcoma.

3. Clinical pharmacology of chemosensitizers. The clinical pharmacology of individual chemosensitizers, i.e., their absorption, distribution, metabolism, toxicity, and drug interactions, is as important for successful in vivo modulation of clinical drug resistance as is pharmacological anti-MDR activity. The identification of many drugs already in clinical use as active chemosensitizers against MDR is a unique situation within the field of anticancer experimental therapeutics, because the clinical pharmacology of many of these drugs is already known. However, the necessity to achieve much higher plasma and tissue levels than ordinarily used presents considerable problems. Thus, knowledge of the dose-related toxicities and clinical pharmacokinetics for chemosensitizers is crucial to the selection of optimal agents for successful use in humans. Although an extensive literature exists for many of these drugs, we will only briefly review the clinical pharmacology of several chemosensitizers, emphasizing those with apparent clinical potential based on pharmacokinetic properties. These data are summarized in table 7.

The calcium channel blocker verapamil, traditionally used in the treatment of coronary artery disease, arterial hypertension, and supraventricular arrhythmias, has vasodilatory effects on vascular smooth muscle, has negative inotropic effects on myocardial cells, and lengthens atrioventricular conduction time (Fleckenstein, 1977). Verapamil is almost completely absorbed after oral administration and is affected by extensive first-pass hepatic metabolism, with P450-mediated N-dealkylation and O-demethylation accounting for the majority of identified metabolites (Eichelbaum et al., 1979). The bioavailability of verapamil is low (10–35%) because of its high total systemic clearance and its half-life of 3–7 h (Echizen and Eichelbaum, 1986). The majority of metabolites lack cardiovascular activity. Whether they possess chemosensitizing activity is an important, but unknown, question. Studies from our laboratory with tumor cells and mice depleted of GSH show a markedly enhanced toxicity of verapamil, suggesting that the GSH pathway may also function to detoxify certain oxidative metabolites of verapamil (Ford and Hait, 1989). These findings are relevant to the proposed use of the GSH inhibitor buthionine sulfoximine in combination with verapamil in humans with resistant cancer.

Plasma concentrations of verapamil have not been routinely monitored, because the cardiovascular therapeutic effects are easily measurable and titratable. Usual therapeutic plasma concentrations for treatment of angina are 150–500 μg/liter (0.4–1.2 μM) (Anderson et al., 1982). Clinical toxicity begins to occur with concentrations greater than these and are characterized by atrioventricular block and hypotension. As discussed in section IV, C, 3, these have been dose limiting in the trials of verapamil in combination with chemotherapeutic drugs for the clinical modulation of MDR.

The dihydropyridine calcium channel blockers, such as nifedipine and nicardapine, have less negative inotropic effects than verapamil and appear to act mainly on vascular smooth muscle. Similar to verapamil, they have a low bioavailability, with extensive first-pass hepatic metabolism and a half-life of 2–8 h, but they have usual plasma concentrations of only 50–100 μg/liter (0.15–0.3 μM) (Echizen and Eichelbaum, 1986).

Thus, the clinical pharmacology of the calcium channel blockers explains their toxicity when used as chemosensitizers in vivo, because they are extensively metabolized and toxic at relevant anti-MDR doses (section IV, C, 3). Although the several dihydropyridine analogs that lack calcium channel-blocking activity but retain chemosensitizing activity (Nogai et al., 1989) may play a role in

TABLE 7
Pharmacokinetic properties of chemosensitizer drugs

Drug	Usual dose (mg)	Protein bound (%)	Vol_d (liters/kg)	Elimination		Active metabolites	Serum levels		Reference
				$T_{1/2}$	Route		Therapeutic	C_{max}	
Verapamil	5–15 i.v. 80–160 Q8 p.o.	85–90	4–8	3–7 H	Hepatic	No		148 μg/liter	Eichelbaum and Echizen, 1984
Nifedipine	1–4.5 i.v. 10–20 Q6 p.o.		1.4	2–8 h	Hepatic			160 μg/liter	Eichelbaum and Echizen, 1984
Quinidine	800–2400 QD p.o.	50–95	0.5	3–14 h	Renal/hepatic	Yes	1–5 μg/ml		Woosley and Funck-Brentano, 1988
Amiodarone	400–600 QD		5–20	13–103 days	Hepatic	Yes	1–2.5 μg/ml		Somani, 1989 Maling, 1988
Fluphenazine	2–60 QD p.o.	>99	20	16 h	Hepatic			18 μg/liter	Balant-Gorgia and Balant, 1987a
cis-Flupenthixol	1–2 i.v. 4–8 p.o.	>99	14–20	34–36 h	Hepatic		1–4 μg/liter	4 μg/liter	Balant-Gorgia and Balant, 1987a
trans-Flupenthixol		>99			Hepatic			10 μg/liter	Balant-Gorgia and Balant, 1987a
Tamoxifen	40 QD p.o.	>99		7–14 h	Hepatic	Yes	300–400 μg/liter	850 μg/liter	Buckley and Goa, 1989
Toremifene	20–400 QD p.o.	90–95		5 days	Hepatic	Yes	1.4–3.5 μg/ml	6.5 μg/ml	DeGregorio et al., 1989 Wiebe et al., 1990
Erythromycin	500–1000 QID	42–90		1.5 h	Hepatic		300–400 μg/liter	12 μg/ml	Brittain, 1987
Cefoperazone	2 g BID i.v.	90	10–14	1.5–2.0 h	Hepatic/renal		100 μg/ml	500 μg/ml	Craig and Middleton, 1980 Funk and Straus-baugh, 1982
Cyclosporin A	10–100	>80	4–8	6–20 h	Hepatic		150–500 μg/liter	800 μg/liter	McMillan, 1989 Kahan, 1989

the modulation of clinical drug resistance, calcium channel blockers, in general, seem poorly suited for use as chemosensitizers in humans.

Two other antiarryhthmic drugs, quinidine and amiodarone, have entered clinical trials (Fojo et al., 1989). Quinidine, a class IA antiarrhythmic, depresses the fast inward depolarizing sodium current and prolongs repolarization and the refractory period of myocardial tissue. Quinidine has excellent bioavailability after oral administration (70–95%), but its clearance and elimination half-life vary widely, from 3–19 h (Woosley and Funck-Brentano, 1988). Although careful monitoring of plasma concentrations is often used, a number of adverse reactions still occur. Recurrent syncopal episodes and *torsade de pointes*, hypotension, gastrointestinal symptoms, and occasional thrombocytopenia are well-documented side effects (Cohen et al., 1977). Nevertheless, therapeutic doses of quinidine usually result in plasma levels of 1–5 mg/liter (2–10 μM), within the range of its anti-MDR activity.

Amiodarone is a class III antiarrhythmic agent used for life-threatening malignant arrhythmias in patients resistant to treatment with other, less toxic drugs. Because of its extreme lipid solubility, it exhibits an unusual pharmacokinetic profile. The oral absorption of amiodarone ranges from 3–100%, with a mean bioavailability of 30–60% due to interpatient variability in first-pass hepatic metabolism (Pourbaix et al., 1985). The major metabolite, desethylamiodarone, is formed within minutes of amiodarone administration, maintains antiarrhythmic activity (although its chemosensitizing activity is not known), and is found in relatively high concentrations in plasma and all tissues (Somani, 1989). Amiodarone has an extremely long elimination half-life, ranging from 13–120 days, due to extremely complex and poorly understood pharmacokinetics. Thus, even with extensive plasma level sampling, adjusting the maintenance dose of amiodarone with chronic administration has been problematic, and a new steady state may take 2–15 months to achieve (Somani, 1989). Plasma drug concentrations of 1–2.5 mg/liter (1.4–4 μM) are generally considered therapeutic (Maling, 1988). Of particular relevance to its use as a chemosensitizer in solid tumors, amiodarone has a very high volume of distribution (66 liters/kg) and achieves concentrations in tissues of ≤180 times greater than in plasma (Marcus, 1984), with concentrations measured in liver and lung of >100 mg/kg (Holt et al., 1983).

Unfortunately, amiodarone is associated with considerable toxicity, including neuropathy, pulmonary fibrosis, phospholipidosis, and hepatitis (Somani, 1989; Riva et al., 1989; Martin et al., 1989). Therefore, although certain of the unique pharmacokinetic properties of amiodarone make it particularly attractive for use as a chemosensitizer in vivo, its severe toxicities, low safety margin, and difficulty in dosing make it suboptimal for high-dose use in humans.

Extensive clinical experience has been accumulated with the phenothiazine and thioxanthene classes of antipyschotic and chemosensitizing drugs. However, their numerous active metabolites, lipophilicity, lack of correlation between plasma concentrations and clinical efficacy, and analytical problems with both chemical and biological assays have made the study of their clinical pharmacokinetics difficult (Dahl, 1986). In general, the tricyclic neuroleptics are well absorbed from the gastrointestinal tract but undergo extensive first-pass hepatic metabolism. Nevertheless, phenothiazines and thioxanthenes have a half-life of approximately 20 h because of accumulation of the drugs in tissues (Balant-Gorgia and Balant, 1987). Tissue penetration by the parent drug and its metabolites is likely to be high because of their lipophilicity. Of interest, phenothiazine metabolites have been shown to possess significant anti-MDR activity (Ford et al., 1989). Neuroleptics are associated with numerous side effects, most notably neurological. Specifically, drug-induced parkinsonism with akinesia or bradykinesia, resting tremor and rigidity, acute or tardive dyskinesia, akathisia, sedation, seizures, and neuroleptic malignant syndrome are associated with all active antipsychotics and are related to both concentration and total dose of drug (Bishop and Tourney, 1984). Indeed, the use of the active antipsychotic trifluoperazine as a chemosensitizer in humans has been limited by these effects (Miller et al., 1988; Hait et al., 1989a).

Preclinical and clinical data reported for the potent thioxanthene chemosensitizer *trans*-flupenthixol suggest that it may be an excellent candidate for the in vivo clinical modulation of MDR. *trans*-Flupenthixol has been shown to be an inactive antipsychotic and has not been associated with any notable side effects at clinically tested doses (Johnstone et al., 1978). In fact, in mouse studies, *trans*-flupenthixol has been shown to be 100- to 1000-fold less potent than the active *cis*-isomer in a number of standard assays used to measure the side effects and neuroleptic potential of antipsychotic drugs, such as inhibition of spontaneous motor activity, cataleptic reaction, ptosis-inducing effects, and inhibition of apomorphine- and amphetamine-induced stereotypy (Nielson et al., 1962, 1973). In addition, in vitro studies demonstrate that [^{3}H]*trans*-flupenthixol completely lacks specific dopamine receptor binding (Hyttel et al., 1984), in contrast to the highly specific, potent dopamine D1 and D2 receptor binding of [^{3}H]*cis*-flupenthixol (Huff and Molinoff, 1984). Also, *trans*-flupenthixol has little or no α-adrenergic, β-adrenergic, or 5-HT$_1$-blocking activity (Hyttel et al., 1984). Clinical trials of the antipsychotic effects of thioxanthene isomers have shown *trans*-flupenthixol to completely lack dopaminergic activity and extrapyramidal side effects (Johnstone et al., 1978). Sensitive chromatographic and radioimmunoassays have been developed to differentiate tissue and plasma concentrations of *cis*- from *trans*-flupenthixol (Jorgensen, 1978; Po and Irwin, 1979; Rowell et al., 1979). Flupenthixol has been shown to undergo extensive first-pass hepatic metabolism and to have a high systemic clearance due to its high hepatic extraction ratio, with mean systemic availability ranging from 30–70% (Jorgensen, 1980). Nevertheless, it possesses an apparent half-life of elimination of 34–36 h due to a large volume of distribution (20 liters/kg) (Jorgensen et al., 1982). Also, flupenthixol's lipophilicity results in selective partitioning into tissues (suggesting high tumor tissue levels may be possible), making correlations between plasma levels and clinical effects notoriously difficult (Balant-Gorgia and Balant, 1987). Reported peak plasma levels for *cis*-flupenthixol treatment (1 mg, i.v., or 4 mg, p.o.) have been 3–4 μg/liters (7 nM) (Jorgensen and Fredricson, 1980), 1000-fold less than concentrations necessary for in vitro chemosensitizing activity. It is possible that larger doses of *trans*-flupenthixol may be safely administered to humans, because of its apparent lack of clinical activity. In addition, in a recent study in which the steady state levels of *cis*- and *trans*-flupenthixol resulting from 4- to 15-mg daily oral doses of a 1:1 mixture of both drugs were measured, *trans*-flupenthixol levels of $\leq$20 nM were reported, indicating that two-fold greater *trans*-flupenthixol steady state plasma concentrations were achieved in comparison to *cis*-flupenthixol (Balant-Gorgia et al., 1987). These data suggest that it may be possible to achieve sufficient tissue levels of *trans*-flupenthixol capable of producing anti-MDR activity in human tumors. Furthermore, the use of depot preparations of flupenthixol may lead to more optimal anti-MDR pharmacokinetics (Jorgensen, 1980).

The *trans*-triphenylethylene isomer, tamoxifen, acts as an estrogen antagonist and partial agonist (Jordan, 1984) and is an important adjuvant chemotherapy in early breast cancer (Fisher et al., 1989). Additionally, tamoxifen and other triphenylethylene derivatives have chemosensitizing activity in tissue culture (Ramu et al., 1984b; DeGregorio et al., 1989). The pharmacokinetics of tamoxifen have not been fully studied, and it is unclear whether plasma concentrations correlate with clinical efficacy for its primary antitumor effect (Legha, 1988). However, plasma concentrations of 300 μg/liter (0.5 μM) are common and levels $\leq$850 μg/liter (1.5 μM) have been measured (Kemp et al., 1983). Furthermore, tamoxifen has been shown to accumulate in tumor tissues, with concentrations of >18 ng/mg protein found in the cytosol and nuclear fractions of human breast tumor cells (Murphy et al., 1987). Tamoxifen undergoes extensive hepatic metabolism, with its active metabolites possessing both estrogen antagonist and agonist properties (Buckley and Goa, 1989). Tamoxifen has been used extensively with combination chemotherapy in breast cancer and is very well tolerated at usual doses (Legha, 1988), with retinopathy associated with high-dose treatment (Kaiser-Kupfer and Lippman, 1978).

Toremifene is another triphenylethylene that has identical in vitro chemosensitizing activity as tamoxifen (DeGregorio et al., 1989). Unbound plasma toremifene and its metabolites from the serum of patients treated with toremifene possess significant chemosensitizing activity when used against cultured cells. Toremifene has a much longer half-life than tamoxifen (Wiebe et al., 1990), and clinically relevant anti-MDR plasma concentrations of ≤10 μM can be maintained with chronic, high-dose treatment and is associated with few, minor side effects (Hietanen et al., 1987; Kangas, 1987). Thus, the pharmacokinetics of the triphenylethylene antiestrogens suggest they may be well-tolerated, effective chemosensitizers for use in combination with other chemotherapeutic agents in clinical MDR in solid or hematological tumors.

The clinically used antibiotics erythromycin and cefoperazone have recently been shown to possess chemosensitizing activity against cultured cells (section IV, A, 7). Although these drugs are far less potent (concentrations of 0.5–1.0 mM are necessary for anti-MDR activity in vitro) and likely less specific than other chemosensitizers, their relative lack of toxicity at high molar concentrations in vivo suggest their possible clinical use as modulators of drug resistance.

Erythromycin is poorly absorbed orally, and intravenous administration leads to higher drug concentrations. Although its half-life is short (1.5 h), serum concentrations of ≤12 mg/liter (17 μM) may be achieved after a single 1-g i.v. dose (Brittain, 1987). The drug is widely distributed through the body and penetrates most tissues except brain and cerebrospinal fluid. Serious toxicity is rare and side effects are mostly gastrointestinal, although ototoxicity and hepatotoxicity can occur (Brittain, 1987).

Cefoperazone, a third generation semisynthetic cephalosporin, is generally administered at a dose of 2 g, i.v., twice a day but has been given safely at doses of 12 g/day. Peak serum concentrations of 500 mg/liter (1.1 mM) have been achieved after 3-g, i.v., bolus infusions (Craig and Middleton, 1980). Cefoperazone penetrates most body tissues, with levels of 4–8 mg/liter (10–20 μM) measured in tonsils, middle ear, and subcutaneous tissues (Hoffstedt and Walder, 1981; Shimizu, 1980). Adequate antimicrobial drug concentrations have also been found in lung, bone, prostate, and kidney, although poor penetration is observed in cerebrospinal fluid (Funk and Strausbaugh, 1982). At usual doses cefoperazone is safe, with <10% of patients treated experiencing the major adverse effect of diarrhea (Funk and Strausbaugh, 1982). Thus, although cefoperazone is not a potent chemosensitizer, high plasma levels are easily achieved within the range necessary for in vitro chemosensitizing activity. Whether such high levels may also be achieved in tumors is not yet known.

One of the most promising classes of chemosensitizers in cultured cells and in animal models are the cyclosporins (section IV, A, 8). As an immunosuppressive agent, CsA is widely used in organ transplantation (Kahan, 1989). Because of its high degree of lipophilicity, most circulating CsA is associated with lipoproteins, although a variable fraction is found unbound. CsA accumulates in fat, as well as liver, pancreas, kidney, and spleen (McMillan, 1989), and is eliminated by first-pass hepatic metabolism. Unfortunately, the toxicities associated with CsA are numerous and common. Neurological, dermatological, gastrointestinal, hepatic, endocrine, vascular, and hematological effects and toxicities have all been reported (Kahan, 1989). Of most concern is its well-known nephrotoxicity, characterized by progressive and only partially reversible renal failure.

Because of its acute and chronic nephrotoxicity, extensive pharmacokinetic studies and modeling have been performed with CsA. However, analytical difficulties in measuring CsA and its ill-defined array of metabolites by radioimmunoassay and high-performance liquid chromatography have made interpretation of these data difficult. A recently introduced radioimmunoassay utilizing a non-cross-reactive monoclonal antibody may allow more reliable measurement of CsA concentrations (Ball et al., 1988). Furthermore, trough rather than peak plasma CsA levels are generally measured (usual therapeutic range is 150–400 ng/ml), because peak concentrations do not occur at uniform times and do not correlate with the occurrence of toxic complications (Kahan, 1985). Finally, it is thought that serum CsA levels do not accurately reflect tissue levels of the drug (Quesniaux, 1989). Therefore, it is difficult to determine whether relevant anti-MDR concentrations of CsA (1–2 μg/ml) are achievable without significant toxicity, particularly in tissues and solid tumors.

With regard to the use of combination chemosensitizers to modulate clinical drug resistance, CsA significantly interacts with numerous drugs, including verapamil, nifedipine, erythromycin, and the cephalosporins (McMillan, 1989). For example, the calcium channel blockers compete with CsA for cytochrome P450 binding and lead to increased CsA concentrations.

The finding that certain nonimmunosuppressive analogs of cyclosporin possess significant chemosensitizing activity in vitro (section IV, A, 8) is encouraging. Although these alternative cyclosporins may lack the nephrotoxicity associated with CsA, little information is available regarding their pharmacokinetics in vivo or their other possible toxicities.

In summary, the previous study of the pharmacokinetics of many chemosensitizers provides essential data in determining which drugs may hold promise as successful anti-MDR agents in humans. It is apparent, however, that further in vivo investigations are necessary to determine their maximal tolerated doses in the presence of chemotheraputic agents, their penetration and

distribution in tumor tissues, and the chemosensitizing activity of their metabolites.

D. Mechanism of Action of Chemosensitizers in Multidrug Resistance

One goal of studying chemosensitizers is to understand the molecular and biochemical mechanism(s) of MDR. Given the extremely broad range of compounds that have been shown to effectively alter MDR and the numerous mechanisms of resistance present in most MDR cells, it is difficult to envision a single common cellular mechanism or target through which these actions are mediated. On the other hand, it is possible that a variety of interactions with different cellular targets may result in a similar effect on cytotoxic drug potentiation. In the following sections we will review a number of mechanisms that have been suggested to be relevant to the anti-MDR action of chemosensitizing drugs.

1. Membrane alterations. Several investigators have found differences in MDR plasma membranes compared to those of sensitive tumor cells. For instance, Ramu et al. (1983, 1984c) demonstrated altered cellular lipid content and composition in P388/ADR cells, produced by an increased ratio of sphingomyelin to phosphatidylcholine. Tritton's group (Bhushan et al., 1989) also investigated the composition of 10 lipid species in five sublines of sarcoma 180 cells selected for progressively increasing doxorubicin resistance and found a variety of differences between resistant lines. However, only sphingomyelin correlated with resistance.

Tritton and colleagues (Siegfried et al., 1983, Bhushan et al., 1989) have also shown that anthracycline resistance in these same sarcoma 180 cells was associated with increased membrane fluidity by electron spin labeling. Other investigators have also found increased membrane fluidity in doxorubicin-resistant MDAY-K2 cells (Wheeler et al., 1982) and P388/DOX cells (Ramu et al., 1983) using measurements of fluorescence polarization. It has been suggested that these membrane differences may contribute to the alterations in drug accumulation seen in MDR. Because cationic, amphipathic drugs, including most chemosensitizers, are known to interact with polar lipids such as phosphatidylserine (Lullmann and Wehling, 1979), it has been suggested that chemosensitizers may cause perturbations in MDR membrane order or fluidity, resulting in further changes in cellular drug accumulation.

As a model of the possible effect of membrane perturbation on MDR, several investigators showed that the surface active nonionic detergent, Tween 80, caused alterations in drug accumulation and cytotoxicity similar to that of verapamil and that this effect was seen in MDR but not in sensitive P388 cells (Riehm and Biedler, 1972; Klohs et al., 1986). However, using a different MDR P388 cell line, Inaba and and Johnson (1977, 1978) could not reproduce this effect. Ramu et al. (1984a) found that perhexiline maleate, a compound known to raise phospholipid content in fibroblasts (Albouz et al., 1981), also reversed MDR. Although this was cited as evidence favoring a membrane-based mechanism of action, perhexiline maleate is also known to block calcium channels (Fleckenstein, 1977) and may share other pharmacological properties with this class of drug.

The nonspecific membrane effects of phenothiazine CaM antagonists have also received a great deal of attention with respect to their anti-MDR activity, antipsychotic effects, and cytotoxic actions (Roufogalis, 1975; Scott et al., 1988). Indeed, a number of other unrelated chemosensitizers are similar to the phenothiazines in that their high degree of lipophilicity results in a preferential partitioning into membranes (Kanno and Susaki, 1982). However, this property does not preclude their significant interaction with membrane proteins such as P-gp, or other intracellular targets, in a specific, if yet undefined, manner. In fact, we have found that the quaternary amino methiodide salts of chlorpromazine and trifluopromazine do not alter drug resistance in MCF-7 Adr^R cells (J. M. Ford and W. N. Hait, unpublished observations), although they interact with CaM and other phenothiazine receptors in a manner similar to phenothiazines with tertiary amino groups (Albanus et al., 1961; Prozialeck, 1984). Because quaternary amino phenothiazines are not able to cross cellular membranes (Huang et al., 1970), this suggests that the target for antagonism of MDR by phenothiazines is either cytosolic or on the cytosolic side of the plasma membrane.

Thus, it is not known at this time how the structure of MDR membranes or their alteration by chemosensitizers may relate, if at all, to changes in drug accumulation. It may be that certain chemosensitizers act within the membrane to alter the local environment in the vicinity of P-gp, leading to structural modification and inhibition of drug transport. Alternatively, overexpression of the membrane protein P-gp may directly cause the alterations in membrane properties noted in MDR cells, and these changes may be unrelated mechanistically to drug accumulation and toxicity.

2. Alteration in calcium homeostasis. The initial discovery that verapamil and other calcium channel blockers could antagonize MDR (Tsuruo et al., 1981, 1982, 1983b) prompted the suggestion that calcium may play a role in the development or maintenance of MDR, perhaps through a calcium-dependent mechanism of drug transport, and that chemosensitizers may function by altering calcium fluxes. However, mounting evidence indicates that altered calcium physiology does not contribute to MDR. For example, several investigators have shown that P388-sensitive and MDR cells have similar levels of total intracellular Ca^{++} content (Kessel and Wilberding, 1985a; Nair et al., 1986). Furthermore, a number of groups have demonstrated conclusively that, unlike excitable tissues, neither MDR cells nor their sensitive counterparts possess voltage-dependent cal-

cium channels, because calcium uptake was not altered by membrane voltage potentials or calcium channel blockers in MDR or P388-sensitive cells, Chinese hamster ovary cells, or C6 glioblastoma cells (Ramu et al., 1984d; Kessel and Wilberding, 1985a; Cano-Gauci and Riordan, 1987; Huet and Robert, 1988). Similarly, whole-cell and single-channel patch-clamp techniques demonstrated that both drug-sensitive CCRF-CEM and MDR CEM/VLB_{100} cells lack voltage-gated calcium channels (Lee et al., 1988).

Additional evidence against calcium fluxes affecting MDR was also provided by two studies in which pharmacological manipulation of calcium concentrations mimicked the effect of calcium channel blockers on excitable tissues. For example, treatment of MDR C6 cells with manganese, which produced a noncompetitive reduction in calcium uptake, or the calcium ionophore A23187, which increased intracellular calcium, had no influence on doxorubicin accumulation or cytotoxicity (Huet and Robert, 1988). Similar results were found by alteration in net intracellular calcium by reduction of extracellular calcium concentrations or chelation of calcium ions by ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (Ramu et al., 1984a). In all of these states of altered calcium homeostasis, the anti-MDR activity of verapamil was not changed. Thus, it appears that alterations in calcium flux do not alter drug resistance in MDR or sensitive cells and that calcium channels are not the primary target of calcium channel blockers in this system.

3. Inhibition of calmodulin-mediated processes. The finding that phenothiazines and other CaM antagonists modulate MDR implicated CaM in the regulation or reversal of MDR. However, the actual role of CaM in MDR is unknown. CaM regulates a wide variety of critical cellular functions (Manalan and Klee, 1984; Hait and Lazo, 1986; Hait and DeRosa, 1988). Particularly relevant to MDR is CaM's ability to activate plasma membrane Ca^{++}-ATPase (Gopinath and Vincenzi, 1977; Niggli et al., 1981) and to stimulate protein kinases and phosphatases (Schulman and Greengard, 1978; Schulman et al., 1980). All of these processes are inhibited by CaM antagonists (Raess and Vincenzi, 1980; Weiss et al., 1982). Whether CaM-mediated phosphorylation may activate or regulate the function of P-gp in MDR is a provocative but unanswered question.

CaM concentrations in sensitive and MDR cells are the same (Nair et al., 1986, Beck, 1984). Whether the primary structure or function of CaM in MDR cells is in any way altered is not known, although we have found no changes in its sensitivity to drugs (Hait and Pierson, 1990).

Several investigators have attempted to correlate the potency of CaM antagonists as inhibitors of CaM-mediated processes with their chemosensitizing activity in MDR cells, with conflicting results. For example, Ganapathi et al. (1984b) studied three phenothiazines, trifluoperazine, prochlorperazine, and chlorpromazine, and the naphthalene-sulfonamides W-12 and W-13 in P388/DOX cells and found that their activity was in general agreement with their relative anti-CaM potency. W-12 and W-13, drugs with similar hydrophobicity and structure (Hidaka and Tanaka, 1983), have different effects on MDR (table 3), implying that nonspecific hydrophobicity alone is not sufficient to alter the MDR process. However, both Tsuruo et al. (1982) and Akiyama et al. (1986) reported that the chemosensitizing activity of trifluoperazine, thioridazine, and chlorpromazine in P388/DOX and KB-ChR-24 cells, respectively, did not correlate with known IC_{50} values for the inhibition of CaM. Furthermore, Akiyama found that the CaM antagonists W7 and N-(6-aminohexyl)-1-chloronaphthalensulfonamide (W5) were equally poor chemosensitizers, although W7 was a significantly more potent CaM antagonist (Hidaka and Tanaka, 1983). However, the sample size in each of these studies was too small to make definite conclusions regarding the role of CaM antagonism in the modulation of MDR.

In a larger study of the effect of CaM antagonists on cellular proliferation and MDR (Ford et al., 1989), we determined the IC_{50} concentrations necessary for inhibition of CaM-dependent phosphodiesterase activation and inhibition of MCF-7-sensitive and MDR human breast cancer cell growth for 28 phenothiazines and structurally related drugs and their degree of chemosensitizing activity in MCF-7 Adr^R cells. A good correlation was found between the potency of these drugs as inhibitors of CaM and as inhibitors of cell growth, supporting and extending previous observations with a limited number of phenothiazines in MDA-MB-231 human breast cancer cells (Wei et al., 1983), C6 astrocytoma cells (Lee and Hait, 1985), HL-60 human leukemia cells, L1210 murine leukemia cells, and HCT-8 human colonic carcinoma cells (Hait et al., 1985). These data are consistent with the known role of CaM in cellular proliferation (Hait and Lazo, 1986; Rasmussen and Means, 1987) and suggest a role for CaM as the target involved in cell growth inhibition by phenothiazines.

However, a complete lack of correlation was found between anti-CaM activity and antagonism of MDR by equimolar concentrations of the 28 phenothiazines, suggesting an alternative mechanism for this effect (Ford et al., 1989). Phenothiazines are generally considered to be nonspecific probes for CaM function in whole cells because of their activity as inhibitors of PKC (Schatzman et al., 1981) and antagonists of dopamine receptors (Creese and Sibley, 1980). We have studied the chemosensitizing effect of several compounds with relatively increased specificity for CaM. Iodo- and cyano-derivatives of the naphthalene sulfonamide W7 possess increased anti-CaM activity associated with less PKC inhibitory activity (MacNeil et al., 1988) and caused little

or no potentiation of doxorubicin toxicity in MCF-7 Adr^R cells (J. M. Ford and W. N. Hait, unpublished observations). Similarly, the CaM antagonist CGS 9343B, which is more than 100-fold less potent an inhibitor of PKC than CaM (Norman et al., 1987), caused only a two-fold potentiation of doxorubicin toxicity in MDR cells (J. M. Ford and W. N. Hait, unpublished observations). Thus, it appears unlikely that the pharmacological reversal of P-gp-associated MDR is mediated through the interaction of drugs with CaM.

4. Inhibition of protein kinase C. Indirect evidence also suggests a possible regulatory role for the phospholipid/Ca^{++}-dependent PKC in MDR. For example, Palayoor and Hait postulated that chronic antagonism of PKC by natural product chemotherapeutic agents would lead to up-regulation of the enzyme, analogous to the physiology of adrenergic receptor modulation. Upon withdrawal of the selecting agent, the increased activity of uninhibited PKC might then result in enhanced outward drug transport (Palayoor et al., 1987), because the enhancement of cellular secretion is known to be a major role of PKC in many tissues (Zawalich et al., 1983; Delbeke et al., 1984; Katakami et al., 1984). This hypothesis was based on the work of Wise et al. (1982) who found that anthracyclines were effective inhibitors of PKC and that of Fine and co-workers (1988) who demonstrated the increased activity of PKC in MCF-7 MDR cells. In fact, there was support for this idea. For example, we found that both anthracyclines and *Vinca* alkaloids were competitive inhibitors of the activation of PKC from P388 MDR cells by phosphatidylserine and that doxorubicin displaced phorbol esters from the phorbol ester receptor of these cells (Palayoor and Hait, 1987). In addition, support for a role of PKC in drug resistance comes from several studies demonstrating at least transient increases in cellular drug resistance as well as changes in drug accumulation when cells are exposed to activators of PKC such as the phorbol ester, TPA. Specifically, treatment of sensitive human KB cells with TPA caused a two-fold protection against the cytotoxicity of VP-16, vincristine, and mitoxantrone, which could be circumvented by coadministration of verapamil (Ferguson and Cheng, 1987). Similarly, phorbol esters increased resistance to doxorubicin and vincristine in both sensitive MCF-7 and MDR MCF-7 Adr^R cells by 4.0- and 1.5-fold, respectively, and decreased [^{3}H]vincristine accumulation by 40 and 10%, respectively (Fine et al., 1988). Both of these TPA-induced changes were reversible with trifluoperazine. Phorbol ester also increased the resistance of P388, although not P388/DOX cells to daunomycin (Kessel, 1988), and reduced [^{3}H]vinblastine accumulation in MDR KB-Vl cells induced by verapamil (Chambers et al., 1990).

Although a role for PKC in MDR is tempting, the preliminary reports are inconclusive. For example, we studied the activity of PKC in P388, HL-60, DC-3F, and MCF-7 cell lines and their MDR clones and found that the activity of PKC was significantly increased in only the MCF-7 MDR line (Palayoor and Hait, 1987). Others have found increased activity in selected MDR lines (Fine et al., 1988; Posada et al., 1989). Also, preliminary evidence suggests that the pattern of expression of PKC isoforms may be altered in MDR cells (Aftab and Hait, 1988; Aquino et al., 1988b).

Results of additional studies have also been conflicting. For example, Tritton and coworkers (Posada et al., 1989) found that a 30-min exposure of sarcoma 180 doxorubicin-resistant cells to TPA induced an increase in PKC activity which was associated with an *increase* in doxorubicin cytotoxicity, whereas chronic exposure of cells to TPA for 24 h resulted in a down-regulation of PKC and a decrease in doxorubicin toxicity. On the other hand, we were unable to show significant changes in accumulation of doxorubicin in HL-60 cells with low, normal, or overexpressed activity of PKC (Hait and DeRosa, 1990). Finally, other drugs that induce the MDR phenotype such as actinomycin D and colchicine had no effect on the activity of PKC (Palayoor and Hait, 1987). Therefore, it is presently unclear whether the chronic antagonism of PKC by chemotherapeutic agents results in regulation of MDR. By identifying specific inhibitors of PKC and PKC isoforms, and developing cell lines highly resistant to these inhibitors, we may be possible to test this hypothesis more precisely.

A number of chemosensitizers, such as the phenothiazines, thioxanthenes, verapamil, tamoxifen, and possibly CsA inhibit the activity of PKC (Mori et al., 1980; Schatzman et al., 1981; O'Brian et al., 1985; Walker et al., 1989), further implying a potential role for PKC in the regulation of MDR. However, we found that 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, a relatively specific, competitive inhibitor of PKC (Hidaka et al., 1984; Ohta et al., 1988), had no chemosensitizing effect on MCF-7 Adr^R cells but rather enhanced the resistance of cells to doxorubicin (J. M. Ford and W. N. Hait, unpublished observations), in agreement with other results reported by Ganapathi and Grabowski (1988) in MDR L1210 cells. Ido et al. (1986) found that 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine inhibited a TPA-induced decrease in vincristine accumulation in sensitive P388 cells.

It has been reported that phorbol esters increase P-gp phosphorylation (Hamada et al., 1987), raising the possibility that PKC modulates the function of P-gp. Verapamil and trifluoperazine also cause hyperphosphorylation of P-gp, although at different serine residues (Center, 1985). Whether the effect of chemosensitizers on the phosphorylation of P-gp is mediated by PKC is unclear. Meyers (1989) studied the phosphorylation of soluble and membrane-bound proteins in two MDR cell lines, DC-38/VCR_d-5L and DC-3F/ADX. Phosphorylation of P-gp was increased by Ca^{++}, enhanced somewhat by

CaM, but was not affected by the combination of Ca^{++}, phosphatidylserine, and diacylglycerol. These data suggest that the phosphorylation of P-gp is calcium dependent, but the calcium-sensitive kinase remains unknown.

The activation of PKC by phorbol esters in MDR and other cell types results in the phosphorylation of proteins, in addition to P-gp, possibly associated with MDR (Aquino et al., 1988a; Fine et al., 1988). For example, Fine et al. (1988) noted an increased phosphorylation of a 20-kDa protein in MCF-7 and MCF-7 Adr^R cells treated with phorbol ester. This was inhibited by trifluoperazine and associated with changes in drug resistance to doxorubicin or vincristine. Tissue from several patients with breast cancer and small cell lung cancer clinically resistant to vinblastine and doxorubicin had increased contents of the 20-kDa phosphoprotein, whereas untreated patients showed little or no phosphorylation of this protein (Fine et al., 1989).

In summary, within the calcium messenger system, PKC is a target for many chemosensitizers and chemotherapeutic agents involved in MDR. In certain MDR cell lines, the activity of PKC and specific PKC isoforms is altered. Because P-gp is phosphorylated, and its phosphorylation level is altered by several chemosensitizers, it is attractive to speculate, but premature to conclude, that PKC has a regulatory or modulatory role in P-gp-associated MDR.

5. Interaction with P-glycoprotein. The most compelling evidence suggests that chemosensitizers act by directly affecting the function of P-gp. For example, Cornwell et al. (1986a) found that [^{3}H]vinblastine accumulated within P-gp-enriched membrane vesicles from KB-C4 MDR cells in a specific, saturable, temperature-dependent manner but did not accumulate in vesicles from sensitive KB-3–1 or revertant KB-R1 cells. Accumulation was inhibited by excess unlabeled vinblastine, vincristine, and verapamil, less so by daunomycin, and poorly by colchicine (Cornwell et al., 1986a).

Safa and others used photoactive analogs of vinblastine ([^{3}H]NABV and ^{125}I-NASV), which irreversibly bind to MDR cell membrane fractions (Cornwell et al., 1986b), to identify vinblastine-binding proteins (Safa et al., 1986). A 150- to 180-kDa photoaffinity labeled membrane protein was found specifically in MDR cell lines and was immunoprecipitated with a polyclonal antibody known to cross-react with P-gp. In addition, verapamil inhibited ^{125}I-NASV photoaffinity labeling of a 170-kDa membrane protein from KB-V1 cells which was immunoprecipitated with a monoclonal antibody to P-gp (Cornwell et al., 1987). Additional studies of the binding of chemotherapeutic agents to P-gp and the competitive inhibition of this binding by chemosensitizers have demonstrated that [^{3}H]vincristine binds to MDR K562/ADM and Ehrlich ascites EHR2/DNR+ membrane preparations in a specific, temperature-, pH-, and ATP/Mg^{++}-dependent manner, with a K_d of 0.24 μM (Naito et al., 1988; Sehested et al., 1989). The chemosensitizers verapamil, nicardapine, quinidine, CsA, progesterone, and to a far lesser extent trifluoperazine competitively inhibit this high-affinity [^{3}H]vincristine binding to K562/ADM membrane preparations (Naito et al., 1988, 1989; Naito and Tsuruo, 1989). ^{125}I-NASV photoaffinity labeling of KB-V1 and CEM/VLB_{1K} P-gp was inhibited by 10–12.5 μM reserpine, cepharanthine, quinidine, the isoprenoid SDB-ethylenediamine, and synthetic dihydropyridine chemosensitizers but was poorly inhibited by $\leq$100 μM concentrations of the phenothiazines, trifluoperazine, thioridazine, and chlorpromazine (Akiyama et al., 1988; Beck et al., 1988; Nogai et al., 1989). ^{3}H- and ^{125}I-labeled photoactive colchicine analogs bind P-gp from DC-3F/VCRd-5L cell membranes in a specific manner, which was inhibited by *Vinca* alkaloids, doxorubicin, and actinomycin D (Safa et al., 1989). ^{125}I-labeled daunomycin (iodomycin) photoaffinity labeled P-gp from membrane vesicles prepared from MDR CHO B30 cell membrane vesicles in a specific manner, which was inhibited by vinblastine, nortrendipine, and verapamil (Busche et al., 1989), although others have failed to demonstrate photoaffinity labeling of P-gp from EHR2/DNR plasma membrane vesicles by a radiolabeled doxorubicin derivative (Sehested et al., 1989). These results suggest that *Vinca* alkaloids, colchicine, and perhaps anthracyclines bind to P-gp and that certain chemosensitizers compete for either a common drug-binding site, for overlapping sites, or for sites that cause allosteric changes resulting in inhibition of the binding of other drugs.

Certain chemosensitizers have been shown to bind directly to membranes enriched for P-gp, and this binding was inhibited by other chemosensitizers and by chemotherapeutic drugs. For example, KB-V1 MDR vesicles bind 15- to 20-fold more verapamil and diltiazem than sensitive KB-3–1 vesicles in a specific and saturable manner (Cornwell et al., 1987). Experiments with photoactive, radiolabeled verapamil analogs, such as [^{3}H] azidopine and ^{125}I-NASAV, demonstrate irreversible binding to P-gp, which is effectively inhibited by nifedipine, nicardapine, verapamil, progesterone, *cis*- and *trans*-flupenthixol, and vinblastine, but poorly inhibited by trifluoperazine, chlorpromazine, fluphenazine, doxorubicin, and colchicine (Safa et al., 1987; Safa, 1988a,b; Yang et al., 1988; Yang et al., 1989; Ford et al., 1990).

Vesicles prepared from MDR cell membranes inwardly transport [^{3}H]vinblastine in an ATP-dependent fashion (Horio et al., 1988), and epithelial cells grown on porous filters express P-gp in a polarized manner and transport anthracyclines and *Vinca* alkaloids in an energy-dependent, saturable manner (Horio et al., 1989; Kamimoto et al., 1989). Using these simplified models of MDR, investigators have shown that vinblastine transport is competitively inhibited by vinblastine, vincristine, actino-

mycin D, verapamil, quinidine, and less effectively by daunomycin and colchicine.

Several studies have shown that chemosensitizers may be substrates for the P-gp multidrug transporter, supporting their possible mechanism as competitive ligands for sites on P-gp. For example, Cano-Gauci and Riordan (1987) found that MDR Chinese hamster ovary cells accumulated less [^{3}H]verapamil than their sensitive counterparts. Similarly, Tsuruo's group showed a threefold decrease in the accumulation of [^{3}H]verapamil in MDR K562/ADM cells as compared with sensitive cells, along with increased [^{3}H]verapamil accumulation when the MDR cells were treated with 5 μM nicardipine or vincristine (Yusa and Tsuruo, 1989). We have found that MCF-7 Adr^R cells accumulate two- to four-fold less *trans*-flupenthixol than sensitive MCF-7 cells after a 3-h incubation with 3–100 μM drug (J. M. Ford and W. N. Hait, unpublished observations).

It appears that certain chemosensitizers may be transported by P-gp in a similar manner to cytotoxic drugs, thus functioning as competitive agonists. However, not all chemosensitizers are handled in this manner. For example, trifluoperazine accumulated identically in sensitive and resistant P388 cells (Hait and Pierson, 1990).

An independent line of evidence that chemosensitizers may interact with P-gp came from the recent purification and partial characterization of P-gp by Hamada and Tsuruo (1988a,b). P-gp was purified from human K562/ADM cells by immunoaffinity chromatography and found to possess ATPase activity (Hamada and Tsuruo, 1988a). Whereas vincristine and doxorubicin did not affect the ATPase activity of immobilized P-gp, the chemosensitizers trifluoperazine and verapamil caused increased ATPase activity (Hamada and Tsuruo, 1988b). This was surprising because phenothiazines inhibit the activity of other ATPases (Pang and Briggs, 1976; Raess and Vincenzi, 1980). Therefore, further studies of the ATPase activity of P-gp should focus on its possible role as a target for chemosensitizers.

It appears that there may be several sites on P-gp through which chemosensitizers interfere with the MDR process. Certain chemotherapeutic drugs encompassed by the MDR phenotype, such as doxorubicin, daunomycin, and particularly colchicine, may not compete for the same binding sites on P-gp as the calcium channel blockers and *Vinca* alkaloids. There may be other drug-binding sites that lead to active transport, or a family of drug-binding proteins may exist, which display different affinities for various classes of compounds. Chemosensitizers such as the phenothiazines may specifically bind to these additional sites and antagonize MDR, although this cannot explain why such agents most often affect the accumulation of *Vinca* alkaloids, anthracyclines, and colchicine in a similar manner. Alternatively, phenothiazines and other chemosensitizers may inhibit P-gp activity by interacting with physically separate sites on P-gp, such as the ATPase or phosphorylation domains, or act by altering the local membrane environment leading to structural or functional changes in P-gp, or finally act by changing the activity of enzymes that alter the function of P-gp.

6. Inhibition of lysosomal function. Akiyama and coworkers (1984, 1985) demonstrated that verapamil affected the function of lysosomes by inhibiting their degradation of cellular proteins. This led to the discovery that certain other "lysosomotropic agents," such as propranolol (Tsuruo et al., 1984) and chloroquine (Shiraishi et al., 1986; Zamora and Beck, 1986), could also partially reverse MDR. Furthermore, cytotoxic concentrations of vinblastine or doxorubicin increased the number of lysosome-like vacuoles in cells (Zamora and Beck, 1986). These observations led to an additional hypothesis for the cellular mechanism of MDR (Beck, 1987, Sehested et al. 1987). According to this formulation, lysosomes or other intracellular acidic compartments in resistant cells would protonate and thereby entrap weakly basic drugs such as doxorubicin or vinblastine, which are then extruded from the cells by vesicular fusion with the plasma membrane. In this scheme, P-gp serves not as a drug transporter but rather to alter or direct membrane turnover or vesicular trafficking (Beck, 1987). Because verapamil and chloroquine disrupt lysosomal functions, they may interfere with the ability of vesicles to transport drug out of cells, therefore increasing the concentrations of drug reaching cytotoxic targets. In support of this hypothesis, cytochalasin B, a drug that disrupts microfilaments, also inhibited outward drug transport and increased cytotoxicity in both sensitive and MDR cells (Tsuruo and Iida, 1986; Klohs and Steinkampf, 1988). However, Beck's group (Zamora et al., 1988) reported that a number of additional lysosomotropic amines, such as methylamine, suramin, trypan blue, and epinephrine, did not potentiate the cytotoxicity of vincristine in MDR CEM/VLB$_{100}$ cells. They concluded that the ability to impair lysosomal function alone was not sufficient to alter MDR. It remains tenable that lysosomes function as an alternative, or additional, mechanism for drug detoxification in both sensitive and MDR cells.

E. Future Directions

A tremendous amount has been learned about MDR at the molecular, biochemical, and cellular levels, but little has yet been translated into clinical therapeutic gains. However, much of the data now emerging from studies of the clinical expression of P-gp and its relevance to drug resistance in humans suggests that there will be a role for chemosensitizers in cancer chemotherapy. Such "resistance modification" strategies may be effective for tumors with intrinsic or acquired drug resistance. However, even if P-gp-associated MDR proves to be a relevant and reversible cause of clinical drug resistance, numerous problems remain to be solved before effective clinical chemosensitization may be achieved. Such fac-

tors as absorption, distribution, metabolism and toxicity, the effect of chemosensitizers on chemotherapeutic drug toxicity to normal tissues expressing P-gp, and the most effective regimens all remain to be studied in vivo. Clearly, the identification of more specific, potent, and less toxic chemosensitizers for clinical use remains critical to the possible success of this approach.

Clinical drug resistance may be so complex, however, that the likelihood of a drug that affects only one mechanism of resistance would have an effect on the clinical course of disease would be small. One must also recall that, although renal cell carcinoma and colonic carcinoma express P-gp, these tumors are resistant to virtually all classes of chemotherapeutic agents, including those known not to be affected by MDR. Indeed, the concept of multiple mechanisms of drug resistance, within the same tumor cells, is one that is finding experimental support. Several examples of this emerging principle have now been demonstrated in cultured cells, such as a Chinese hamster lung cell line with independent, dissociable alterations in topoisomerase II activity and P-gp expression (Larsen and Jacquemin-Sablon, 1989) and a series of L1210 cells selected for increasing resistance to doxorubicin that display progressively increasing P-gp expression and decreasing topoisomerase II activity (Ganapathi et al., 1989). Future chemotherapeutic regimens, therefore, may need to include combination chemosensitizers or the rational use of drugs to circumvent the likely cause of resistance.

This approach may not be simple. For example, Kramer (1988) reported that the combination of buthionine sulfoximine, an inhibitor of GSH production, with the P-gp chemosensitizer verapamil could completely sensitize the MCF-7 Adr^R cell line to doxorubicin, which contains alterations in GSTπ activity and an overexpressed and amplified *mdr1* gene (Batist et al., 1986; Fairchild et al., 1987), whereas each agent individually could not. We found that the combination of buthionine sulfoximine with verapamil alone, i.e., in the absence of doxorubicin, was extremely toxic to tumor cells, because of a specific enhancement of the toxicity of verapamil by buthionine sulfoximine (Ford and Hait, 1989). Furthermore, otherwise nontoxic doses of verapamil were found to be lethal to animals receiving standard doses of buthionine sulfoximine in their drinking water. These findings emphasize the complexity involved in altering natural detoxification mechanisms utilized by both normal and neoplastic tissues. Clearly, the simultaneous clinical modulation of multiple mechanisms of drug resistance will present significant challenges.

In conclusion, the finding that a number of pharmacological agents can antagonize a well-characterized form of experimental drug resistance provides promise for potential clinical applications. The further study of chemosensitizers in animals and in man and the rational search or design of novel chemosensitizers with improved activity should define the importance of MDR to clinically resistant cancer.

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