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Minireview

Altered Drug Translocation Mediated by the MDR Protein: Direct, Indirect, or Both?

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Overexpression of the MDR protein, or p-glycoprotein (p-GP), in cells leads to decreased initial rates of accumulation and altered intracellular retention of chemotherapeutic drugs and a variety of other compounds. Thus, increased expression of the protein is related to increased drug resistance. Since several homologues of the MDR protein (CRP, ltpGPA, PDR5, sapABCDF) are also involved in conferring drug resistance phenomena in microorganisms, elucidating the function of the MDR protein at a molecular level will have important general applications. Although MDR protein function has been studied for nearly 20 years, interpretation of most data is complicated by the drug-selection conditions used to create model MDR cell lines. Precisely what level of resistance to particular drugs is conferred by a given amount of MDR protein, as well as a variety of other critical issues, are not yet resolved. Data from a number of laboratories has been gathered in support of at least four different models for the MDR protein. One model is that the protein uses the energy released from ATP hydrolysis to directly translocate drugs out of cells in some fashion. Another is that MDR protein overexpression perturbs electrical membrane potential ($\Delta\Psi$) and/or intracellular pH (pH_i) and thereby *indirectly* alters translocation and intracellular retention of hydrophobic drugs that are cationic, weakly basic, and/or that react with intracellular targets in a pH_i or $\Delta\Psi$-dependent manner. A third model proposes that the protein alternates between drug pump and Cl^- channel (or channel regulator) conformations, implying that both direct and indirect mechanisms of altered drug translocation may be catalyzed by MDR protein. A fourth is that the protein acts as an ATP channel. Our recent work has tested predictions of these models via kinetic analysis of drug transport and single-cell photometry analysis of pH_i, $\Delta\Psi$, and volume regulation in novel MDR and CFTR transfectants that have not been exposed to chemotherapeutic drugs prior to analysis. This paper reviews these data and previous work from other laboratories, as well as relevant transport physiology concepts, and summarizes how they either support or contradict the different models for MDR protein function.

KEY WORDS: Multidrug resistance; intracellular pH; membrane potential.

INTRODUCTION

A central theme in tumor MDR[2] research is the altered intracellular drug retention that accompanies

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[2] Abbreviations: MDR, multidrug resistance; p-GP, p-glycoprotein; CRP, chloroquine resistance protein; ltpGPA, *Leshmenia tarantolae* p Glycoprotein; PDR5, pleiotropic drug resistance protein 5; sapABCDF, *Salmonella typhimurium* ABC transporter complex; bR, bacteriorhodopsin; $\Delta\mu_H^+$, proton electrochemical potential; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, free inorganic phosphate; $\Delta\Psi$, electrical membrane potential; pH_i, intracellular (cytoplasmic) pH; TPP^+, tetraphenylphosphonium; BCECF-AM, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxy methyl ester; LUV, large unilamellar vesicle; CFTR, cystic fibrosis transmembrane conductance regulator; ΔpH,pH gradient across the plasma membrane; ABC, ATP-binding cassette; PKC, protein kinase C.

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overexpression of MDR protein in MDR tumor cells; however, the mechanism whereby MDR protein overexpression leads to altered retention is unresolved. This review highlights the remaining questions and controversies. Although it is hoped this review will be of use to the diverse group of investigators that have studied tumor cell chemotherapeutic drug resistance and MDR phenomena over the past twenty five years, it is directed primarily at students and others that have more recently become interested in the function of the MDR protein and that study membrane transport phenomena from a biophysical or physiological perspective.

The study of tumor drug resistance phenomena is as old as chemotherapy (Burchenal *et al.*, 1950) and has its origins in the pharmacology, oncology, and cell biology disciplines. Study of tumor multidrug resistance (MDR) began in earnest when investigators began to model drug resistance in tissue culture (Biedler and Riehm, 1970), and it has more recently attracted the intense interest of investigators concerned with details of molecular membrane transport mechanisms (Ames, 1986) because the MDR question has evolved into a detailed membrane biophysics problem. As with most highly interdisciplinary fields, analysis of MDR protein function and the complex cell biology of MDR cells has followed several experimental paths. In analyzing the variety of data obtained from these diverse studies, it can prove useful to address the studies in chronological order. This provides historical perspective and may help to explain why some techniques and ideas have been favored over time and others not. Alternatively, it is also valuable to address the sum total of years of data from the perspectives of different scientific disciplines, perhaps even presumptuously "re-interpreting" data using the principles and language of those disciplines. This carries its own unique blend of risk and benefit, but can be an important mechanism for resolution of remaining controversy.

It is the thesis of this paper that models that envision some type of direct drug translocation by the MDR protein (e.g., "pump," "flippase," or "vacuum cleaner" models; see Gottesman and Pastan, 1993; Reutz and Gros, 1994b; Higgins and Gottesman, 1992) are certainly not the only viable models one can use in attempting to understand altered drug retention in MDR cells and to further explore the complex cell biology mediated by overexpression of the MDR protein. Via our biophysical analysis, pump models are not even the models that are best supported upon consideration of all data and arguments from the variety of different disciplines we feel impact upon the field of MDR phenomena, but the reader should be aware that this is currently not the favored view in the MDR field for a variety of reasons (see also Gottesman and Pastan, 1993; Reutz and Gros, 1994a, b; Roninson, 1995; Shapiro and Ling, 1995b). Nonetheless, through analyzing data decidedly *out* of historical context, but rather within the framework of the language and traditions of the field of transport physiology and biophysics, we and others have independently reached conclusions presented in this paper (see also Simon and Schindler, 1994; Wadkins and Houghton, 1993, 1995; Roepe *et al.*, 1993; Roepe, 1994, 1995; Wadkins and Roepe, 1996). This paper further brings these antidogmatic conclusions more up to date in light of recent provocative data.

Thus, the reader should note that several major differences of opinion exist in the MDR field. We cannot, in one paper, present the sum of 25 years of data and arguments in favor of all different opinions; we present those that we feel best represent analysis of MDR protein function from a traditional biophysical and transport physiological perspective. The reader is therefore cautioned and also referred to other recent reviews of MDR protein function that present data from different perspectives (e.g., Gottesman and Pastan, 1993; Reutz and Gros, 1994b; Shapiro and Ling, 1995b). Upon consideration of the arguments in these papers along with ours, our interpretation may or may not turn out to be that of the reader. Nonetheless, detailed consideration of the data and arguments summarized in this paper raises many critical questions that will ultimately need to be resolved if we are to truly understand MDR protein function, regardless of which model for function turns out to be correct.

THE PUMP MODEL: ESSENTIAL FEATURES, SUPPORTING DATA, AND APPARENT CONTRADICTIONS

Since it was proposed before the other models, and is championed more vigorously by more investigators, it makes some sense to start a discussion of MDR protein function by reviewing the data and arguments that are used to support various versions of a "direct pump" model. Most early (prior to 1992) studies of MDR protein function were primarily initiated due to an intense interest in understanding the cellular pharmacology of chemotherapeutic drugs, or the unique cell biology and genetics of drug-resistant tumor cells. These studies thus did not, in general, necessarily

emphasize emerging practices and concepts from the molecular membrane transport fields, because at the time the focus of the MDR field was different and these practices and concepts were relatively new and hence not universally well known. In reviewing these data, as mentioned in the introduction, we could follow the studies historically, starting with Danø's classic study that examined drug accumulation and nuclear association for MDR tumor cells created by selection with anthracycline (Danø, 1973) and finishing with recent detailed kinetic (Roepe, 1992; Spoelstra *et al.*, 1992; Bornmann and Roepe, 1994; Stein *et al.*, 1994; Robinson and Roepe, 1996) and thermodynamic (Reutz and Gros, 1993; 1994a; Sharom *et al.*, 1993; Shapiro and Ling, 1995a; F. Fritz and P. D. Roepe, unpublished) investigations of altered drug translocation mediated by MDR protein using cells, vesicles, and proteoliposomes. We would also need to include analysis of Skovsgaard's work (1978) from both a pharmacologic and detailed physical chemical perspective. Different conclusions can be drawn from these data, depending on the perspective that is chosen. However, this style of analysis has been followed previously (Roepe, 1995). Let us therefore step back and first review the fundementals of pumps, as transport physiologists and biophysicists have traditionally defined them, and then the important predictions of this definition with respect to the features that should be exhibited by MDR protein in order for a pump model to be valid. Then, we will re-examine recent data that is sometimes used to support a pump hypothesis, as well as the results from other experiments explicitly designed to test predictions that come from these fundementals. Such a "hindsight" approach is also useful.

Pumps are polytopic integral membrane proteins or protein complexes that perform vital cell functions. A pump transports substrate against a concentration gradient (i.e., "uphill" thermodynamically). The transport process catalyzed by the pump thus requires energy, and must proceed faster than the rate of passive diffusion of substrate if gradients in osmotically sensitive substrate are to be maintained. Some investigators avoid using the term "enzyme" when discussing pumps, since one definition of an enzyme is that it changes the chemical identity of its substrate (i.e., breaks/forms chemical bonds), and many pumps do not break/form substrate chemical bonds. However, if a pump is translocating substrate against a gradient, the chemical potential of the substrate on one side of the membrane is different from the other; thus, the pump does indeed formally change the chemical identity of its substrate. Therefore, many transport physiologists/biophysicists frequently refer to pumps as enzymes. Also, pumps exhibit exquisite substrate specificity, another characteristic of enzymes (i.e., they conform to the law of enzyme specificity). If pumps were not substrate specific, gradients across membranes would eventually collapse, destroying the integrity of the cell. In one other sense it is useful to think of pumps as enzymes, that is, the Michaelis–Menten language that is frequently used to describe the kinetic behavior of enzymes is often quite satisfactory for describing the kinetic behavior of pumps. Saturation, V_{max}, K_m, etc. are well-defined terms for many well-studied pumps, although their meaning can sometimes be ambiguous for pumps with complex regulation or energetics. In any case, when these caveats are considered, it is useful to consider that pumps are simply specialized enzymes that catalyze "vectorial" chemistry, that is, chemistry in a directional sense across a membrane.

Many pumps have been studied in detail over the past twenty years. Two of the "archetypes" are the bacteriorhodopsin (bR) H^+ pump, and the lac permease lactose/H^+ symporter. These two pumps use light energy and the energy stored in the H^+ electrochemical potential ($\Delta\mu_H{}^+$), respectively, to drive uphill transport (pumping) of their substrates. Pumps can sometimes be forced to operate in "nonphysiologic" modes that do not require energy and where substrate is thus not being translocated uphill. A third archetype, the "Na^+ pump," or Na^+/K^+ ATPase, uses a third form of energy, hydrolysis of ATP, to drive uphill Na^+ transport. In all three cases, another fundemental concept in the study of pumps is introduced, namely, the concept of "coupling." To illustrate, one photon of light energy catalyzes the pumping of one H^+ (at about 20–30% efficiency, due to the photochemistry of the retinal chromophore) for bR, and energy released from the downhill movement of one H^+ in response to $\Delta\mu_H{}^+$ drives the pumping of precisely one lactose molecule via the lac permease. One ATP is hydrolyzed to pump precisely 3 Na^+ out of the cell via the Na^+ pump (with 2 K^+ translocated into the cell in the same cycle). When one experimentally forces one lactose molecule "downhill" by artificially setting up a lactose concentration gradient, one H^+ is actively translocated by the permease; analogously, one ATP is formed from ADP and P_i when energy is released by promoting downhill movement of 3 Na^+ through the Na^+ pump. Via the coupling principle the photoisomerization of bR that releases energy stored as charge separation should be reversed upon forcing H^+ downhill through bR under the appropriate conditions, and recent data that analyzes M, N, and O

intermediate conversion vs. pH for D85N mutant bR supports this (see Turner *et al.*, 1993). In any case, the point is that pumps exhibit "tight" stoichiometric coupling to an energy source in either direction for obvious reasons that we shall not review here.

Therefore, discussion of a pump from a biophysical perspective should include four points: (1) thermodynamics of the transport process it is believed to catalyze (e.g., is it "uphill"), (2) substrate specificity, (3) kinetics of the transport process (i.e., how much faster than passive diffusion does it operate and can one calculate V_{max}, K_m, etc.), and (4) the energetics and coupling (what provides the energy and what is the "stoichiometry" of the pump). In the case of the MDR protein, if we assume it is a drug pump, it has proven difficult to address point 1, because the putative substrates (chemotherapeutic drugs and other compounds) are typically quite hydrophobic (they generally have octanol/saline partitioning coefficients between 10 and 1000 that are also very pH dependent). Therefore, determining the precise magnitude of any experimentally generated gradient in osmotically sensitive putative substrate is very difficult and can be ambiguous due to aggregation, partitioning, and binding phenomena, as pointed out in a recent proteoliposome study (Shapiro and Ling, 1995a) that calculated only downhill translocation of substrate by MDR protein. However, as reviewed previously (Roepe, 1995), several other recent studies have provided data that reveals apparent concentration of substrate by the MDR protein (Reutz and Gros, 1994a; Sharom *et al.*, 1993; see also below). If MDR protein is a drug pump, it clearly violates point 2 in an unprecedented fashion; cells overexpressing the MDR protein exhibit decreased intracellular retention and rates of accumulation (interpreted by many investigators to be the manifestation of outward-directed pumping) of over a hundred structurally divergent compounds, including anthracyclines, oxacarbocyanines, tetraphenylphosphonium, vinca alkaloids, verapamil, and yohimbine, to name a few. The kinetics of the putative pumping believed to be mediated by MDR protein (point 3) have proven elusive to define, since the rate of passive diffusion of the compounds believed by some to be pumped by the MDR protein are so fast that novel methods need to be developed to kinetically separate passive diffusion from any pumping believed to exist. Several studies using novel methodology have concluded that the kinetics of drug efflux from MDR cells are inconsistent with an outward pumping process (see Roepe, 1992; Bornmann and Roepe, 1994), but at least one study claims the converse (Spoelstra *et al.*, 1992) based on the effects of putative pump inhibitors. With respect to point 4, the energy for putative drug pumping by the MDR protein is envisioned to come from the hydrolysis of ATP since the protein harbors two predicted ATP binding sites, as elucidated by Walker motifs. Moreover, the protein has been shown to be an ATPase by several laboratories (Sharom *et al.*, 1993; Al-Shawi *et al.*, 1994; Shapiro and Ling, 1994) and catalyzes the hydrolysis of about 600 ATP/minute. Thus, in theory the protein has the capacity to provide energy for an active transport process. However, when stoichiometries of ATP hydrolysis:drug translocation are calculated from the best available data (e.g., Sharom *et al.*, 1993; Schlemmer and Sirotnak, 1994) the estimates range from 10^5:1 to 10^4:1 (ATP hydrolyzed:drug molecules translocated). Note that overexpression of MDR protein typically decreases the initial rate of drug accumulation by 30–90%, or even more (Hammond *et al.*, 1989; Stein *et al.*, 1994; Robinson and Roepe, 1996). Thus, when the kinetics of chemotherapeutic drug passive diffusion under initial rate conditions are considered (see Bornmann and Roepe, 1994; Roepe, 1995), it is clear that active drug translocation would present a severe to impossible burden on the ATP generating capability of the cell, unless the putative drug pump also violates the coupling principle and can "choose" widely different ATP hydrolysis:drug translocation stoichiometries, depending on the conditions, or constitutively hydrolyzes ATP independent of substrate translocation for some reason.[3] Either scenario would be unprecedented for a pump.

[3] Assuming the coupling principle is valid (as it is for all other pumps), and assuming published pumping data are correct within an order of magnitude or so (e.g., Sharom *et al.*, 1993 from which one can conservatively calculate an apparent ATP hydrolysis:drug translocation stoichiometry of about 36,000:1), then to explain, for example, a 50% reduction in the initial rate of vinblastine accumulation as is typically observed (see Hammond *et al.*, 1989) via a pump model, on the order of 10^9–10^{10} ATP per cell per second would be consumed (hypothetically), due to the fast rates of passive diffusion of chemotherapeutic drugs under initial rate conditions. We are thus left with a putative pumping process that represents a major bioenergetic challenge for the cell; 10^9 ATP per cell at steady state is a reasonable estimate of cellular ATP concentration, and even if this were sufficient ATP for a few seconds of pumping, it is difficult to imagine that a cell could generate sufficient ATP per second to maintain putative drug pumping at these stoichiometries. Thus, if we entertain the notion that MDR protein is a drug pump, current data argue that it must violate the coupling principle and either hydrolyze ATP without coupling the hydrolysis to translocation of substrate, or dramatically alter hydrolysis:translocation stoichiometries under some conditions.

Thus, if the MDR protein is a drug pump, it violates two fundamental tenets of active transporters, namely, the law of specificity and the coupling principle, and it would be the first example of a pump to do so in such dramatic fashion. The kinetics of putative active drug efflux are either difficult or impossible (e.g., Roepe, 1992; Bornmann and Roepe, 1994) to measure, depending on which interpretation is followed (see also Spoelstra *et al.*, 1992; Stein *et al.*, 1994; Roepe, 1995) and thermodynamic evidence for translocation against an apparent drug concentration gradient, although it does exist (Reutz and Gros, 1994a), is scarce and depends upon calculations of intracompartmental volume and estimates of osmotically sensitive concentrations of hydrophobic compounds on either side of a membrane. Since these hydrophobic drugs also generally tend to aggregate at concentrations < 2 μM, these estimates are far from a trivial endeavor. The largest accumulation of substrate by the MDR protein that has been reported with a well-defined system is about 6–7 fold (see Reutz and Gros, 1994a) but several important potential complications to interpretation of this study have been pointed out (Roepe, 1994, 1995; Wadkins and Roepe, 1996). Mild accumulation for the vast majority of compounds believed to be "pumped" by the MDR protein can also be easily explained via mechanisms that do not invoke an active pumping process (see Roepe, 1995 and below).

Another important consideration from analysis of the best available data (Reutz and Gros, 1994a; Schlemmer and Sirotnak, 1994; Sharom *et al.*, 1993) that addresses the feasibility of a pump model is quantitative estimate of "turnover" of the pump. The most explicit calculations that have been published can be found in Schlemmer and Sirotnak (1994), where it is clear that transport is assessed at concentrations of putative substrate that straddle an apparent K_m of 215 nM (for vinblastine). Under conditions where substrate is not limiting, a hypothetical turnover of about 1 vinblastine molecule/pGP molecule/40 min is calculated (see Table I and Roepe, 1995). Similarly, we calculate a turnover of about 1 colchicine molecule/pGP molecule/hour from the data in Sharom *et al.* (1993), although it has been stressed that these investigators estimate the K_m for colchicine pumping to be near 50–100 μM (F. Sharom, personal communication), so in theory this hypothetical turnover estimate is low. However, it is difficult to understand the significance of a hypothetical K_m in the 100 μM range (3 orders of magnitude over that measured for vinblastine by Schlemmer and Sirotnak [1994]), since hydrophobic drugs exhibit complex behavior at these high concentrations (see also Wadkins and Houghton, 1993). Regardless, the point is, can turnovers at physiologically meaningful levels of putative substrate explain the altered drug partitioning and retention that is the hallmark of the MDR phenotype? As has been pointed out previously (Roepe, 1995) it is estimated that these turnovers are 3–6 *orders of magnitude* too slow to explain typical decreased rates of drug accumulation under intial rate conditions (Hammond *et al.*, 1989) or altered retention of drug (see Demant *et al.*, 1990 for explicit calculation of hypothetical pump turnover that woud be required). Our recent survey of a variety of published data (Table I) demonstrates that the low turnovers calculated from these recent data are not an aberration; no studies that we are aware of have obtained evidence for active transport at anywhere near a sufficient turnover to explain the decreased drug accumulation and retention for MDR cells that is generally attributed to MDR protein overexpression.

On the other hand, other data, namely photolabelling studies (reviewed in Beck and Qian, 1992; Tew *et al.*, 1993) and phenotypic peculiarities of cells expressing certain mutant MDR proteins (discussed in Roepe, 1995), as well as the persistence of drug accumulation in the presence of nitrate in the study by Reutz and Gros (1994a), are often cited as support for the drug pump hypothesis, and it has sometimes been claimed that other models for MDR protein (like the altered partitioning model, see below) cannot explain some of these data. However, we feel the altered partitioning model can indeed explain these data (Roepe, 1995 and see below). Regardless, the summary presented above brings up important points to consider even if one chooses to interpret photolabelling and mutant data to indicate that the MDR protein actively pumps drugs, as these points will need to be incorporated into further refinement of pumping models. Also, it is important to consider that there are other possible interpretations of photolabelling analysis (see Tew *et al.*, 1993; Wei *et al.*, 1995; Roepe, 1995) and that many pumps and channels bind a variety of hydrophobic probes but do not necessarily transport them (see Roepe, 1995). Thus, it is certainly conceivable that the MDR protein might be efficiently photolabelled with azido derivatives of certain chemotherapeutic drugs and other hydrophobic compounds but not actively transport them, particularly if one uses membranes with very high levels of MDR protein in these experiments, as is typically the case

Table I. Quantitative Estimates of the Putative Drug Pumping Turnover of p-Glycoprotein Derived from a Number of Recent Publications[a]

Reference	Model system	Substrate	[S] (μM)	[ATP] (mM)	Turnover drug/ p-GP/sec
Sharom *et al.* (1993)	PL, hamster p-GP	COL	0.16	1.0	High, $2.7 \cdot 10^{-4}$ Low, $5 \cdot 10^{-5}$
Lelong *et al.* (1992)	PMV, human p-GP	VBL	0.0053	0.33	High, $1.1 \cdot 10^{-5}$ Low, $6.6 \cdot 10^{-6}$
Doige and Sharom (1992)	PMV, hamster p-GP	COL	1.6	1–3	High, $1 \cdot 10^{-3}$ Low, $1 \cdot 10^{-4}$
Shapiro and Ling (1995a)	PL, hamster p-GP	Hoechst 33342	25	1.5	$1.6 \cdot 10^{-2}$
Schlemmer and Sirotnak (1994)	ISO PMV, murine MDR protein	VBL	0.07	5	High, $4 \cdot 10^{-3}$ Low, $4 \cdot 10^{-4}$
Ruetz and Gros (1994a)	Secretory vesicles, murine mdr 1, murine mdr 3	VBL	2.5	2.25	High, $5 \cdot 10^{-3}$ Low, $5 \cdot 10^{-4}$
Ruetz *et al.* (1993)	Yeast ISO PMV, murine mdr 3	VBL	1	2.25	High, $2.7 \cdot 10^{-2}$ Low, $2.7 \cdot 10^{-3}$
Horio *et al.* (1988)	ISO PMV	VBL	0.0045	0.3	High, $1 \cdot 10^{-4}$ Low, $3 \cdot 10^{-6}$

[a] PL, proteoliposome preparation; PMV, plasma membrane vesicles; ISO, inside-out; COL, colchicine; VBL, vinblastine. In some of these estimates, assumptions regarding site density of p-GP and percent of substrate associated to PMV or PL that is actually transported need to be made, in which case a high estimate (assuming 1% of integral membrane protein is p-GP and all substrate associated is transported) and a low estimate (approximately 10% of vesicle membrane protein is p-GP and total association minus apparent background association [if it is presented] is assumed to represent transported substrate) are presented. If two estimates are not presented, then the number shown is that calculated by the authors of the study. The only study that explicitly calculates an apparent K_m is Schlemmer and Sirotnak (1994); thus, in all other cases, turnover is calculated at the substrate concentration used in the transport assay, which is above K_m reported by Schlemmer and Sirotnak (about 215 nM) in 5 of the 9 cases. In all other respects, the estimates are generated based on the optimal rate of transport possible from the published data; however, most of these studies do not present formal initial rate analysis; the rates are calculated based on a linear best fit to the presented transport data and they are therefore crude quantitative estimates. High and low estimates provide some insight into the possible turnover that is reasonable based on the best available data.

(see Wei *et al.*, 1995). Importantly, no one has yet demonstrated photolabelling of MDR protein above background using membranes expressing physiological levels of the protein (see Wei *et al.*, 1995) and no studies have yet been published that unequivocally demonstrate specific drug binding to the MDR protein via equilibrium binding measurements followed by Scatchard analysis. In addition, analysis of mutant MDR proteins has been performed using cell lines selected with chemotherapeutic drugs under different conditions (either different concentrations of drug or different drugs altogether) relative to cell lines harboring the wild type protein (e.g., Gros *et al.*, 1991). It is conceivable that the different drug selection conditions, not the different MDR proteins themselves, cause the phenotypic peculiarities for cells harboring mutant MDR proteins relative to cells harboring the wild type protein (see Roepe, 1995). It is not absolutely necessary to invoke "mutation of the drug binding site" as an explanation for these data (Roninson, 1995).

Thus, in light of the caveats described above, it is valid and necessary to consider the possibility that the MDR protein does not *directly* translocate substrate, but *indirectly* alters the efficiency of intracellular retention of a variety of hydrophobic drugs and other compounds. In this case, models can be envisioned wherein MDR protein would not be required to violate the law of enzyme specificity or the coupling principle, and would not exhibit a kinetic signature for pumping and thus be consistent with a variety of recent kinetic data (Roepe, 1992; Bornmann and Roepe, 1994; Hammond *et al.*, 1989). Via these models, overexpression of the protein could still lead to mild accumulation of substrate in vesicle/proteoliposome studies, and even more significant altered cellular retention of the compounds over time.

MORE RECENT PERMUTATIONS OF THE PUMP MODEL

Before discussing these alternative models that envision MDR protein indirectly affects drug translocation, it has also been proposed that further refinement

of the pump hypothesis might possibly explain the lack of substrate specificity, poorly defined coupling, and vague kinetics for the drug transport mediated by MDR protein. Two of these modified versions of the pump hypothesis are referred to as the "flippase" (Higgins and Gottesman, 1992) and "vacuum cleaner" models (Gottesman and Pastan, 1993). The first proposes that MDR protein does not perform conventional cytoplasm to extracellular pumping, but "flips" hydrophobic drugs from the inner to outer leaflet of the membrane bilayer. The predicted kinetic manifestations of this process that would be required such that net movement of drug out of the cell to the extracellular aqueous phase is possible and could compete against passive diffusion under initial rate conditions are also not immediately apparent in currently available data (Roepe, 1992; Bornmann and Roepe, 1994), but "flippases," if they exist, have not yet been well studied; thus, there may be essential features yet to be discovered.

The second permutation proposes that MDR protein does not translocate cytoplasmic drug, but pumps "intramembranous" drug. That is, MDR protein is envisioned to "vacuum" drug from the membrane bilayer before it actually reaches the cytoplasm. Some data on the kinetics of drug accumulation for MDR cells has been interpreted to be consistent with this idea (see Stein *et al.*, 1994), but there are perhaps other explanations for decreased initial rates of drug accumulation (see below and Robinson and Roepe, 1996; Wadkins and Roepe, 1996). Since under zero-trans initial rate conditions the rate of passive diffusion of chemotherapeutic drugs is very fast and since initial rates of accumulation are generally decreased by 30–90% for MDR cells, the vacuum cleaner model either predicts an enormous affinity of the drugs for MDR protein, or that the drug molecules are somehow "trapped" in the bilayer. Since the relative volume of the plasma membrane is $<0.1\%$ the volume of the cell, significant concentrations of these drugs in the plasma membrane are not predicted from physical chemical theory and also not observed experimentally (e.g., Coley *et al.*, 1993). Although it is dangerous to predict affinities from photolabelling data, available competition experiments do not appear to be consistent with the affinities that are necessitated by this model (Safa 1988; Cornwell *et al.*, 1986).

However, in contrast to the analysis presented above it has also been argued that these models offer attractive features. For example, some investigators have reasoned that if the putative pump is actually a flippase, perhaps lipid "complexed" with hydrophobic drug is the actual entity recognized by MDR protein, and not drug directly, thereby explaining the apparent lack of substrate specificity for the putative pump. In addition, it has been reasoned that if the putative pump translocates membranous drug but not cytoplasmic, this might explain the lack of efflux kinetics attributable to MDR protein but still accommodate the frequent observation of decreased initial rates of drug influx for cells overexpressing the MDR protein. We do not find these arguments and several others along these lines convincing due to the implied unusual thermodynamics and kinetics of binding and translocation, but for a complete appreciation of the arguments in favor of these models the reader is urged to review them elsewhere (e.g., Gottesman and Pastan, 1993; Reutz and Gros, 1994b; Shapiro and Ling, 1995b).

THE ALTERED PARTITIONING MODEL

It is also possible to view the vast majority of data collected to date within the framework of a completely different model for MDR protein. This model has several major advantages, but also some unresolved complexities that remain puzzling. It envisions that overexpression of the MDR protein leads to some combination of altered electrical membrane potential ($\Delta\Psi$) and/or elevated intracellular pH (pH_i), and that these perturbations directly (through electrostatic mechanisms) and indirectly (through other effects on cellular physiology) then alter the accumulation, partitioning, and retention of drugs. Changes in $\Delta\Psi$ and pH_i that would be necessary and sufficient (see Wei *et al.*, 1995; Hoffman *et al.*, 1996) to cause levels of drug resistance unequivocally mediated by the MDR protein (see Guild *et al.*, Devault and Gros, 1990; Hoffman *et al.*, 1996) could be caused by MDR protein functioning as an ion channel, channel regulator, or perhaps some type of electrogenic ion co-transporter/exchanger. Since lower $\Delta\Psi$ and altered pH_i will affect the diffusion and partitioning of *any* hydrophobic compound that is either charged/weakly basic and/or that binds to an intracellular target in a pH- or $\Delta\Psi$-dependent fashion (that is, drugs to which MDR cells are resistant), the altered partitioning model also does not violate the law of enzyme specificity as does the pump model.

Data that most strongly argues against the altered partitioning model are drug transport studies with secretory vesicles harboring murine MDR protein performed under conditions where $\Delta\Psi$ (measured by TPP^+ distribution) is believed to be collapsed via addi-

tion of nitrate to the transport medium (Reutz and Gros, 1994a), but a variety of other data detailing important pH_i and $\Delta\Psi$ changes in MDR cells and their strong effects on drug translocation and retention strongly support the model (Roepe, 1995; Wadkins and Roepe, 1996). A variety of studies reporting altered Cl^- and ATP translocation in MDR cells likely hold clues as to how $\Delta\Psi$ and pH_i alterations are produced in MDR cells (see "Altered Ion Transport in MDR Cells ..." below), but a molecular mechanism has not yet been unequivocally determined. However, the altered partitioning model should not be neglected out of hand simply because it harbors unresolved complexities. In fact, although their ultimate resolution may require significant advances in our understanding of eukaryotic plasma membrane bioenergetics, it could be argued that these complexities are less problematic than the specificity, kinetic, and coupling principle difficulties associated with the pump hypothesis. At the very least, viewing the MDR problem in the context of the altered partitioning model suggests interesting experiments that are not suggested by the pump model and that will therefore further test predictions of hypotheses for MDR protein function in novel ways (e.g., Wei and Roepe, 1994; Wei *et al.*, 1995).

The altered partitioning hypothesis (Roepe *et al.*, 1993; Roepe, 1995) is based on $\Delta\Psi$ and pH_i measurements, several curious and paradoxical observations for MDR cells regarding pH_i regulator activities and expression (Roepe *et al.*, 1993, 1994; Luz *et al.*, 1994), and a substantial amount of physical chemical data relating to the passive diffusion of chemotherapeutic drugs and other hydrophobic weak bases/cations (e.g., Mayer *et al.*, 1985, 1986; Praet *et al.*, 1993). The model focusses on measured reduced rates of drug *accumulation* for MDR cells (not increased rates of efflux) and release of a higher *percentage* of intracellular drug in efflux assays under zero-trans conditions (not necessarily increased pumping against a concentration gradient), since these phenomena have been more firmly documented by more laboratories.

Several studies have shown that MDR cells exhibit decreased plasma membrane electrical potential (lower $\Delta\Psi$) and several studies have quantitatively estimated depolarization via K^+/valinomycin "null point" titration (see Roepe *et al.*, 1993; Luz *et al.*, 1994; Hoffman *et al.*, 1996). A variety of studies from several laboratories have measured altered pH_i homeostasis in MDR cells (reviewed in Roepe, 1995). In contrast to many observations of alkaline pH_i, one report of acid and unchanged pH_i for two different MDR cell lines derived by protocols that included exposure to chemotherapeutic drug has been published (Altenberg *et al.*, 1993). However, it may be important to note that Altenberg *et al.* performed pH_i measurements in an atmosphere of 95% O_2 after loading cells with BCECF-AM (10 μM) for a relatively long time (60 min) in the presence of detergent (the pluronic F-127), procedures which were not followed in the other studies. Also in this paper, putative connections between drug transport and pH_i were assessed using the mitochondrial dye rhodamine, which is really not representative of chemotherapeutic drugs, since its principle target is not tubulin or nucleic acid and the pK_b of the compound is high (> 10). Thus, it is not expected that altered pH_i near physiologic values would necessarily affect the partitioning of rhodamine over the time period measured in Altenberg *et al.* However, since rhodamine is a lipophilic cation, changes in $\Delta\Psi$ would be predicted to alter its partitioning, and $\Delta\Psi$ was not examined in Altenberg *et al.* It is also possible that other membrane changes induced by the presence of chemotherapeutic drug in the growth medium for the cells used in this study alter the cellular partitioning of rhodamine, which can be displaced from various membranes in a variety of ways (see Wadkins and Houghton, 1993). In any case, importantly, the altered partitioning model does not exclude the possibility that changes in pH_i may not be found for some MDR cells, particularly if they have been selected for long periods of time with chemotherapeutic drugs that likely induce additional drug resistance mechanisms and other phenomena that could conceivably "mask" pH_i perturbations caused by MDR protein overexpression (e.g., altered expression of either the Cl^-/HCO_3^- exchanger or Na^+/H^+ exchanger; see Roepe *et al.*, 1993; Luz *et al.*, 1994).

An important point in analyzing data that might initially appear to disagree with the altered partitioning model is that chemotherapeutic drugs have enormously complex and potent effects. Therefore, the long-term drug selection typically used to derive a MDR cell line, or the exposure to drug typically used to "maintain" MDR protein expression in most transfectants, likely induces many phenomena along with MDR protein overexpression (Roepe, 1995; Hoffman *et al.*, 1996). These additional phenomena make elucidation of the specific role of MDR protein in many model systems extremely difficult.

Thus, as is the case in most drug transport studies, interpretation of all $\Delta\Psi/pH_i$ data currently published is sometimes difficult due to the very different drug

selection conditions that have been used to generate the vast majority of model MDR cell lines. The altered partitioning model is not solely based on pH_i perturbations, but is a more general model encompassing both $\Delta\Psi$ and ΔpH perturbations. Since regulation of pH_i overlaps with volume regulation mechanistically, subtle volume perturbations should not be neglected either, and altered cytoplasmic pH_i regulation may have effects on ΔpH across compartmental membranes that might then affect subcellular partitioning and traffic of drugs. At this point, until we understand more, further development of the altered partitioning model should entertain a variety of possibilities for different cell types because the molecular details of $\Delta\Psi$ and ΔpH regulation vary widely in different systems. It is not yet known if MDR protein overexpression directly causes $\Delta\Psi$ perturbations (as a channel, channel regulator, or other ion transporter) which then indirectly alters pH_i regulation in some cell types for thermodynamic reasons, or whether the protein perturbs both parameters directly (e.g., as an ion co-transporter or anion channel that translocates HCO_3^- along with some other ion). A third possibility consistent with the idea that MDR protein is an ion transporter regulator (Hardy *et al.*, 1995) is that MDR protein perturbs neither $\Delta\Psi$ nor pH_i directly, but regulates other ion channels/transporters that do by some biochemical mechanism (e.g., as a kinase or via translocating a regulatory entity).

Because of the complexities induced by selecting model MDR cell lines with potent drugs, a recent study (Hoffman *et al.*, 1996) that measures elevated pH_i and decreased $\Delta\Psi$ for several different, independently isolated clones of LR73 fibroblasts overexpressing hu MDR 1 protein, but not previously exposed in any way to chemotherapeutic drugs (see Table II), is important. From these data, as well as similar data for dozens of other nonchemotherapeutic drug-selected transfectants recently created in our laboratory (L.-Y.W., M.M.H., and P.D.R., unpublished data) that express high levels of the MDR protein (Fig. 1), it appears that MDR protein overexpression in and of itself typically elevates pH_i and lowers $\Delta\Psi$ for these fibroblasts. Other preliminary data suggest hu MDR 1 overexpression also depolarizes embryonic kidney cells (M.M.H. and P.D.R., unpublished) and previous work (Roepe *et al.*, 1993) strongly suggests that the protein depolarizes cells of B-cell lineage; thus, these effects are not peculiar to one cell type. Moreover, recent work suggests

Table II. Relative MDR protein (Via Western Blot and Densitometry), Size (by the Single Threshold Coulter Method), $pH_i \pm$ S.D. (by Single-Cell Photometry), $\Delta\Psi \pm$ S.E. (by K^+/val Titration with di-4-ANEPPS; See Roepe *et al.*, 1993; Luz *et al.*, 1994; Wei *et al.*, 1995), and Drug Resistance (by Colony Formation; See Wei *et al.*, 1995) for LR73-Derived hu MDR 1 Transfectants, as well as Parental LR73 cells and a Negative Control Transfectant (LR73/neo) Also Selected with G418[a]

Cell line	Relative MDR protein	Size (μm)	pH_i	$\Delta\Psi$ ($\pm \leq 4$ mV)	Fold resistance to		
					Dox	Vncr	Col
LR73	0	12.04 ± 0.27	7.14 ± 0.03	45	—	—	—
LR73/neo	0	11.91 ± 0.19	7.16 ± 0.03	46	—	—	—
LR73/21	1	11.87 ± 0.74	7.38 ± 0.06	38	+	+	+
LR73/24	11.7	11.91 ± 0.11	7.48 ± 0.04	24	+	+++	++
LR73/27	9.7	11.86 ± 0.26	7.51 ± 0.05	26	+	+++	+
LR73/71	2.7	11.72 ± 0.06	7.42 ± 0.04	29	+	++	+
LR73/88	2.1	11.85 ± 0.21	7.40 ± 0.03	31	+	+	+
LR73/95	2.8	11.57 ± 0.17	7.37 ± 0.05	35	+	++	+
EX4N7 (LR73/mu MDR 1)	5.1	N.D.	7.31 ± 0.04	23	++	+++	++

[a] As described in detail elsewhere (Hoffman *et al.*, 1996), these clones were created without any exposure to chemotherapeutic drug whatsoever; thus they represent the "naked" effects due unequivocally to MDR protein overexpression alone. Also shown for comparison purposes is similar data for EX4N7, a nonchemotherapeutic drug-exposed mu MDR 1 transfectant created in the Gros laboratory (see Luz *et al.*, 1994 and references within). Relative MDR protein is the average of three determinations; note that clone No. 21 is arbitrarily assigned a value of 1. Levels of expression for clones Nos. 24 and 27 are comparable to, for example, DC3F-ADX and are thus among the highest levels of overexpression yet recorded (Hoffman *et al.*, 1996; see Fig. 1). Resistance is expressed as + (1–3-fold), ++ (3–5-fold), or +++ (>5 fold); data shown is the average of two determinations (Wei *et al.*, 1995). Note that all MDR transfectants are depolarized and alkaline. However, in quantitative estimation of $\Delta\Psi$ by K^+/valinomycin titration (Laris and Hoffman, 1986), it is not prudent to place emphasis on the *exact* value of $\Delta\Psi$ calculated for a given cell, only on relative differences between closely related cells [e.g., transfectants derived from the same parent (e.g., Luz *et al.*, 1994 and these data) or cell lines selected from one another in a clonal fashion (e.g., Roepe *et al.*, 1993)]. N.D. denotes not done.

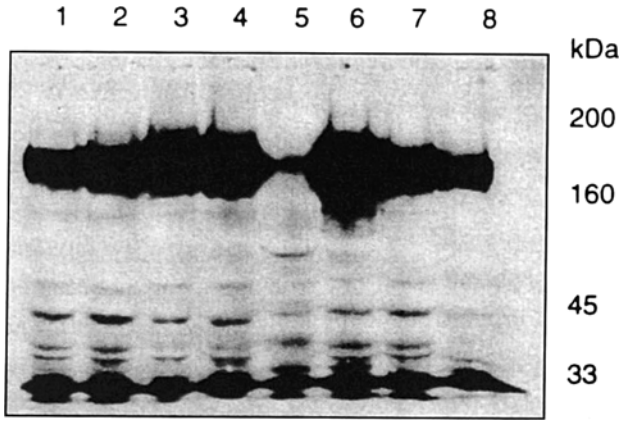

Fig. 1. Western blot analysis of hu MDR 1 protein expression in "pure" transfectants recently created in our laboratory without any exposure to chemotherapeutic drugs whatsoever. Also shown is the level of MDR protein expression in several well-studied drug-selected MDR cell lines. The primary antibody was the monoclonal C219, image resolution was with the peroxidase method, and 100 μg total cellular protein is electrophoresed in each lane of the gel. Lanes 1–4 are clones created in our laboratory (lanes 3 and 4 are clones Nos. 24 and 27 listed in Table II, respectively) and their construction is reported in detail in another paper (Hoffman *et al.*, 1996). Lane 5 is MCF 7-ADR created in the Cowan laboratory (expressing human MDR 1), lane 6 is DC3F-ADX created in the Biedler laboratory (expressing hamster p-GP), lane 7 is LR73 1-1 created in the Gros laboratory (expressing murine MDR 1), and lane 8 is 8226/Dox_{40} created in the Dalton laboratory (expressing human MDR 1). These other cell lines were selected with the chemotherapeutic drugs doxorubicin, actinomycin-D, vinblastine, and doxorubicin, respectively, and exhibit approximately 100-fold, 10,000-fold, 100-fold, and 60-fold levels of resistance to the selecting agent, respectively. Thus, note that much of the resistance exhibited by these cells may not be related to the overexpression of MDR protein (compare to levels of resistance for clones Nos. 24 and 27 in Table II, as well as data in Guild *et al.*, 1988 and Devault and Gros, 1990).

that elevation of pH_i in two cell types may be due at least in part, to inhibition of anion exchanger (AE or Cl^-/HCO_3^- exchanger) (Roepe *et al.*, 1993, 1994; Luz *et al.*, 1994; M.M.H. and P.D.R., unpublished data). Since several isoforms of AE are known to exist and to have different properties, and since very different levels of AE isoforms are found in different cell types, it is anticipated that MDR protein overexpression will produce different pH_i effects in different cell types.

ARE OBSERVED $\Delta\Psi/pH_i$ PERTURBATIONS SUFFICIENT TO CAUSE MDR?

Kinetic and thermodynamic studies of chemotherapeutic drug translocation using large unilamellar vesicles (LUVs) with defined lipid composition, $\Delta\Psi$ and pH_i/pH_o ratios (Mayer *et al.*, 1985, 1986; Praet *et al.*, 1993; Adam Wos, Anthony Scotto, and P.D.R., unpublished) indicate that observed $\Delta\Psi$ and pH_i perturbations in MDR cells could potentially lead to huge decreases in the rates of passive diffusion and the extent of intracellular accumulation of the chemotherapeutic drugs to which MDR cells are resistant. These data and other data (Wei *et al.*, 1995; Robinson and Roepe, 1996) are relevant for understanding slow rates of drug accumulation for MDR cells.

However, whether these $\Delta\Psi$ and pH_i changes are sufficient to cause the MDR *phenotype* (i.e., drug resistance, etc.) is another issue altogether. To address this, we first need to determine precisely how much drug resistance is unequivocally due to MDR protein overexpression and not other events caused by chemotherapeutic drug selection of model MDR cell lines. In this regard, we have only three reports upon which to base our conclusions (Guild *et al.*, 1988; Devault and Gros, 1990; Hoffman *et al.*, 1996), along with the data presented in Table II. All of these data suggest that the level of drug resistance unequivocally mediated by MDR protein overexpression alone is substantially less than that typically found for MDR cells created by selection or maintenance on chemotherapeutic drugs. Thus, importantly, $\Delta\Psi$ and pH_i perturbations caused by MDR protein overexpression do not need to explain levels of drug resistance that are greater than about 10-fold (cf. Table II).

Second, it would be informative to produce changes in $\Delta\Psi$ and/or pH_i of the magnitude observed for MDR cells that are MDR purely via overexpression of the MDR protein (e.g., cells such as those described in Table II) via some other mechanism that does not entail overexpression of the MDR protein or selection with chemotherapeutic drug and then measure whether any resistance exhibited is similar to that reported on in Table II and elsewhere (Guild *et al.*, 1988; Devault and Gros, 1990; Hoffman *et al.*, 1996). In this regard there are only two studies (Wei *et al.*, 1995; Hoffman *et al.*, 1996) wherein similar stable $\Delta\Psi$ or pH_i perturbations are produced. In one example (Wei *et al.*, 1995) stable depolarization was accomplished in NIH 3T3 fibroblasts via overexpression of constituitively active cystic fibrosis transmembrane conductance regulator (CFTR), a well-known Cl^- channel. Levels of chemotherapeutic drug resistance exhibited by the CFTR overexpressors as well as decreased rates of drug accumulation are similar to, but slightly lower than, the levels exhibited by the cell lines summarized in Table

II. However, several CFTR transfectants used in this study were found to exhibit mildly acidic pH_i, which is predicted to sensitize some cells to the toxic effects of some chemotherapeutic drugs (see Wei *et al.*, 1995). Therefore, unless one wishes to envision that the CFTR is also a drug pump, then decreased $\Delta\Psi$ alone is likely sufficient to cause a low-level MDR phenotype. Thus, as alluded to above, the altered partitioning models opens up the possibility for complex patterns of drug resistance; relative changes in pH_i (and even their direction) could be variable in different cell types that have different pH_i regulation, if MDR protein is in any way similar to the CFTR. It will become necessary to define these possibilities for different cell types, and also to determine whether $\Delta\Psi$ and pH_i changes of the magnitude observed are additive or synergistic with respect to conferring drug resistance, if we are to fully appraise the possibilities suggested by the altered partitioning model.

INDIRECT EFFECTS OF $\Delta\Psi$ AND pH_i THAT FURTHER CONTRIBUTE TO RESISTANCE

One final important point with respect to the altered partitioning model is that the effects on drug partitioning and retention caused by MDR protein overexpression do not necessarily need to be, in total, direct electrostatic effects (e.g., altered cytoplasm/extracellular equilibrium distribution of a weak base upon establishing an alkaline inside ΔpH, or reduced rates of passive diffusion of a lipophilic cation upon reducing $\Delta\Psi$). In fact, it has recently been shown (Wei *et al.*, 1995) that decreased electrical potential alone is sufficient to confer resistance to colchicine, which is (an exception to the rule) an *uncharged* drug to which MDR protein overexpression confers resistance (albeit very mild resistance, see Hoffman *et al.*, 1996). Thus, although the precise mechanism(s) have not yet been elucidated, there must be perturbations in cell properties upon membrane depolarization that confer resistance to some drugs via nonelectrostatic effects. For example, perturbations in $\Delta\Psi$ could conceivably translate into altered tubulin organization near the plasma membrane (Aszalos *et al.*, 1986). Since monomeric, but not dimeric, tubulin is a binding target for colchicine as well as vinca alkaloid drugs, changes in $\Delta\Psi$ might contribute to resistance in some cases by altering organization of the *target*. Although this idea is somewhat speculative, the point is that it may be worthwhile to consider less obvious (nonelectrostatic) mechanisms whereby perturbations in $\Delta\Psi$ and pH_i alter the pharmacology of chemotherapeutic drugs. As another important example, it is well known that monomeric tubulin is efficiently titrated into polymerized tubulin by small changes in pH_i, and that the binding of colchicine to monomeric tubulin is very pH dependent. Thus, subtle changes in pH_i can affect the efficiencies of some drug/target interactions in several ways.

Thus, we predict that the effects of $\Delta\Psi$ and pH_i are multifaceted. There is likely a complex relationship between the altered partitioning that occurs in seconds to minutes in some drug transport assays and the longer-term cellular retention of drugs that is observed in hours to days in other experiments. We should not overinterpret possible connections between these types of data. There are likely even more complex relationships between partitioning, retention, and resistance to particular compounds that are yet to be elucidated. That is, we cannot explicitly predict what level of resistance to, for example, doxorubicin, would be measured in a 72-hour growth inhibition assay for a cell line that exhibits a 2-fold decrease in cytoplasmic concentration of the anthracycline as measured in several minutes. Since chemotherapeutic drugs are concentrated enormously (50–100-fold) in cells, relative to external incubating concentrations, it may be more important to consider how small changes in "equilibrium" cytoplasmic concentrations of drug affect binding to target when addressing how altered drug accumulation and intracellular retention translate into drug resistance.

ALTERED ION TRANSPORT IN MDR CELLS AND THE THIRD MODEL: CHANNEL, CHANNEL REGULATOR, OR ION CO-TRANSPORTER?

Pharmacologic complexities aside, is it logical to predict that overexpression of MDR protein causes lowered $\Delta\Psi$ and altered pH_i? Indeed it is, if the protein is hypothesized to transport ions at a significant rate and/or alter the activity of an ion transporter to a significant extent. For example, as mentioned (Wei *et al.*, 1995) overexpression of the CFTR (a Cl^- channel) is predicted to lower $\Delta\Psi$ by increasing the significance of the Cl^- permeability term in the Goldman/Hodgkin/Katz expansion that approximates equilibrium $\Delta\Psi$, and thereby decrease the significance of the K^+ term, which normally dominates eukaryotic plasma mem-

brane $\Delta\Psi$. This is of course experimentally observed to be the case (Stutts *et al.*, 1993; Wei *et al.*, 1995). Higgins and colleagues (Valverde *et al.*, 1992) recently proposed that the MDR protein might be a Cl^- channel, a notion that strongly supports the altered partitioning model. However, note that the Cl^- channel hypothesis has been strongly challenged (Ehring *et al.*, 1994). Also, with regard to pH_i changes in MDR cells, some investigators might predict that depolarization could lead to acidification of pH_i for some cell types, not alkalinization as is typically observed in MDR cells (see also Wei *et al.*, [1995]). Thus, if we are to explain both lower $\Delta\Psi$ and elevated pH_i in several cell types overexpressing MDR protein, we need to consider the possibility that MDR protein functions as more than a Cl^- channel. Higgins and colleagues (Hardy *et al.*, 1995) more recently have suggested that MDR protein is not a Cl^- channel, but rather a channel regulator. In principle, the altered partitioning model is consistent with either scenario.

A different channel regulator hypothesis for ABC proteins such as the MDR protein and the CFTR has more recently been proposed by Al-Awqati and by Guggino and colleagues (Al-Awqati, 1995; Schwiebert *et al.*, 1995), and, like the Cl^- channel model, it has also proved controversial. It is known that the function of several ABC homologues of the MDR protein also appears to include modulation of either $\Delta\Psi$, pH_i, or both (e.g., the CFTR and sulfonyl urea receptor [SUR] proteins). As described by Al-Awqati (1995) and Schwiebert *et al.*, (1995) one possible mechanism for diverse channel regulation by different ABC proteins, that then effects $\Delta\Psi$ and pH_i in different systems, could be via activation of purinergic receptors upon translocation of ATP by the ABC proteins. Cantiello and colleagues (Abraham *et al.*, 1993) originally suggested that MDR protein might directly translocate ATP, based on electrophysiology studies at high ATP concentrations. The key observations were then confirmed for CFTR by the Guggino group and others (Schwiebert *et al.*, 1995). However, the measured ATP conductance in Abraham *et al.*, (1993) and Schwiebert *et al.* (1995) is very small, and the high concentrations of ATP necessary to observe ABC protein-mediated ATP conductance in these experiments (as well as some other experimental details regarding liquid-junction electrodes) aroused considerable skepticism almost immediately. Nonetheless, this model has enjoyed considerable appeal because it could connect several important but very curious observations in ß-cells (site of SUR expression) and lung epithelia (site of CFTR expression) noted upon addition of extracellular ATP to the function of the ABC homologues. That is, effects elicited by addition of extracellular ATP to cells or membranes lacking functional CFTR (e.g., Knowles *et al.*, 1991) are known to at least partially mimic the effects of restoring functional CFTR; thus, transport of ATP by CFTR would make some "physiologic" sense. However, importantly, at least two recent studies (Reddy *et al.*, 1996; Li *et al.*, 1996) strongly challenge the ATP channel hypothesis. In this work, several groups fail to find experimental evidence consistent with direct ATP transport by the CFTR, even in experiments using purified CFTR protein. Thus, if ATP translocation is mediated by ABC protein overexpression in some cells, it may be an indirect effect.

To compare Cl^- transport to ATP transport theories, review of several other recent studies that have measured ion conductances in MDR cells is also important. Several laboratories find evidence to support the contention that MDR protein is either a volume-regulated Cl^- channel or a regulator of a Cl^- channel that is "triggered" in some cells (but not all cells) by hypotonic challenge (Valverde *et al.*, 1992; Gill *et al.*, 1992; Altenberg *et al.*, 1994; Bear, 1994). A recent study reports that this activity may be modulated by PKC (Hardy *et al.*, 1995) which introduces an additional level of complexity, since PKC activity varies among different cell types. Other studies challenge facets of this hypothesis and suggest that MDR protein is not activated by hypotonic challenge (Ehring *et al.*, 1994; Dong *et al.*, 1994), does not play a role in volume regulation (Altenberg *et al.*, 1994), or is in reality perhaps confused with other effects due to chemotherapeutic drug selection (Luckie *et al.*, 1994). A point worth remembering is that 15–20 years of controversy accompanied elucidation of the fact that the protein defective in cystic fibrosis (later identified as CFTR) is a low-conductance, cAMP-dependent Cl^- channel with very complex regulation (Bear *et al.*, 1992), and we are just now beginning to understand its effects on other ion conductances mediated by other channels (e.g., Stutts *et al.*, 1995). If MDR protein is a channel or channel regulator, and if the well-known structural homology between MDR protein and the CFTR (Riordan *et al.*, 1989) predicts any functional similarity at all, sufficient data may not yet have been collected to clearly resolve the issues. We propose that, due to the complex effects of drug selection of most model systems, additional studies with transfectants not exposed to chemotherapeutic drugs (see below) are needed.

In any case, via either the ATP-transporter model, a Cl^--channel model (Valverde *et al.*, 1992), a channel regulator model (Hardy *et al.*, 1995), or a dual function model (Gill *et al.*, 1992), it is clear that intracacies of pH_i regulation for a particular cell type Cl^-/HCO_3^- and Na^+/H^+ exchanger isoforms and their regulation) as well as regulation of any Cl^- conductance mediated directly or indirectly by the MDR protein might lead to subtleties and complexities for $\Delta\Psi$ and pH_i regulation for individual cell types that may not be immediately apparent from simple biophysical theory. The "MDR problem" is rapidly evolving from a fascinating pharmacology problem into an extraordinarily interesting membrane bioenergetics problem.

COMPLEXITY OF THE MODEL SYSTEMS CONFOUND REMAINING ISSUES

All of this may appear confusing, but it is important to note that we are essentially left with two hypotheses for altered drug transport and resistance in MDR cells. That is, MDR protein either directly or indirectly alters drug partitioning and retention. A third possibility that has been proposed is essentially the sum of these two hypotheses (the dual function hypothesis; see Gill *et al.*, 1992). In studying a variety of provocative but sometimes conflicting data, several different logical arguments can be followed by any investigator in judging which model for MDR protein is more or less valid; however, in analyzing all of this, the objective cell biologist, biophysicist, or pharmacologist immediately concludes that matters would be much less confusing if model cell lines were not exposed to chemotherapeutic drugs as a method for causing MDR protein overexpression, since these drugs cause many effects. Thus, transfectants not exposed to chemotherapeutic drugs (e.g., Table II) are most desirable, and better characterization of their drug transport properties is needed. Paradoxically, most MDR researchers do not use this type of model system for a simple and very valid reason; they are extremely difficult to create and when they are manufactured they exhibit much lower levels of drug resistance than is desirable for routine pharmacology studies as discussed above. Nonetheless, cell lines like those in Table II will prove essential for further advances and for a detailed understanding of the biophysics of MDR.

SUMMARY

We propose that the drug pump hypothesis for the MDR protein is an important conceptual simplification of a rather complex phenotype that is likely to have very different character in different cell types. Since $\Delta\Psi$ and pH_i are different and regulated in different ways for various cell types, it is not expected that overexpression of an ion transporter (channel/exchanger/co-transporter or pump), ion transporter regulator, or ATP transporter would have identical effects in every model system. However, it is suggested that after the myriad of effects caused by drug selection of MDR cell lines are eliminated, several important common threads will be apparent. Continued thorough, critical analyses of data from a biophysical, biochemical, and pharmacological perspective is essential. More data is needed with regard to (1) effects of $\Delta\Psi$, pH_i, and volume perturbations on the drug transport measured with model systems, as well as precise calculation of the kinetic and thermodynamic character of that transport, (2) the mechanism of $\Delta\Psi$ and pH_i perturbations in MDR cells, and (3) "calibration" of drug resistance and other properties of MDR cells due unequivocally to MDR protein overexpression and not other effects caused by drug selection. Regardless of whether one prefers the pump or altered partitioning hypothesis, $\Delta\Psi$ and pH_i perturbations in MDR cells will indeed contribute to altered drug partitioning in very important ways. Even if some version of a pump hypothesis (that manages to explain vague kinetics, unprecedented coupling, and an explanation for lack of specificity) is ever proved, further investigation of the molecular basis of $\Delta\Psi$ and pH_i perturbations will then uncover yet unidentified transporters that contribute to this critical aspect of the MDR phenotype. These studies will perhaps identify new chemotherapeutic drug targets and offer insight into the important cell biology mediated by homologues of the MDR protein.

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