Are ion-exchange processes central to understanding drug-resistance phenomena?

Paul D. Roepe and James A. Martiney

Drug resistance in malarial parasites is arguably the greatest challenge currently facing infectious disease research. In addressing this problem, researchers have been intrigued by similarities between drug-resistant malarial parasites and tumour cells. For example, it was originally thought that the role of pfMDR (*Plasmodium falciparum* multidrug resistance) proteins was central in conferring antimalarial multidrug resistance. However, recent work has questioned the precise role of MDR proteins in multidrug resistance. In addition, recent ground-breaking work in identifying mutations associated with antimalarial drug resistance might have led to identification of yet another parallel between drug-resistant tumour cells and malarial parasites, namely, intriguing alterations in transmembrane ion transport, discussed here by **Paul Roepe and James Martiney.** This further underscores an emerging paradigm in drug-resistance research.

The similarities and differences between multidrug resistance (MDR) phenotypes exhibited by tumour cells, certain bacteria and malarial parasites (*Plasmodium falciparum*) have intrigued many researchers for a long time[1–3]. A key role for ATP-binding cassette (ABC) proteins has dominated the drug-resistance literature in recent years; however, it appears that these proteins are unable to explain all phenomena, including levels of resistance and the biophysics of altered drug traffic in many examples of resistant cells[2–7]. In fact, it can be argued convincingly that the role of ABC proteins in conferring MDR phenomena has been overemphasized[8,9]. Recently, several reports that initially appear to be unrelated underscore an unanticipated and interesting role for ion exchange processes in drug-resistance phenomena[10–12]. Dysregulation of ion exchange might be central to the evolution of drug resistance in tumour cells, malarial parasites and some bacteria. The implications of this observation are important because the findings confirm a central prediction of the 'indirect' drug-partitioning model[3,5,13]. This indirect model suggests that altered drug accumulation in resistant cells and microorganisms is fundamentally a result of alterations in pH gradients, electrical membrane potential and perhaps other biophysical parameters, and is not necessarily a result of direct drug trafficking by putative nonspecific hydrophobic drug pumps. If confirmed, the observations predict an important new set of 'targets' for improved therapy of drug-resistant cells and microorganisms, and propose a new way to envisage the emergence of drug-resistance phenomena.

P. D. Roepe, Member, Lombardi Cancer Center Program in Tumour Biology, Assistant Professor, Department of Chemistry, Georgetown University, Washington, DC 20057, USA, and **J. A. Martiney,** Member, The Kenneth S. Warren Laboratories, 765 Old Saw Mill River Road, Tarrytown, NY 10591, USA.

Background

It is estimated that drug-resistant tumour cells are responsible for 500 000 deaths annually in the USA alone, and that deaths from malaria worldwide could approach ten times that number shortly after the millennium. The rapid geographical spread of drug-resistant malaria, as well as the continued rapid evolution of drug-resistant parasites and bacteria is arguably the most pressing concern in pharmacology today.

Thus, there is great excitement about the realization that some common features appear to be shared among drug-resistant tumour cells, malarial parasites and certain gram-negative bacteria. The most important of these features are that drug resistance is typically accompanied by altered intracellular accumulation of the drugs to which cells or microorganisms are resistant, and that certain 'chemomodulators' (such as the Ca^{2+} channel blocker, verapamil) appear to reverse more than one type of drug-resistance phenomenon (for example, both tumour and antimalarial MDR). Thus, although it is still not understood precisely how verapamil reverses MDR in these systems, it is reasonable to expect that molecular concepts from the study of drug-resistant tumour cells might also apply to drug-resistant malarial parasites, and vice versa. Such was the hope when *pfMDR* (*P. falciparum* multidrug resistance) genes were first cloned[14,15] and shown to be similar to the *hu MDR 1* gene, which is overexpressed in a variety of model drug-resistant tumour cell lines[6], and which has been proposed to encode a nonspecific drug pump. Initially, it was thought that drug pumps for chloroquine and other antimalarials must therefore exist in drug-resistant *P. falciparum*, and that these would be similar to the drug pump proposed for tumour cells (thought to be encoded by *hu MDR 1*) that is believed by some investigators to translocate vinblastine, doxorubicin and other anti-tumour drugs. Moreover, this logic predicts that similar compounds might interact with the homologous pumps (for example, verapamil), providing a pharmacological paradigm for reversal of drug resistance in both systems.

However, progress in capitalizing on this idea has been painfully slow. Along with poor progress in understanding the mechanism of verapamil 'chemomodulation', subsequent work on antimalarial drug resistance showed that many drug-resistant *P. falciparum* are not genetically linked to mutation or increased expression of *pfMDR* genes[16,17] and, consequently, other genetic events must be linked to the evolution of drug resistance. At around the same time, an increasing importance for overexpression of other genes was beginning to be

 0165-6147/99/$ – see front matter PII: S0165-6147(98)01282-6

recognized for drug-resistant tumour cells[13,18]. Thus, as in the case of the pfMDR protein and malarial MDR, the precise role of the hu MDR 1 protein in conferring MDR in some tumour cells has been questioned in recent years, as more and more predictions of 'drug pump' models have failed to be substantiated in several laboratories, and as more and more data reflecting additional important consequences of overexpression of the gene encoding hu MDR 1 has been gathered[3,4,6,11,13].

The presence of nonspecific drug pumps in the plasma membrane of drug-resistant tumour cells and the vacuolar membrane of drug-resistant *P. falciparum* is a controversial topic. For example, although many pharmacologists favour the interpretation that the hu MDR 1 protein 'pumps' drugs inside a tumour cell to the extracellular aqueous phase, this has still not been measured directly, and neither is it clear at this point whether this model will ever satisfy critical thermodynamic and kinetic considerations[3,6]. For these reasons, and the fact that drug pump models appear to violate the law of enzyme specificity, the coupling principle for pumps and kinetic principles, some investigators now openly discuss the possibility that the hu MDR 1 protein might cause drug resistance by alternative 'indirect' means[3,19]. Alternatively, based on measurements of ATP-dependent uptake of chemotherapeutic drugs into inside-out vesicles harbouring MDR protein[20], there remains considerable interest in active drug-transport models for the hu MDR 1 protein.

Similar debate and questions regarding the biophysical merits of drug pumps have been raised in the antimalarial drug-resistance field[2,5,16,17], and even in the literature summarizing drug resistance in some bacteria[21]. Although there certainly appears to be altered chloroquine accumulation in drug-resistant *P. falciparum*, some investigators[5,22] have strongly questioned whether altered drug transport by drug-resistant malarial parasites necessarily exhibits the specific thermodynamic and kinetic features of drug pumping. That is, pumping is defined as transport against a substrate concentration gradient with a rate constant faster than passive diffusion, and as pointed out in several more lengthy reviews[3,6], outward pumping of drugs from resistant *P. falciparum* (or tumour cells or bacteria) has yet to be measured directly. While these debates have proceeded, new data from several laboratories have been obtained that might help to resolve some questions by identifying a previously unrecognized similarity in the molecular alterations that accompany the evolution of drug resistance in tumour cells, malarial parasites and some bacteria; namely, dysregulation of ion exchange.

Recent results

Drug resistant P. falciparum *and cg2*

Ground-breaking work on elucidating the molecular alterations that accompany antimalarial drug resistance was published recently by Wellems and colleagues[23] in their report on the primary sequence of cg2. In subsequent work, Lanzer and co-workers[12] examined a number of features of drug-resistant malarial parasites linked to expression of a mutant cg2 allele. They found: (1) a raised pH in the cytosol of drug-resistant *P. falciparum*; (2) what appears to be constitutively activated Na^+–H^+ exchange in resistant parasites; and (3) interesting effects of amiloride derivatives on the altered drug accumulation noted in these resistant parasites. Amiloride compounds are well-known inhibitors of Na^+–H^+ exchangers and some ion channels. Thus, as with verapamil (another ion transport inhibitor that also affects drug accumulation in *P. falciparum*), a compound typically recognized for its effects on ion transport now also appears to have important effects on drug resistance and drug transport. Along with an analysis of interesting amino acid similarities between cg2 and the Na^+–H^+ exchangers of yeast, *Caenorhabditis elegans*, human, rat, rabbit, pig and hamster, these data have recently led Lanzer and colleagues to conclude[24] that mutated cg2 might represent a dysregulated ion exchanger (but see also note added in proof at the end of this article). Other conclusions from this work (for example, that this result indicates cg2 is both an ion exchanger and a chloroquine drug pump) are somewhat controversial[5] and are again strikingly reminiscent of an additional facet to recent debate in the tumour MDR field; namely, it has also been proposed that the hu MDR 1 protein might be both a chemotherapeutic drug pump and an ion transporter or ion transport regulator[25]. However, it might be unnecessary to include direct drug pumping as a component of models for drug resistance protein function, as defined ion transport functions might be sufficient to explain the resistance conferred solely by these proteins[3]. Confusion on this point leads to similar debate in both the tumour and antimalarial MDR literature.

Drug resistant Bacillus subtilis *and* tet *B(L)*

However, this is not where recent curious parallels between altered ion transport and drug resistance end. In a paper that has not attracted the attention it probably deserves, Krulwich and colleagues report a physiological function for the *tet* B(L) locus of *B. subtilis*[10]. This long misunderstood genetic element, linked to the emergence of tetracycline resistance in this bacterium, turns out to catalyse Na^+–H^+ exchange[10]. Perhaps infrequent discussion of this result in the drug resistance community is due to the fact that ion exchangers are usually thought of in terms of more mundane cellular 'housekeeping' tasks, such as intracellular pH (pH_i) and volume regulation, not crucially important clinical phenomena like drug resistance. Thus, it is sometimes argued[26] that ion transport alterations in drug-resistant cells are mere epiphenomena unrelated to the 'more essential' biochemistry regulating drug resistance (that is, drug pumping). Could the putative participation of two ion-exchange processes in two different drug-resistance phenomena (chloroquine resistance in malarial parasites and tetracycline resistant in *B. subtilis*) be mere coincidence?

Tumour drug resistance and hu MDR 1

One might be tempted to say 'yes' to the above question, but perhaps not after analysing recent observations of Roepe and colleagues[11,27,28]. This group has been trying to elucidate the molecular mechanism of well-known alterations in membrane potential and pH_i in MDR tumour cells[13]. Some investigators believe that these perturbations might be sufficient to explain the altered drug accumulation in MDR tumour cells that is specifically a result of P-glycoprotein synthesis[3]. In recent work, detailed single-cell photometry experiments with living cells under constant perfusion reveal a striking and completely unexpected Cl^-–H^+ exchange process in 'true' hu MDR 1 transfectants overexpressing the gene for the hu MDR 1 protein[11]. Once again, an altered ion exchange process has been linked to drug-resistance phenomena.

An important caveat to understanding this novel result in the context of a variety of other hypotheses regarding the function of hu MDR 1 protein in tumour cells is that virtually all previous studies of hu MDR 1 effects in intact tumour cells or transfectants (including most analysis of drug resistance and drug transport) have been performed using cell lines selected or conditioned on potent, complex chemotherapeutic drugs. By contrast, these recent biophysical studies of Roepe and colleagues have been performed solely with true transfectants, not adulterated by selection on chemotherapeutic drugs before analysis. This is important because chemotherapeutic drug exposure causes a variety of effects that can affect drug resistance and events at the cell plasma membrane[28]. Thus, in the work reported in Ref. 11, the 'unadulterated' biophysical effects of hu MDR 1 in whole cells are studied for the first time. These effects appear to include rather remarkable perturbations in apparent ion exchange. This third example of dysregulated ion exchange linked to a drug resistance phenomenon drives home an important point for pharmacologists: we believe the common thread in these observations with *P. falciparum*, *B. subtilis*, and true hu MDR 1 transfectants might be more than mere coincidence, and that it points to a new paradigm in drug resistance research.

To illustrate this further, it has always perplexed us that in one of our first studies with drug-resistant myeloma tumour cells[13] we found that the overexpression of the gene for hu Na^+–H^+ and hu Cl^-–HCO_3^- exchangers were more dramatic events in the evolution of doxorubicin resistance than even the overexpression of the gene for hu MDR 1. Since that time, other perturbations in the synthesis of ion exchangers or their activity have been noted in other cell models of chemotherapeutic multidrug resistance[27–30]. Moreover, although Cl^- and HCO_3^- transport is altered in MDR tumour cells with altered levels of Cl^-–HCO_3^- exchanger gene expression, the alterations are in the wrong direction. That is, increased expression of Cl^-–HCO_3^- exchanger mRNA has been associated with decreased apparent Cl^-–HCO_3^- exchange activity in MDR tumour cells[13,29], which suggests the presence of an additional interesting ion transport process that 'competes' with Cl^-–HCO_3^- exchange. One curious possibility strongly supported by recent studies[11] is an additional Na^+- dependent Cl^-–H^+ exchange process that must be mediated directly or indirectly by hu MDR 1 protein. That is, we suggest that the overexpression of the hu MDR 1 gene is one common example of several possible ion-transporter gene overexpression or mutational events that occur in tumour cells upon selection with chemotherapeutic drugs. By analogy, the same might be true for cg2 and antimalarial selection of *P. falciparum*, and tet B(L) and antibiotic selection of *B. subtilis*, etc. These events might represent attempts by the cell or microorganism to change key biophysical parameters of the cell (such as pH and electrical gradients) to manipulate physical–chemical interactions between hydrophobic drugs and the cell to the cell's 'advantage'.

Discussion

In summary, recent data from different laboratories working on unrelated drug resistance problems involving bacteria, malarial parasites and tumour cells all reveal surprising alterations in ion exchange linked to the emergence of drug resistance. Much can be learned by asking the following questions: (1) what are the possible consequences of altered ion exchange with regard to relevant pharmacological issues (such as decreased interaction with the drug target in the resistant cell or microorganism), and (2) why do these alterations occur in ion exchangers and not in ion pumps or channels? The first question is easier to answer, as much work relating to this has already been done[3,6,27,31–33]. Depending on the transported ions and their transport stoichiometry, ion exchangers could conceivably influence both pH gradients and membrane potential. Starting with experiments using model liposomes and artificial pH gradients or membrane potentials, it has been shown that these biophysical parameters could have tremendous (in some cases, not obvious) overlapping effects on transmembranous partitioning of chemotherapeutic drugs and other compounds[31–33]. A detailed theoretical analysis of some of these effects was recently presented[3] but not every effect is completely understood. In particular, drug 'aggregate' formation at dielectric boundaries, and the effects of membrane potential and pH, or both, on these phenomena are not well understood, but need to be if the pharmacology of chemotherapeutic drugs, antimalarials, and antibiotics, as well as the complete role of membrane potential and pH perturbations (as a result of altered ion transport) in drug resistance are to be understood. Beyond direct biophysical effects, other recent studies also hint at a role for these biophysical perturbations in influencing signal transduction, which is critically important in pharmacology (for example, signal transduction associated with the apoptotic cascade[34]). This adds an additional 'layer' to understanding the complete role of these perturbations in determining drug resistance.

The second question is more difficult to answer at this time, but is perhaps just as interesting, as exchangers are less common than pumps and channels. Although there is currently only a small amount of data in favour of the ideas, some suggestions that might be helpful in future work can be offered. In these three cases (altered Na^+–H^+ exchange in resistant malarial parasites and *B. subtilis*, and altered Cl^-–H^+ exchange in tumour cells) an ion that is conceivably important for regulating membrane potential or volume (such as Na^+ or Cl^-) as well as an ion that is important for regulating pH (H^+), are both being translocated in a different way by the resistant cell or parasite. Thus, the possibility for concomitant perturbations in membrane potential, volume and pH exists. Because these parameters have overlapping and complementary effects on altering drug distribution[3,31–33], altering ion exchange might provide more possibilities for evading the toxic effects of these compounds. Alternatively, there might be common elements in promoter regions of ion-exchanger genes that respond to the stress induced by selection with toxic hydrophobic drugs. Some of the earliest changes in cells induced to undergo apoptosis are changes in cell volume and pH (Ref. 34), and these might be causal or permissive for subsequent events in the apoptotic cascade. A cellular response by cells attempting to avoid 'death' (for example, apoptosis) that entails altered expression/activity of the ion transporters particularly important for the control of pH and volume (such as ion exchangers) appears to us to be quite logical, if not obvious.

Concluding remarks

Recent data from three different branches of the drug-resistance community (bacterial, malarial parasite and tumour cell) point to an intriguing coincidence; namely, alterations in ion-exchange processes appear to be involved intimately in the evolution of distinct drug-resistance phenotypes. This mirrors another recent coincidence in these three fields, namely, emphasis on the putative participation of nonspecific drug pumps in conferring drug resistance in these three systems, and the subsequent difficulty in confirming many predictions of the drug pump models[3,6]. As more data has been gathered, detailed thermodynamic and kinetic evidence for drug pump processes has proved difficult to obtain. One frequently argued possibility is that pumping of these compounds is extremely difficult to measure because of high lipid/saline partitioning coefficients. Another is that alterations in ion transport, including these preliminary indications of altered ion exchange, play a more important role in altering drug transport and conferring drug resistance than was previously thought to be the case. We suggest that more precise quantitation of the levels of resistance specifically conferred by overexpression or mutation of the genes for key drug resistance proteins [cg2, MDR 1, tet B(L)], along with determining whether these levels of resistance are compatible with the biophysical perturbations that accompany overexpression or mutation (that is, changes in pH, membrane potential and perhaps volume) are important areas for additional work. Ultimately, the precise molecular details of these alterations in ion transport will need to be understood to design chemotherapeutic strategies for circumventing resistance phenomena. Drug-resistance research is rapidly expanding to include key questions in cell physiology which, when answered, will strongly complement the great progress made in recent years with the isolation of various drug-resistance genes.

Note added in proof

A recent short report from Wellems and colleagues[35] questions the conclusion that cg2 could directly catalyse ion exchange, because immunoelectron microscopy reveals that cg2 protein is apparently localized to vesicle-like structures in the parasitophorus space and the food vacuole, and perhaps not within the plasma membrane itself. Thus, cg2 might regulate pH and ion exchange, but may not directly catalyse Na^+–H^+ transport across the plasma membrane.

References

1 Karcz, S. and Cowman, A. F. (1991) *Exp. Parisitol.* 73, 233–240
2 Bray, P. G. and Ward, S. A. (1998) *Pharmacol. Ther.* 77, 1–28
3 Wadkins, R. M. and Roepe, P. D. (1997) *Int. Rev. Cytol.* 171, 121–165
4 Bornmann, W. G. and Roepe, P. D. (1994) *Biochemistry* 33, 12665–12675
5 Martiney, J. A., Cerami, A. and Slater, A. F. G. (1995) *J. Biol. Chem.* 270, 22393–22398
6 Roepe, P. D. *et al.* (1996) *J. Bioenerg. Biomembr.* 28, 541–555
7 Bray, P. G., Hawley, S. R. and Ward, S. A. (1996) *Mol. Pharmacol.* 50, 1551–1558
8 Martin, S. K. (1993) *Parasitol. Today* 9, 278–279
9 Houghton, P. J. and Kaye, S. B. (1994) *J. Natl Inst. Health Res.* 6, 55–61
10 Cheng, J., Guffanti, A. A. and Krulwich, T. A. (1994) *J. Biol. Chem.* 269, 27365–27371
11 Hoffman, M. M. and Roepe, P. D. (1997) *Biochemistry* 36, 11153–11168
12 Wünsch, S. *et al.* (1998) *J. Cell Biol.* 140, 335–345
13 Roepe, P. D. *et al.* (1993) *Biochemistry* 32, 11042–11056
14 Wilson, C. M. *et al.* (1989) *Science* 244, 1184–1186
15 Foote, S. J., Thompson, J. K., Cowman, A. F. and Kemp, D. J. (1989) *Cell* 57, 921–930
16 Wellems, T. E. *et al.* (1990) *Nature* 345, 253–255
17 Barnes, D. A., Foote, S. J., Galatis, D., Kemp, D. J. and Cowman, A. F. (1992) *EMBO J.* 11, 3067–3075
18 Cole, S. P. C. *et al.* (1992) *Science* 258, 1650–1654
19 Schindler, M. *et al.* (1996) *Biochemistry* 35, 2811–2817
20 Ruetz, S. and Gros, P. (1994) *J. Biol. Chem.* 269, 12277–12284
21 Parra-Lopez, C., Lin, R., Aspedon, A. and Groisman, E. A. (1994) *EMBO J.* 13, 3964–3972
22 Ginsburg, H. and Stein, W. D. (1991) *Biochem. Pharmacol.* 41, 1463–1470
23 Su, X-Z., Kirkman, L. A., Fujioka, H. and Wellems, T. E. (1997) *Cell* 91, 593–603
24 Sanchez, C. P., Horrocks, P. and Lanzer, M. (1998) *Cell* 92, 601–602
25 Hardy, S. P. *et al.* (1995) *EMBO J.* 14, 68–75
26 Sharom, F. J. (1997) *J. Membr. Biol.* 160, 161–175
27 Hoffman, M. M., Wei, L-Y. and Roepe, P. D. (1996) *J. Gen. Physiol.* 108, 295–313
28 Wei, L-Y., Hoffman, M. M. and Roepe, P. D. (1997) *Am. J. Physiol.* 272, C1642–C1653
29 Luz, J. G. *et al.* (1994) *Biochemistry* 33, 7239–7249
30 Roepe, P. D. *et al.* (1994) *Biochemistry* 33, 11008–11015
31 Mayer, L. D. *et al.* (1985) *Biochim. Biophys. Acta* 816, 294–302
32 Mayer, L. D., Bally, M. B. and Cullis, P. R. (1986) *Biochim. Biophys. Acta* 857, 123–126
33 Praet, M., Defrise-Quertain, F. and Ruysschaert, J. M. (1993) *Biochim. Biophys. Acta* 1148, 342–350
34 Robinson, L. J. *et al.* (1997) *Biochemistry* 36, 11169–11178
35 Wellems, T. E. *et al.* (1998) *Cell* 94, 285–286

Acknowledgements
Work reviewed here that was performed in the Roepe and Martiney laboratories was supported by the NIH (RO1 GM54516, and RO1-AI-30660-S1, respectively) and grants to P. D. R. by the Wendy Will Case fund and the Cystic Fibrosis Foundation. We thank our laboratory colleagues for their hard work and many helpful discussions.