changes cannot follow from changes in fluidity. More recently, we have shown that nonylphenol, which strongly inhibits ATPase activity, reduces the equilibrium level of phosphorylation of the ATPase by phosphate, again a result which cannot be explained in terms of effects of fluidity (F. Michelangeli, S. Orlowski, P. Champeil, J. M. East & A. G. Lee, unpublished work).

The idea that the activity of membrane-bound enzymes should be sensitive to membrane fluidity is intrinsically attractive for, after all, we know that enzymes undergo conformational changes and it seems reasonable that the more fluid the environment the easier will be these changes. Nevertheless, for the $(Ca^{2+}-Mg^{2+})$-ATPase there is no evidence that the fluidity of the surrounding lipid has any significant effect on activity, while there is evidence that other effects must be important. There seems to be no reason to suppose that the ATPase is in any way unusual in this respect.

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Interaction of anthracyclines with plasma membranes from tumour cells: implications on drug resistance

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Clinical oncologists are much too familiar with situations in which cancers that responded initially well to chemotherapy, became completely unresponsive upon relapse. Changes in the dose administered or in the drug or drugs being used, would not result in significant improvement, as the unresponsive tumour seems to be cross-resistant even to drugs that were never used during the initial treatment. This acquired cross-resistance, referred to as 'multidrug resistance' (MDR), constitutes a major obstacle to the success of chemotherapy programmes and thus the elucidation of its molecular basis is of great interest.

MDR is associated with a decreased intracellular accumulation of chemotherapy agents and to the over-expression of a high molecular mass glycoprotein, known as P-glycoprotein, which has a high degree of sequence similarity to bacterial haemolysin export protein, and it has been suggested that it acts as an ATP-dependent, protein pump to actively eliminate drugs from cells.

Anthracycline antibiotics, such as daunomycin (DNM), are among the most potent cytotoxic drugs currently used as antitumour agents. The classical model for explaining anthracycline cytotoxicity has been based upon binding to intracellular sites and interference with nucleic acid function [1]. However, based upon the cytotoxicity demonstrated for non-penetrating, polymer-immobilized drugs [2], effects at the level of the cell membrane have also been proposed.

The previous paragraphs strongly suggest that both the mechanisms of drug cytotoxicity and MDR should have in common a central role for the plasma membrane of the tumour cell as either a cytotoxic site itself and/or regulating the transport of the drug in and out of the cell. To test the above hypothesis, we began by studying binding of anthracyclines to isolated plasma membrane fractions from anthracycline-sensitive and -resistant P388 murine leukaemia cell lines. Use of isolated plasma membranes in binding studies reduces to a minimum difficulties encountered when using whole-cell systems, such as simultaneous drug metabolism, internalization or binding to other cellular components. Equilibrium binding studies using ultracentrifugation and fluorescence anisotropy techniques, indicate that plasma membranes from drug-sensitive cells exhibit an approximately 2-fold higher affinity for binding of DNM than those derived from drug-resistant cells.

The observed binding of DNM to plasma membranes does not appear to involve protein components of the membranes. This conclusion arises from equilibrium binding experiments in which the plasma membrane fractions from both cell lines are previously heated at temperatures at which membrane proteins are denatured or treated with trypsin under conditions at which extensive proteolysis of membrane proteins occurs. Heat-denatured or trypsin-treated membranes used in the drug-binding assays, exhibit binding parameters which are very similar to those from untreated samples, thus indicating that the protein components of the membranes do not have a major role in determining drug binding or in establishing the observed resistance-related differences in drug-binding affinity. However, this is in contrast with reports by Pastan's group [3], in which binding of vinblastine to plasma membrane from drug-sensitive and -resistant KB cells appeared to be exclusively mediated by a resistance-related, high-molecular-mass membrane glycoprotein. We do not have a satisfactory explanation for this apparent discrepancy, although it might be possible that cellular drug resistance could be differently manifested in different cell lines. Nevertheless, it should be noticed that Pastan's group measured drug binding by using an ultrafiltration assay in which dilution, filtration and wash steps took approximately 15 s [3]. Under those conditions, with a reported K_d of 1.5 μM, and assuming a moderate rate for the dissociation of drug–acceptor complexes [4], it is likely that a good amount of the bound vinblastine would have dissociated in less than 15 s and, therefore, the published data could represent drug binding remaining upon dissociation from a high-affinity,

Abbreviations used: MDR, multidrug resistance; DNM, daunomycin; PS, phosphatidylserine; PC, phosphatidylcholine.

low-capacity site (i.e. the high-molecular-mass glycoprotein), rather than the total drug binding to the plasma membrane.

A comparison between the lipid composition of plasma membranes from drug-sensitive and -resistant P388 cells indicates differences in the relative abundance of several lipid components, namely phosphatidylserine (PS) and also cholesterol. To test whether the different lipid components present in our membrane fractions could be partly responsible for the observed differences in drug binding, artificial liposomes were made from egg phosphatidylcholine (PC), which contained PS and/or cholesterol at different molar ratios. This hypothesis appeared reasonable, since it was previously shown that anthracyclines were able to bind to artificial liposomes [5], to natural heart mitochondrial [6] and to *Torpedo* postsynaptic membranes [7], where the observed binding of the drugs is preferentially, if not exclusively, mediated by membrane lipids. Equilibrium binding data using the liposomes from above, indicate that both the drug binding constant and the stoichiometry were decreased and increased, respectively, when the molar fractions of cholesterol and PS within the liposomes were increased. Furthermore, the effects caused by increasing PS can be emulated by the presence of dicetyl phosphate in 'negatively charged' liposomes [8], thus indicating that electrostatic interactions between the drug and the liposome surface are important in determining total drug binding to membrane sites. On the other hand, cholesterol appears to be able to compete with the drug for the occupation of internal sites within the bilayer, as indicated by fluorescence-energy transfer and fluorescence-quenching techniques. This is consistent with the observation that this and other techniques, including circular dichroism [9], indicate that drug binding to membranes occurs by accommodation of the drugs into both surface- and lipid-intercalated domains of the membranes. Considering all of the above, the observed differences in drug binding to plasma membranes from drug-sensitive and -resistant P388 cells seem compatible with the observation that plasma membranes from drug-resistant cells contain less PS and more cholesterol than those from drug-sensitive cells. In fact, when liposomes are made from PC/PS/cholesterol mixtures at molar ratios resembling those found in plasma membranes from drug-sensitive or -resistant cells, our results indicate that the binding parameters for the interaction of DNM with these liposomes are almost indistinguishable from those exhibited by the corresponding native plasma membranes. Our conclusion is, therefore, that PS and cholesterol have an important role in modulating the binding of the drug to entire plasma membranes and in establishing the observed resistance-related differences.

Our observations on DNM binding to plasma membranes may also offer some insight into the possible mechanism(s) of transport of anthracyclines across plasma membranes. Three possibilities have been entertained to explain drug accumulation in intact cells: passive diffusion through the plasma membrane, with or without the selective removal of the drug via a metabolic energy-dependent efflux pump, or facilitated diffusion involving a protein carrier [10]. The preferential binding of the drug to membrane lipids and its ability to interact with both surface and core domains of the bilayer [7, 11, 12] suggests that anthracyclines may diffuse through the membrane passively and that such diffusion would be partly controlled by properties of the bilayer. However, for passive diffusion to explain more fully a differential drug uptake by drug-sensitive or -resistant cells, other factors such as the intracellular pH and the pK of the drug need to be taken into consideration. DNM has a pK in the range 7.6–8.2, corresponding to ionization of an amino group at the daunosamine sugar residue and, therefore, it exists in neutral and ionized forms at physiological pH. From these, the neutral, uncharged species moves more readily across membranes and therefore is preferentially taken up by cells. Once inside the cells, an equilibrium would be established which would be dependent upon intracellular pH: the more acidic the intracellular pH, the more ionized forms of the drug would be present. These more abundant ionized species, present under more acidic conditions, are preferentially bound by intracellular sites, including DNA [13], nucleotides [14] or phospholipids [6–8, 11] and thus an increase in intracellular drug content would result because the equilibrium between intracellular and extracellular free drug would be shifted. Intracellular pH measurements in P388-sensitive and -resistant cells, using pH-sensitive fluorescence probes, have been carried out in our laboratory and, in fact, a more acidic intracellular pH has been detected in drug-sensitive cells. Furthermore, this rationale has been exploited to increase the efficiency of anthracycline trapping into liposomes as carrier systems, in response to a transmembrane pH gradient (interior acidic) [15].

Our conclusion, based on passive diffusion phenomena, is that the observed differences in plasma membrane lipids and in intracellular pH between drug-sensitive and -resistant cells, may partly explain why drug-resistant cells exhibit a reduced intracellular drug accumulation. The proposed ATP-dependent, drug-efflux pump activity of the P-170 glycoprotein [16] would be an added mechanism to secure efficient elimination of the drug once it gets inside the cells.

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