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COMMENTARY

THE CELL BIOLOGY OF MULTIPLE DRUG RESISTANCE

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A major problem in the treatment of cancer is the development of resistance to chemotherapy and subsequent relapse. Ultimately, patients may fail to respond to any therapy and exhibit clinical resistance to many drugs. Despite the appearance of a form of *multiple drug resistance* (MDR) in these patients, little is known about its biochemical expressions. In contrast to the clinical situation, broad cross-resistance to "natural product" anticancer drugs, such as Vinca alkaloids and anthracyclines, by tumor cells in culture is a well-documented and well-studied observation. *This* MDR is associated with specific alterations in tumor cell membranes and tumor cell pharmacology, namely, increases in a high molecular weight cell surface glycoprotein and decreases in the ability of cells to accumulate and retain drug respectively. Although overexpression of the MDR-associated "marker" glycoprotein has been demonstrated in some specimens of tumor cells taken directly from patients [1], the relationship and function of the biochemical and pharmacologic features to experimental and, especially, clinical MDR are presently unknown.

Since the general features of MDR have been detailed in recent reviews [2–4], they will be considered here only with regard to the aims of this commentary. My goals are to discuss the cell biology of MDR in the context of the transport of natural product drugs by cells; to re-examine the altered pharmacology that is characteristic of MDR in light of the various membrane changes reported in MDR cells; to point out what I perceive to be controversies and contradictions in the field; to offer alternative explanations for diminished drug retention seen in MDR; and to suggest future research directions that might clarify some of the unresolved questions.

MEMBRANE ALTERATIONS IN MDR

Increased amounts of a high molecular-weight (≈170–190 kDa) surface glycoprotein(s), variously termed P-glycoprotein (P-gp) [5], gp180 [6], or gp150–180 [7], are the hallmark of MDR in cultured cell lines. Other biochemical changes have also been reported, but whether they are directly related to MDR is not clear. For example, decreases in a lower molecular-weight (≈70–100 kDa) glycoprotein(s) have been observed in several MDR cell lines [8–10], and alterations in glycosyltransferase and glycosidase activities [11, 12], as well as ganglioside simplification (increase in hematoside, G_{m3}) [9, 13], have been seen in certain drug-resistant rodent cell lines. A recent study from Biedler's laboratory [7] suggested that MDR Chinese hamster lung cells overexpress EGF receptors on their surfaces. We could find no overexpression of several other cell surface antigens on our MDR cells, including OKT1, OKT3, and the transferrin receptor OKT9 [14], indicating that some of the reported membrane changes probably do not cause MDR but rather may be consequences of the primary alteration, i.e. increased amounts of gp180 in the plasma membrane. Finally, various changes in membrane lipid fluidity have been described in MDR cells [15–18], but no consistent pattern has emerged regarding the structural order, fluidity, or even the composition of such MDR membrane lipids. While this inconsistency may reflect, in part, the different methods used for these measurements, it may also reflect the cell type, the degree of resistance of the cells, or the amount of P-gp associated with the membrane.

Recent studies of the molecular biology of MDR have provided insights into the likely basis for these apparently ancillary membrane alterations in MDR cells. The primary structure of the *mdr*1 gene product (P-gp) has been resolved recently by three laboratory groups [19–21]. The protein has a significant number of hydrophobic domains and spans the plasma membrane at least twelve times. Thus, it is likely, as suggested by Riordan and Ling [2], that insertion of large quantities of this membrane-spanning protein could have profound effects on the packing and structural order of lipids in the plasma membrane. In this regard, biochemical studies have shown that changes in membrane lipid composition, which would also alter membrane fluidity, may play a key role in the passage of drugs across the plasma membranes of MDR cells. In support of this notion, Guffy *et al.* [22] demonstrated that doxorubicin cytotoxicity in L1210 cells could be altered significantly, presumably as a consequence of altered drug transport, by supplementing the cells with docosahexaeinoic acid (22:6), which was shown to be incorporated into membranes. Thus, while many different types of membrane changes have been described in MDR cells, the only consistent finding is the increased quantity of P-gp in the plasma membrane; the very presence of this protein may explain some of the other reported membrane changes.

CELLULAR PHARMACOLOGY OF MDR

The pharmacological determinants of MDR, which have been characterized in experimental tumor systems for various natural product compounds, are somewhat better defined than the biochemical determinants, but they are by no means clear. Decreased drug cytotoxicity in MDR most likely stems from a decrease in net drug accumulation, ascribed to either decreased uptake [23] or reduced drug retention [24, 25]. Several laboratories have attributed this decreased retention to the action of an "active efflux pump" of "broad specificity" [25–27], but others have suggested that altered binding to cellular protein(s) or organelles may be a primary mechanism for this effect [28, 29].

Transport of natural product drugs by tumor cells. I reviewed the transport of natural product compounds such as the Vinca alkaloids and anthracyclines by mammalian tumor cells several years ago [30], but too little was known then about the systems to permit any conclusions. There are still surprisingly few studies on the mechanism of natural product entry into either drug-sensitive or drug-resistant mammalian tumor cells. Results from early experiments suggested that vincristine (VCR) accumulates in Ehrlich ascites cells by an active transport mechanism [31], but subsequent studies have not confirmed these findings. For example, Dalmark and coworkers [32, 33] demonstrated that anthracyclines accumulate in erythrocytes and Ehrlich cells by simple diffusion. By contrast, a recent study by Sirotnak *et al.* [34] suggested that Vinca alkaloids and anthracyclines may cross plasma membranes by a carrier-mediated process, with the putative carrier having a very low affinity for these structurally diverse classes of drugs. In that study, however, it was difficult to distinguish carrier-mediated transport from passive diffusion and weak drug-binding to some cellular proteins or organelles. Finally, the initial uptake of anthracyclines [25] or Vinca alkaloids [28] by MDR cells was shown to be decreased, possibly because of alterations in membrane lipid fluidity, structure or composition, as suggested above. Thus, the mechanism(s) by which natural product anticancer drugs cross cell membranes in drug-sensitive or MDR tumor cells has not been resolved. Any experiments designed to distinguish between mediated transport or passive diffusion should be able to demonstrate how a carrier (or carriers) can recognize such vastly different drug structures. Also, it would be important to know the natural substrate(s) for such carriers, if any exist.

Decreased drug retention. If the mechanisms of drug *accumulation* are poorly understood, the situation is even more complex for drug *retention.* Although drug uptake may be reduced in MDR, it appears that the diminished steady-state drug levels are due primarily to decreased drug retention. This conclusion comes from two types of experiments, originally done by Danø [24], and subsequently repeated in many laboratories. When resistant cells were depleted of ATP energy, either by removing glucose from the incubation buffer or by adding a metabolic inhibitor (azide, cyanide, or iodoacetate), the steady-state level of drug increased by comparison with that in metabolically intact cells. Conversely, when glucose was added back to the poisoned cells, the cell-associated drug rapidly decreased to a lower (original) steady-state level. In drug-sensitive cells, this glucose-mediated drug loss usually is absent or much smaller [24–27]. Such results have been used by many investigators to argue for the existence of an "active efflux pump" of "broad specificity" [24–27].

By contrast, experiments in our laboratory showed that the addition of glucose to azide-treated, drug-*sensitive* cells led to a *higher* steady-state drug level [28]. Initial drug release from preloaded MDR cells was independent of the metabolic state of the cell [28]. Finally, preincubation of azide-treated MDR cells with 100-fold molar excesses of "cross-resistant" drugs such as doxorubicin (DOX), teniposide (VM-26), or colchicine (CLC) did not prevent the subsequent accumulation of labeled vinblastine (VLB) or its later release from cells upon subsequent addition of glucose [28]. These experiments led us to conclude that the decreased drug retention in MDR cells was due to diminished drug binding by certain proteins [28] or cellular organelles [35].

EFFLUX PUMP VERSUS ALTERED DRUG BINDING

Whether the decreased drug retention of MDR cells is due to an active efflux pump or to an altered drug binding is an unresolved issue that merits close examination. Any conclusion that the so-called pump *actively* extrudes drug from the cell seems premature, in that there have been no studies unequivocally demonstrating drug loss from MDR cells against a concentration or electrochemical gradient. Early attempts to test this hypothesis [36] were complicated by the method used to establish the concentration of intracellular free (osmotically active) drug (see Ref. 30, pp. 590–3, for a detailed discussion of this point).

Support for an efflux pump concept would be strengthened if the following questions were addressed: Can a pump protein(s) be purified from MDR cells and be reconstituted into transport-competent vesicles? How might a "pump" protein recognize drug molecules of widely different structures? Do these molecules have common structural features? Is there one "promiscuous" pump of "broad specificity", or are there a series of pumps, each recognizing primarily one class of drugs with reasonably high affinity, with perhaps lower affinity for drugs of different classes? Is there a natural substrate(s) for such a pump or is it part of a general detoxification mechanism to protect a cell from chemical insult?

Likewise, experiments to support an altered binding mechanism as a basis of MDR should be able to locate the drug binding site(s), explain their "broad specificity" with respect to ligands, and address the fact that more drug appears to be bound by energy (ATP)-depleted resistant cells than by metabolically intact cells [2, 28].

Recent insights into these apparently distinct mechanism have come from experiments by Pastan and coworkers [37, 38], who studied the binding of

[^{3}H]VLB to plasma membrane vesicles of both drug-resistant and drug-sensitive cells. The binding of VLB to the vesicles from resistant cells was *increased* compared with that of membranes from sensitive cells, and it was both temperature-dependent and saturable. Of considerable interest was the ability of unlabeled VLB, VCR and, to a lesser extent, daunorubicin (DNR) to compete with [^{3}H]VLB for its binding to the plasma membrane vesicles of MDR cells, whereas other "cross-resistant" compounds, such as actinomycin D and CLC, could not [37]. Other work from this group showed that verapamil, which apparently enhances drug retention by intact MDR cells (see below), also competed with [^{3}H]VLB for binding to isolated plasma membrane vesicles [38].

These competition studies do not preclude differences in membrane lipids or in the lipid solubilities of the drugs. They also raise questions as to whether all of the various "cross-resistant" natural product compounds bind to a particular membrane protein, but with very different affinities, or whether a cell produces a family of drug-binding proteins, each having a high affinity for a distinct class of compounds. Indeed, the fact that *several* genes seem to be either amplified or overexpressed [39, 40] in MDR cells supports the notion that a *gene family* may be involved in the MDR phenotype. Ultimately, however, resolution of the "efflux pump" hypothesis will require isolation of the drug-binding protein(s), comparison of the binding affinities of many different classes of "cross-resistant" compounds, and molecular characterization of the mRNAs and proteins made in cell lines selected for primary resistance to different drugs.

INVOLVEMENT OF P-GLYCOPROTEIN IN MDR

The results described in the preceding section indicate that certain drugs can bind specifically to a component of the plasma membrane of MDR cells. To examine this idea, Felsted and colleagues recently developed an iodinated azido-derivative of VLB ([^{125}I]NASV) [41] that bound to plasma membrane fractions of drug-resistant cells but not to those of drug-sensitive parents [38, 41]. Moreover, the major protein specifically labeled by [^{125}I]NASV had an apparent molecular weight of ≈150–180 kDa, and the covalently-linked drug–protein complex could be precipitated by a polyclonal antiserum that recognized the resistance-associated protein, gp150–180 [41]. These results strongly suggest that the resistance-associated glycoprotein is a drug-binding protein that may be involved in the decreased accumulation of certain natural product drugs by MDR cells.

Evidence for a role of P-gp in MDR also comes from transfection experiments in which MDR, including expression of P-gp, could be transferred to drug-sensitive recipient cells by the addition of DNA prepared from MDR cells [42–44]. However, since these experiments were performed with total genomic DNA, the gene(s) responsible for the expression of MDR in the transfectants could not be identified. This problem was recently resolved by Gros and coworkers [45], who inserted a cDNA containing the entire coding sequence of the *mdr* gene into an expression vector. They then transfected the recombinant vector into drug-sensitive recipient cells and demonstrated the subsequent overexpression of the *mdr* cDNA and, importantly, resistance and cross-resistance to VCR, DOX, and CLC in the recipient cells. Presumably, the transfected cells also overexpress the protein encoded by the *mdr* gene, but this was not demonstrated. Also not shown was whether cells transfected with the *mdr* cDNA displayed other biochemical changes (e.g. lipid composition, enzyme activity, membrane lipid fluidity, etc.) known to be associated with MDR, as discussed earlier. Of considerable importance, however, was the fact that the cDNA was isolated from a drug-sensitive cell line, indicating that expression of the MDR phenotype does not require mutations in the primary sequence of the *mdr* gene.

Finally, recent studies from the laboratories of Roninson and Pastan [19] and others [20, 21] elucidating the coding sequence of the *mdr*1 (P-gp) gene have revealed a structure with remarkable sequence homology to certain bacterial peripheral membrane proteins involved in the periplasmic transport of histidine, lysine, ornithine and arginine (*his*P), maltose and maltodextrins (*mal*K), phosphate (*pst*B), and oligopeptides (*opp*D). It is suggested that these proteins are the energy-coupling components of their respective transport systems, based on their capacity to bind ATP and consensus nucleotide-binding sequences. Although not shown, Chen *et al.* [19] found that ≈33% of all the *his*P residues matched with identical residues of *mdr*1. The highest homology and most conserved regions of the two proteins was seen in the nucleotide-binding sites, of which *mdr*1 has two. Based on its transmembrane localization, potential nucleotide-binding sites, and homology of the hydrophilic regions of the *mdr*1 product with bacterial transport proteins, the authors concluded that the P-glycoprotein functions as an efflux pump that can remove drugs from cells via an ATP-dependent mechanism [19].

The above proposal is both plausible and attractive, but, as indicated by Chen *et al.* [19], it has several weaknesses. First, the bacterial transport proteins are separate entities that form a quaternary complex with the binding protein and the integral hydrophobic membrane protein, whereas the proposed hydrophilic nucleotide binding sites of P-gp are part of a larger protein containing its own hydrophobic membrane-spanning regions. Second, functionally, the direction of the bacterial transport is from the periplasmic space to the inside of the cell, whereas the P-gp is presumably involved in the movement of compounds *from* the cell. Third, the bacterial proteins are limited in their substrate specificity, whereas P-gp appears to recognize a wide range of lipophilic structures. Moreover, while the bacterial proteins specifically recognize and transport anions, cations or neutral molecules, the drugs presumably bound by P-gp are lipophilic cations. Fourth, specificity is apparently achieved in the bacterial proteins through recognition of the substrate by the specialized periplasmic binding proteins, which then transmit it to the membrane components, whereas P-

gp itself is apparently capable of substrate binding, although the authors could not preclude the possibilty of additional drug-binding cytoplasmic components.

Thus, P-gp appears to be responsible for MDR, although we do not yet have a clear understanding of the mechanism(s) involved. Whether P-gp binds drug to prevent its entry into cells or whether it either extrudes drug or opens channels in membranes through which drug passes is not known. The model discussed above, based on the protein structure, does not adequately account for the lack of substrate specificity in MDR, nor does it explain the energetics of decreased drug accumulation and retention in MDR (see next section). Experiments with transport-competent membrane vesicles might be of value in addressing some of these questions, as would reconstitution experiments in which purified P-gp is incorporated into either artificial membranes or membranes prepared from drug-sensitive cells. Finally, drug transport experiments with cytoplasts ("bags" of cytoplasm with intact membranes, but lacking cellular organelles such as nuclei, mitochondria and lysosomes) might be particularly useful in distinguishing membrane from cytoplasmic drug-binding components and providing insights into the mechanism of decreased drug retention in MDR.

WHAT IS THE ROLE OF ATP IN DRUG BINDING AND DECREASED DRUG ACCUMULATION IN MDR?

Experiments with metabolic inhibitors indicate that the diminished accumulation and retention of drug by intact MDR cells is energy-dependent, ATP energy being required to maintain diminished steady-state drug levels [28, 46]. However, our studies indicate that, in fact, there are two VLB-binding compartments in intact MDR cells, one requiring metabolic energy and the other being energy independent [4, 28]. Moreover, the recent studies from Pastan's laboratory suggest that the binding of VLB to plasma membrane vesicles [37], and presumably the P-gp [38], is energy-independent. (Although temperature dependency for VLB binding was shown in that study, it likely reflected lipid phase-transitions rather than a requirement for metabolic energy.)

Thus, one can speculate that in intact MDR cells there is an energy-*independent* component of drug-binding and an energy-dependent component that may be responsible for maintaining the diminished steady-state drug levels. One possibility is that the energy required for this latter component may work directly on the P-gp. Accordingly, ATP binding or hydrolysis by or at the consensus nucleotide-binding sequences [19, 20] of this protein could change its conformation in the membrane, releasing bound drug. Such a conformational change might cause (secondary) changes in membrane lipid fluidity, thereby permitting ready passage of drug across the membrane and out of the cell. Alternatively, an *indirect* effect of ATP on P-gp might be on structures either in the cytoplasm or associated with the plasma membrane whose energy-dependent alteration may cause conformational changes in the P-gp sufficient to release bound drug. For example, ATP is required for various "membrane trafficking" events such as endocytosis, acidification of endosomes and lysosomes, and possibly for exocytosis [47]. These acid compartments may be involved in maintaining reduced drug levels in MDR.

ARE ACIDIC COMPARTMENTS INVOLVED IN MDR?

We [35, 48] and others [17] have noted that MDR cells contain more vacuoles than do their drug-sensitive counterparts. While it has been reported that these entities contain lipids [17], we could not confirm this claim.* In our cells, the vacuoles appear to be lysosomes, based on their staining for acid phosphatase [48]. Furthermore, we found that treatment of the MDR cells with cytotoxic concentrations of VLB or DOX produced an increase in the number of these vacuoles [48]. This increase was also seen after treatment with verapamil or chloroquine (CLQ) plus non-toxic concentrations of the natural product drugs [35, 48]. While it is likely that the vacuoles are lysosomes or endosomes that become enlarged after trapping protonated cationic compounds [49], this possibility remains to be proven. However, it is well-known that organic cationic compounds impair the pH gradients in acid compartments [50, 51] and, as a consequence, interfere with such "membrane trafficking" processes as recycling, endocytosis, vesicle fusion, and exocytosis [50].

To be accumulated or retained in acid compartments, a drug molecule would simply have to meet some general criteria of size, solubility, and charge; rigorous structural requirements for "binding" would not be necessary. Our preliminary data indicate that drugs involved in the MDR phenotype as well as "modulators" of MDR are all lipid-soluble organic bases at physiological pH. This lipid solubility is decreased dramatically in an acid pH (≈4.5–5.0),† as would be found in endosomes and lysosomes. The low pH would cause protonation of the drugs, thereby trapping them in these compartments, permitting more drug to be retained for ultimate distribution to its cytotoxic target(s). A modest increase in the pH of these compartments could have a profound effect on the amount of drug accumulated and retained (i.e. "bound") by a cell, without the need for true ligand–receptor interactions [52].

A "membrane trafficking" model that accounts for the data as well as the notion that acid compartments may be involved in MDR is presented below and in Fig. 1. While not meant to be anything more than the hypothetical scheme that it is, its value lies in its ability to serve as both a focus for discussion and a starting point for future experimentation. How might this model be compatible with the P-gp and its apparently rather specific binding of VLB, verapamil and other drugs? A testable hypothesis is that the presence of P-gp in the plasma membrane acts to "destabilize" it, altering its trafficking patterns or turnover.

* J. M. Zamora and W. T. Beck, unpublished observations.

† J. M. Zamora and W. T. Beck, manuscript in preparation.

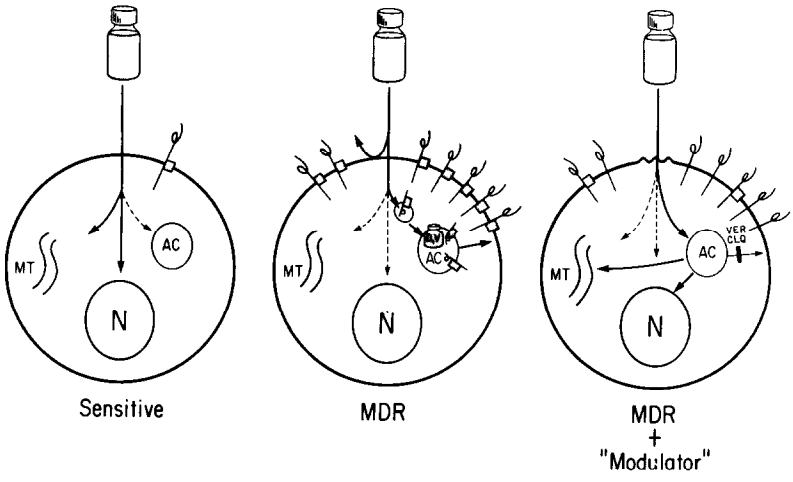

Fig. 1. Hypothetical flow of cytotoxic agents in drug-sensitive and -resistant cells. Drugs such as VLB (V) and DOX (D) would cross the plasma membrane of the drug-sensitive cell (left side) by passive diffusion and arrive at their cytotoxic targets such as the microtubules (MT) or the nucleus (N) respectively. Acid compartments (AC) and P-gp ( ), while present, would have a minimal effect on drug distribution in these sensitive cells. By contrast, drug does not readily enter the MDR cell (middle figure) because of a decreased plasma membrane permeability, but that which does would be shunted to some cytoplasmic, metabolically inert site (possibly an acidic compartment) where it might be removed from the cell via exocytosis. The P-gp may bind drug that enters the cell, permitting its ready release. It may also serve to destabilize the plasma membrane and alter membrane trafficking patterns; this could cause alterations in intracellular drug distribution and rapid loss of drug from the cell. Finally (right side), "modulators" of MDR such as verapamil (VER) or chloroquine (CLQ) might interfere with membrane trafficking or entrapment of drug by acidic compartments, permitting more antitumor drug to be available for its cytotoxic target(s). By interfering with the binding of drug to the P-gp, these agents might also alter the ability of this protein to destabilize the plasma membrane. See text for further details.

In this regard, preliminary results of Sehested *et al.* [53] suggest that membrane recycling is increased in MDR cells. While it is not known if P-gp is recycled with the plasma membrane or if it has a high rate of synthesis, this protein is likely to be present in the Golgi, exocytic fusion vesicles and, possibly, in acidic organelles. Drugs such as VLB are weak bases, highly lipid soluble, and known to interact with membranes [54]. While natural product drugs do not readily enter the MDR cell because of a reduced rate of permeation [25, 28], those that do might be bound by P-gp (possibly at its hydrophobic domain) in either plasma membrane or vesicles. Drug could be released down its concentration gradient, a process that might be enhanced by conformational changes in P-gp caused by ATP hydrolysis, P-gp phosphorylation, or fusion of the P-gp-containing vesicle with the plasma membrane. Furthermore, any drug in the cell might be shunted to a different site, perhaps a metabolically inert *cul-de-sac* [28], because of the altered pattern of membrane trafficking.

Although it has yet to be shown that P-gp is present in sites other than the plasma membrane, there is some experimental support for these ideas. The subcellular distribution of anthracyclines is altered in MDR cells compared to their drug-sensitive counterparts [55, 56], and we have made similar observations in our resistant cell lines.* Rather than being localized in the nucleus, the anthracycline localizes in cytoplasmic vesicles of MDR cells [55, 56]. Verapamil enhances the cytotoxic actions of Vinca alkaloids [14] and anthracyclines [57] in MDR cells (see next section), and it appears to block the redistribution of the latter agents from the nucleus to discrete cytoplasmic compartments [56, 58]. Moreover, verapamil has been shown to affect lysosomal function [59, 60] and enzyme activities [61], and by inference would disrupt membrane trafficking patterns. Our recent studies [48] and those of Kuwano and colleagues [62] demonstrate that the lysosomotropic agent CLQ also potentiates the cytotoxicity of Vinca alkaloids and anthracyclines. CLQ is known to raise the pH of acid compartments [51] and to disrupt membrane trafficking patterns [49, 50]. Finally, the microfilament-disrupting agent, cytochalasin B, enhances the cytotoxic activity of Vinca alkaloids and anthracyclines in MDR cells [63], and it, too, impairs membrane flow (endocytosis) in some systems [64]. Thus, in terms of the above model, these agents would alter membrane traffic, allowing more drug to be available to its cytotoxic target(s). Furthermore, if drug is carried or sequestered in acidic compartments, the increase in their pH caused by CLQ or the other amines [49, 50] would have the effect of decreasing drug retention, allowing more drug to be available to its cytotoxic target(s). If membrane turnover in MDR cells is faster than that in drug-sensitive cells [53], additional support would be gained for the notion that P-gp alters membrane trafficking patterns and,

* J. M. Zamora and W. T. Beck, unpublished.

consequently, impairs the amount of drug retained by the resistant cell.

This model, then, would account for the following: decreased accumulation and retention of drug by energy-intact MDR cells compared to metabolically-deprived cells; "specific binding" of certain lipophilic drugs to (the hydrophobic portions of) P-gp; and increased energy-*independent* drug binding in MDR cells (possibly due to increased amounts of a highly hydrophobic protein). The degree to which drugs are retained by the resistant cell could be explained simply by their physical and chemical features [52].

MODULATORS OF MDR

Ca^{2+}-channel blocking drugs, typified by verapamil and nifedipine, and calmodulin inhibitors, typified by fluphenazine and trifluoperazine, are effective in enhancing the cytotoxicity of natural product anticancer drugs, primarily Vinca alkaloids and anthracyclines, in MDR cells [35, 57, 65–67]. These agents have their major clinical effects in excitable tissue or on the calcium-binding protein, calmodulin. However, since MDR cells apparently have neither inhibitable voltage-dependent Ca^{2+} channels [68, 69,*] nor differences in calmodulin levels [70,†], the mechanism behind the ability of these agents to enhance anticancer drug cytotoxicity must be due to other factors. Indeed, the so-called "Ca^{2+}-channel blockers" have many other actions (reviewed in Ref. 71), including inhibition of voltage-gated K^+ channels [72] and certain calmodulin-dependent activities [73].

Recent experiments from Pastan's laboratory, described above, indicate that verapamil competes with labeled VLB for binding to isolated plasma membrane vesicles from MDR cells [38]. Since VLB binds to P-gp [41], it appears that verapamil might also bind to this protein. We have found that verapamil and the vindoline moiety of VLB share physical and structural features‡ that could explain this competition as well as the enhanced accumulation and retention of VLB in verapamil-treated cells. However, we do not yet known if the same structural relationships obtain for other "modulators" and natural product drug classes. Alternatively, verapamil might exert its action in terms of the "membrane traffic" model proposed above. This is another testable hypothesis. Clearly, studies with these types of modulators may provide insights into the mechanisms of MDR.

Finally, it has been shown by Yalowich and Ross [74] that the "Ca^{2+} channel blocker"-type modulators are also effective enhancers of the cytotoxic actions of drugs such as the epipodophyllotoxins in drug-*sensitive* cells. These findings suggest that either the modulators do not exert their effects directly on P-gp, or the "sensitive" cell lines possess enough P-gp to be inhibited.

Specificity of modulators of MDR. We showed in our vinblastine-resistant human leukemic cell line, CEM/VLB_{100}, that, while verapamil can enhance to a certain extent the cytotoxicity of most natural product drugs, its effect is greatest for the Vinca alkaloids [35]. By contrast, Sikic and co-workers [57] reported that in their doxorubicin-resistant human sarcoma cell line, Dx5, the effect of verapamil was greatest for the anthracyclines and, in fact, had essentially no effect on the Vinca alkaloids. The basis for this discrepancy is unknown, but it may be related to the differences in tumor cell type, primary selection agent, assay conditions (growth inhibition vs cloning), drug exposure time (48 hr vs 1–2 hr), culture conditions (suspension vs monolayer), or degree of primary resistance.

Is calcium important in MDR? Enhancement of the cytotoxic effectiveness of Vinca alkaloids and anthracyclines by the Ca^{2+}-channel blockers has prompted the suggestion that calcium may have a role in MDR, despite the apparent lack of voltage-dependent Ca^{2+} channels [68, 69] or differences in calmodulin content [70] between MDR and drug-sensitive cells. The finding of Tsuruo *et al.* [70] that several MDR cell lines had a higher Ca^{2+} content than did the drug-sensitive cells was not confirmed in subsequent work from Krishan's laboratory [75]. Part of this discrepancy may be related to the different methods used to measure cellular Ca^{2+} levels in the two studies. Although one [75] measured total, free and bound Ca^{2+}, the other [70] did not. Furthermore, it is possible that the bound Ca^{2+} is distributed differently in MDR cells, compared to drug-sensitive cells, despite having the same apparent proportion of bound and free ion [75]; this remains to be determined. Clearly, measurement of cellular Ca^{2+} content by different methods and accurate assessment of the subcellular distribution of Ca^{2+} is necessary to resolve the apparent controversy of the role of Ca^{2+} in MDR.

Koch *et al.* [76] demonstrated by a modified Western blot procedure that the MDR C5 cells of Ling had more of a $^{45}Ca^{2+}$-binding protein of ≈21 kDa than did the drug-sensitive parent line. This is of considerable interest since the apparent molecular weight of this putative Ca^{2+}-binding protein is similar to that of a protein shown by Meyers and Biedler [77] to be overexpressed in some of the VCR-resistant cell lines. However, careful examination of the $^{45}Ca^{2+}$-blots shown by Koch *et al.* reveals that other apparent Ca^{2+}-binding proteins are present in different amounts in the MDR and drug-sensitive cells. Moreover, there is no indication that there is a relationship between the amount of the 21 kDa protein and the degree of resistance of the cells, so it is difficult to draw definitive conclusions without further investigation.

Collateral sensitivity of MDR cells to "modulators" of MDR. Several laboratory groups have observed that MDR cells are collaterally sensitive to the cytotoxic effects of such "modulators" as detergents, local anesthetics, steroids [78, 79], verapamil [80], and CLQ [48]. The basis of this effect is not clear; it is also not known whether these agents work through the same or separate mechanisms. Consider this paradox: while both VLB and verapamil apparently

* C. Deutsch, S. Lee and W. T. Beck, unpublished observations.

† A. Tallent and W. T. Beck, unpublished results.

‡ J. M. Zamora, H. Pearce and W. T. Beck, unpublished observations.

compete for binding to some (common) site of P-gp [38], verapamil is more toxic to the MDR cells than to the drug-sensitive cells, whereas VLB is, by definition, less toxic to the MDR cells. It is likely that the modifiers and the anticancer drugs have different cytotoxic targets. For example, one could postulate that the binding of VLB to P-gp is not a cytotoxic event, whereas binding of the "modifier" is. One possibility is that the two drugs have overlapping but different "binding" sites on P-gp. (Recall that both verapamil and VLB are highly lipid-soluble amines that share some structural similarities.)* Another possibility may relate to the fact that the effective critical cytotoxic concentrations of the two agents differ: the IC_{50} values for VLB are in the nanomolar range [35], whereas those for verapamil are in the micromolar range.† Finally, the collateral sensitivity of the drug-resistant cells to the "modulator" may be related to the fact that its cytotoxic action is a general membrane perturbing effect, achieved only at high drug concentrations. Clearly, the mechanism of collateral sensitivity of the MDR cells to these "modulators" is not known, but understanding its basis should provide a significant clue to the pharmacological basis of modulation of MDR.

FUTURE CONSIDERATIONS

It should be apparent that much of the cellular pharmacology and biochemical basis for altered membrane transport in MDR remains unsettled. A major consideration in future research will be to establish the function of the resistance-associated protein (P-gp, etc.) in MDR as well as in normal tissue. Is it a pump capable of actively extruding drugs from the cell across an electrochemical gradient or against a concentration gradient? Alternatively, does it bind and release some drugs (and modulators) indirectly, possibly by forming openings or pores in membranes? Such openings might be created because of changes in membrane structure due to alterations in protein packing, lipid fluidity, or membrane trafficking. Are there a *series* of "pump" proteins, each having its greatest binding affinity for a different class of antitumor drugs?

The energetics and substrate specificities for diminished drug accumulation and retention in MDR cells are not known. This would seem to be an important area of study, not only to discern basic transport mechanisms, but also to begin to develop rational modes of chemotherapy to increase the amount of antitumor drug in the MDR cells.

Such studies make the assumption that MDR as studied in the laboratory is important clinically. The marker protein, P-gp, has been demonstrated to be present in some tumor specimens taken directly from some therapeutically unresponsive patients [1]. It will be important to establish that tumors of some of these patients overexpress MDR markers such as P-gp and/or its mRNA that correlate with disease or its outcome. Studies underway in several laboratories should yield some preliminary answers about clinical correlations during the next year.

Not addressed here is the notion that there are other forms of natural product MDR that may have partially overlapping cross-resistance patterns with the MDR cells discussed here, but do not overexpress the P-gp. In preliminary studies from our laboratories [81], we have established and characterized a teniposide (VM-26)-resistant human leukemic cell line that is cross-resistant to etoposide, anthracyclines, and other agents, but is *sensitive* to the Vinca alkaloids. This cell line is not altered significantly in the membrane transport of epipodophyllotoxins [81] or of VCR [82], compared to the drug-sensitive parent line, and, significantly, it does not overexpress either gp180 (P-gp) or its messenger RNA.‡ The clinical relevance of this "atypical" MDR remains to be established.

Finally, the "normal" function of P-gp also needs to be established. Is the protein involved in a normal cellular xenobiotic defense mechanism or is it only (over)expressed by selection in a hostile chemical environment? Alternatively, it may have nothing to do with protection from xenobiotics, but rather may be involved normally in the transport, possibly via endocytosis or exocytosis, of physiological substrates in and out of the cell. Indeed, P-gp may function normally in some entirely unrelated cellular activity that *coincidentally* serves to prevent the toxic accumulation of charged, lipophilic xenobiotics in the cell. Work currently in progress in several laboratories should soon provide answers to some of the questions posed here and should provide insights into the complex problem of MDR.

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* J. M. Zamora, H. Pearce and W. T. Beck, unpublished observations.

† W. T. Beck and M. C. Cirtain, unpublished result.

‡ W. T. Beck, M. C. Cirtain, M. K. Danks, R. L. Felsted, A. R. Safa, J. S. Wolverton, D. P. Suttle and J. M. Trent, manuscript submitted for publication.

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