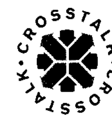
Can prenylcysteines be exploited as ligands for mammalian multidrug-resistance transporters?



Lynne M Hurwitz and Patrick J Casey

The overexpression of specific transport proteins in the membrane of many cancer cells renders these cells resistant to many therapeutic drugs. Some lipid-modified cysteine compounds inhibit one drug-transporting protein, indicating the potential of developing such compounds as therapeutic agents.

Address: Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham NC 27710-3686, USA.

Correspondence: Patrick J Casey
E-mail: pjc@galactose.mc.duke.edu

Chemistry & Biology October 1997, 4:711–715
http://biomednet.com/elecref/10745521004007 11

© Current Biology Ltd ISSN 1074-5521

Introduction

The presence of drug-resistant neoplastic phenotypes, particularly those that are resistant to multiple classes of pharmacotherapeutics, is a significant problem in medicine [1]. These phenotypes render standard medical treatments ineffective and, as a result, have increased the rates of mortality in patients who have such drug-resistant neoplasms. The resistance of tumor cells to distinct anticancer agents is known to be mediated by several different mechanisms. The ability of a neoplasm to escape the toxic effects of chemotherapy depends on the capacity of the neoplastic cells to reduce the effectiveness of chemotherapeutic drugs. This has been shown to occur by various cellular mechanisms that can reduce the intracellular concentration of the drug, alter the intracellular target of the drug, modify cellular repair mechanisms and/or alter the signaling pathways that allow the cell to escape from apoptotic death [2,3]. It is the first of these mechanisms, the reduction of the intracellular drug concentration, that we will discuss here.

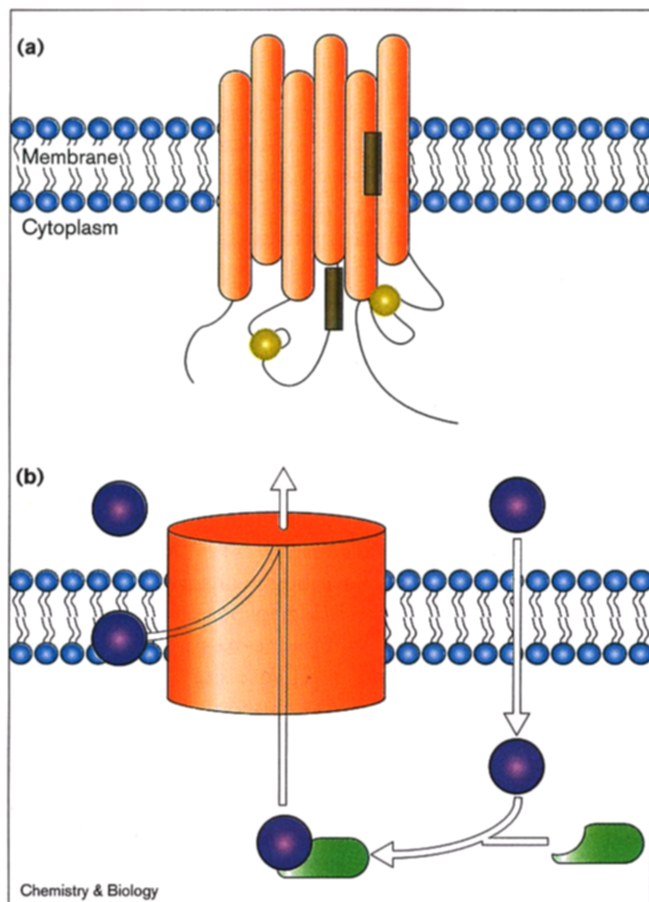
The resistance of cancer cells to a wide variety of therapeutics derived from natural products is one particularly prevalent type of resistance, termed multidrug resistance (MDR). It is generally characterized by the overexpression of cell-surface transporters that actively transport the drug out of the cells [3,4]. Research in a number of laboratories over the past decade has led to the identification of at least two distinct transporters that are involved in this process and to several theories about how multidrug resistance arises. As a result of these studies, pharmacological intervention to overcome this problem is becoming a real possibility. Although several classes of pharmacological agents have been developed that can reverse drug resistance in model cell systems, clinical studies using these agents have not fared well because the compounds cause significant side effects [5]. Novel inhibitors that are highly specific for the cell-surface transporters are now needed in order to overcome the drug-resistant phenotypes. In this article, we address how cell-surface transporters confer drug resistance in neoplastic diseases. We also discuss recent results from our laboratory concerning a novel class of ligands directed against the transporters, and the potential use of these ligands to prevent drug resistance.

Multidrug transporters

The transporters involved in multidrug resistance belong to a family of transmembrane proteins termed ATPase binding cassette (ABC) proteins that are found in both prokaryotic and eukaryotic cells [6]. The basic structure of these proteins appears to have been conserved during evolution. Members of the ABC transporter family characteristically contain a membrane-spanning region, a ligand-binding domain and an ATPase site (Figure 1a). The membrane-spanning region is composed of hydrophobic amino acids and typically contains six transmembrane spans, whereas the ATPase site and the ligand-binding site are located on the cytoplasmic domains of the protein. The characteristics of the ligand-binding site determine the substrate-binding specificity, and presumably the transport specificity, of the protein. All these proteins transport substrates in an ATPase-dependent fashion [4,6].

Two cell-surface proteins belonging to the ABC transporter superfamily have been shown to function effectively in the removal of chemotherapeutic drugs from neoplastic cells. These two proteins, termed the type 1 multidrug-resistance transporter (MDR1, also known as P-glycoprotein) and the multidrug-resistance-associated protein (MRP), were originally identified as proteins that were highly overexpressed in mammalian cells that have the multidrug-resistant phenotype [7,8]. There are theories about how this increased expression of transporters develops. For example, it has been proposed that the expression or amplification of genes encoding these transporters is a consequence of a cell's response to chemotherapeutic agents. A second theory is that a small population of the initial neoplastic cells has this phenotype of amplified expression, and this population then expands after escaping the toxic effects of chemotherapy [1]. In addition to their involvement in drug resistance in neoplasms, members of the ABC transporter family have also been implicated in the emergence of drug resistance in infectious diseases such as leishmaniasis and malaria. Elucidation of the role of these transporters in conferring drug resistance in these parasitic infections is still in its early stages [9,10].

Figure 1



The transporters involved in drug resistance. **(a)** The topology of ABC transporters. Red, membrane-spanning region; yellow, ATP-binding site; brown, potential ligand-binding site(s). **(b)** The proposed mechanisms of drug transport by MDR1 and MRP. Substrates (purple) could interact with the transporter while the substrate is within the phospholipid bilayer (the so-called 'vacuum phenomenon') or after the substrate has entered the cytoplasm. In the latter case, the substrate may be directed to the transporter after binding an intracellular target (green).

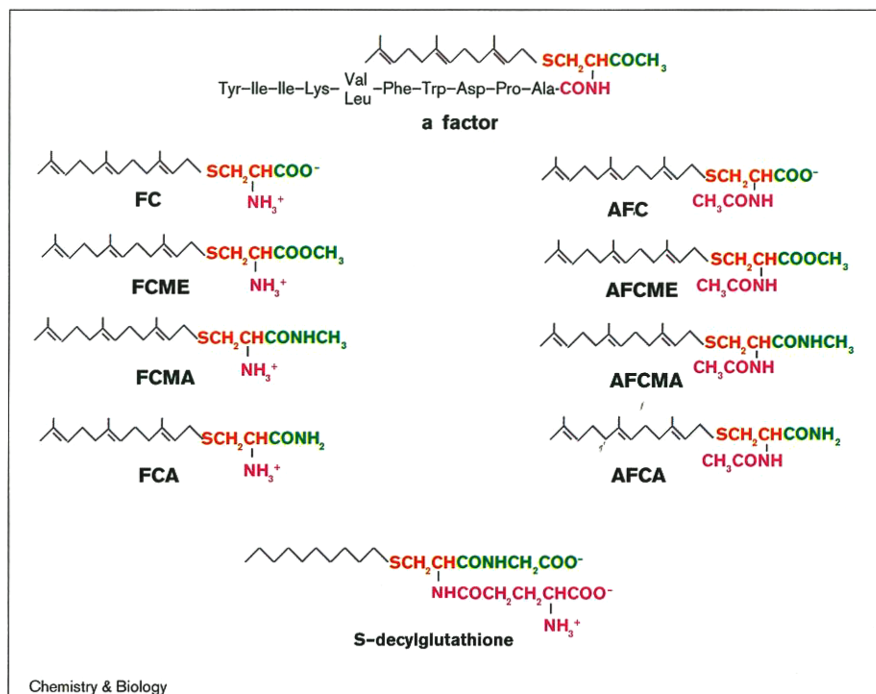
The exact mechanisms by which MDR1 and MRP recognize and transport chemotherapeutic drugs are not yet clear, but the main routes through which it is thought to occur are shown in Figure 1b. Drugs may interact with the transporter when they initially diffuse into the phospholipid bilayer, so that the drug never actually enters the cytoplasm of the cell. Alternatively, the drug may pass through the phospholipid bilayer, and the transporter could then expel the drug from the cytoplasm. In this situation, an as-yet-unidentified cellular process may exist that directs the chemotherapeutic drug to the transporters after the drug has entered the cell. Another cellular mechanism to reduce intracellular concentrations of certain cytotoxic drugs is for the cell to chemically modify the drug in such a way as to allow more efficient excretion of the drug; the most characterized modification of this type is the conjugation of a drug to glutathione by the enzyme glutathione-S-transferase [11,12].

MDR1 is by far the best-characterized transporter that is involved in drug resistance; amplified expression of MDR1 has been implicated in conferring drug resistance in a wide variety of solid tumors as well as several types of leukemia and lymphoma [3,4]. Biochemical characterization of the protein showed that it requires both ATP and magnesium for its transport functions, and that ATP hydrolysis is essential for the transport process [4]. MDR1 recognizes several chemotherapeutic drugs, including taxol, colchicine, vinblastine, doxorubicin, verapamil and cyclosporine A. The MDR1 protein is expressed intrinsically in several mammalian organs and tissues including the adrenal cortex, the proximal tubules of the kidney, the liver, the intestinal epithelium, the pancreatic ducts, specific lymphocytes subclasses, the ovarian follicles, and the capillaries of the testis and of the central nervous system [3]. This distribution of MDR1 in normal human tissue has led to the proposition that MDR1 has a physiological role in tissue detoxification. One recently elucidated potential function of MDR1 depends on its ability to act as a 'flippase' that can translocate short chain lipids, such as phosphatidylcholine, across a membrane bilayer; this process is thought to be particularly important in the production of bile [13]. In addition, the disruption of the gene encoding the MDR1 homolog in mice leads to damage of the nervous system early in life; this has been interpreted as indicating that MDR1 acts as an essential toxin exporter along the blood–brain barrier [14].

MRP was first identified in 1992 in a lung-cancer cell line that was cultured in the presence of the chemotherapeutic agent adriamycin [8]. The MRP protein, which is somewhat larger than MDR1, has been found to be overexpressed in several solid tumor and leukemia cell lines. Like MDR1, it can transport chemotherapeutic drugs out of cells in an ATPase-dependent fashion. MDR1 and MRP share limited specificity in drug recognition; MRP recognizes and transports etoposide, doxorubicin, daunorubicin, vincristine and epirubicine. Unlike MDR1, however, MRP does not interact with taxol, colchicine or vincristine with high specificity [15]. MRP is expressed under normal conditions in several mammalian cell types, most notably in the testis, the lung and peripheral blood mononuclear cells.

Although the physiological functions of MRP are currently unknown, this transporter has been shown to be important in human inflammatory reactions and in drug detoxification in the liver. Its role in inflammatory reactions has been inferred primarily from the finding that it can transport leukotriene C4, a known modulator of inflammatory reactions in humans [16]. The presence of jaundice in mice that have a mutation in a gene encoding a homolog of MRP led to the proposal that MRP is involved in drug

Figure 2



The structures of some lipid-modified cysteine compounds studied in association with multidrug transporters. Shown are the parent 15 carbon farnesylcysteine (FC) and a variety of its derivatives. FCME, FCMA and FCA, which are the methyl ester, methyl amide and amide versions of FC respectively, are all substrates for MDR1, whereas their N-acetylated counterparts, AFCME, AFCMA and AFCA, are inhibitors of this transporter. For comparison, the structures of the prenylcysteine prototype (the yeast mating pheromone **a** factor) and S-decylglutathione are also shown. The thiol backbone is shown in red, the α-amino group and its modifications in pink and the α-carboxyl group and its modifications in green.

detoxification in the liver [17]. MRP has also been reported to transport S-decylglutathione [18], a compound that is similar in structure to the prenylcysteines that interact with MDR1 (see below). Importantly, S-decylglutathione, like other identified transport substrates of MRP, such as leukotriene C4 and glutathione-S-conjugates [19,20], has a net negative charge. In contrast, MDR1 has a preference for molecules that contain a positively charged group [3,4].

Eukaryotic cells can be made to produce high levels of MDR1 and MRP through cDNA expression, allowing a detailed assessment of the interactions of the proteins with various compounds. The baker's yeast *Saccharomyces cerevisiae* has proven especially useful for examining the biological consequences of such interactions [21,22], and high levels of expression of MDR1 and MRP in the Sf9 insect cell system have allowed their biochemical properties to be studied in detail [18,23,24]. Our laboratory has exploited the Sf9 expression system in particular. Using membranes from Sf9 cells that are infected with recombinant baculovirus encoding MDR1, we have identified a new class of compounds that interact with MDR1 — specific lipid-modified cysteines, termed prenylcysteines, and their derivatives [25,26].

Prenylcysteines as ligands for multidrug transporters

The covalent attachment of lipids to proteins is a process that occurs in essentially all cells. One of the most prevalent types of lipid modification in eukaryotic cells is prenylation, which involves the attachment of the 15 carbon farnesyl or 20 carbon geranylgeranyl isoprenoid to cysteine residue(s) close to the carboxyl terminus of a protein. In most cases, the attached isoprenoid directs the protein to specific membranes within the cell and may also be involved in critical protein–protein interactions [27].

Studies on a yeast MDR1 homolog, the Ste6 transporter protein, provided the first evidence for the potential of MDR1 to function as a transporter of S-prenylated compounds. Ste6 is dedicated to the export of a specific mating pheromone peptide, **a** factor, from yeast cells that produce it [28]. Yeast cells lacking Ste6 are unable to mate because they cannot secrete **a** factor into the medium. Mature **a** factor contains a post-translationally modified carboxyl terminus in which the carboxy-terminal cysteine is S-prenylated by the farnesyl isoprenoid and methylated on the carboxyl group (Figure 2). This modified carboxy-terminal cysteine is a key structural feature that is recognized by Ste6, because yeast strains defective in carboxy-terminal cysteine processing are unable to produce an exportable **a** factor peptide [29,30]. The finding that expression of MDR1 in yeast cells lacking Ste6 could complement the mating defect and at least partially restore mating activity suggested that the similarities between MDR1 and Ste6 extend to function as well as structure [21]. Later studies revealed that MRP could also function in this regard [22]. Collectively, these studies implied that the modified carboxyl terminus of **a** factor

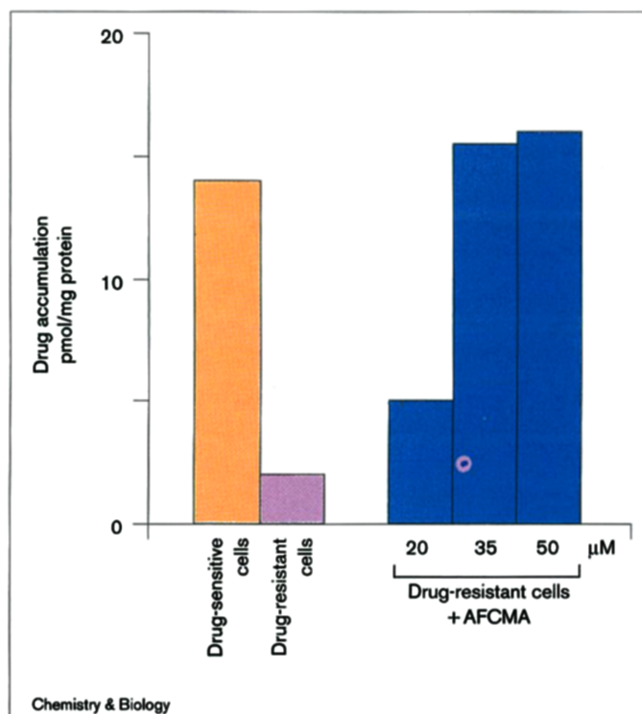
that is recognized by Ste6 is also involved in the specific interaction of **a** factor with both MDR1 and MRP, and indicated that an understanding of the mechanisms involved in this process could lead to important insights into both drug transport and the physiological functions of MDR1 and MRP.

We have found that specific prenylcysteine-containing compounds that correspond to the carboxy-terminal structures of prenylated proteins interact with MDR1 in a characteristic substrate-like way [25]. The structural features that are required for the specific interaction between prenylcysteine methyl esters and MDR1 were strikingly similar to those required for the interaction of **a** factor with Ste6. In both cases, carboxyl methylation of the prenylcysteine was essential, whereas neither transporter could apparently distinguish between a 15 carbon farnesyl or 20 carbon geranylgeranyl isoprenoid on the sulfhydryl group of cysteine. Prenylcysteine-containing compounds have provided intriguing evidence for a potential physiological link between MDR1 and prenylcysteine production in mammalian cells; they are also simple compounds that allow investigation of the essential structural elements that are recognized by MDR1.

Prenylcysteines containing a carboxyl derivative, such as a methyl ester, a methyl amide or an amide group (Figure 2), all activated the ATPase activity of MDR1 in a similar fashion. In contrast, acetylation of the α-amino group of a prenylcysteine rendered the compound inactive. Interestingly, a doubly modified prenylcysteine that was both carboxy-methylated and amino-acetylated (i.e. AFCME; Figure 2) had activity that was consistent with it being an inhibitor of the transporter. In studies using MDR1-transfected human breast cancer cells, AFCME inhibited MDR1-mediated drug transport and enhanced the steady-state accumulation of vinblastine, colchicine or taxol [26]. Two other prenylcysteine analogs that are structurally related to AFCME, namely AFCA and AFCMA (Figure 2), also inhibited MDR1 and restored drug accumulation in the cancer cells in an essentially identical fashion to AFCME (Figure 3) [26]. Consistent with these findings was the ability of the three compounds to inhibit the drug-stimulated ATPase activity of MDR1. Initial structure–activity analyses indicated that the most important features of these molecules for their inhibitory activities were the acetylated nitrogen atom and the lack of a negative charge on the α-carboxyl moiety [26]. These studies suggest a novel approach to designing inhibitors of MDR1-mediated drug efflux based on the structures of prenylcysteines.

Although the specific prenylcysteine-based inhibitors of MDR1 that have been developed to date are quite effective *in vitro*, they have many properties that limit their use *in vivo*. The first limitation is their potency; the IC_{50} values for inhibiting MDR1 activity are in the range of 20–40 μM, which is certainly much higher than one would like for compounds to be used as therapeutic agents. The second concern is that, although the compounds are quite effective at restoring drug accumulation in MDR1-overexpressing tumor cells in short-term (i.e. 2–3 h) assays [26] (Figure 3), this effect is lost when the studies are conducted for longer time periods (L. Zhang and P.J.C., unpublished observations). A likely explanation for this phenomenon is that the prenylcysteines are being metabolized in the cell; indeed, a novel enzyme capable of degrading prenylcysteines has recently been identified [31]. Medicinal chemists could help solve this problem, since it may be possible to design much more potent and stable analogs of the active prenylcysteines by chemically modifying those portions of the molecules responsible for recognition by the multidrug transporters, namely the isoprenoid, α-carboxyl and α-amino moieties. Given the flexibility of MDR1 in ligand recognition, such an approach could well lead to the discovery of compounds with properties that make them more amenable for use as therapeutic agents.

Figure 3



The ability of a specific prenylcysteine, AFCMA, to restore accumulation of drug (in this case taxol) in human breast cancer cells overexpressing MDR1. For comparison, the typical level of taxol accumulation in the parental cell line that does not express significant levels of MDR1 (drug-sensitive cells) is shown. See [26] for further details.

Prospects

Overcoming multidrug resistance is a daunting problem. Pharmacological interventions are one approach to this problem, and the development of highly specific inhibitors

of the multidrug transporters is essential for the success of such therapy. Information obtained from studies directed at understanding the physiological role of the multidrug transporters could lead to the design of compounds with enhanced specificity for these transporters. We feel that such information is indeed coming from an examination of the role of lipid-modified cysteine compounds as substrates of the transporters. Although the interaction of prenylcysteine compounds with MRP still needs to be characterized, the present data indicate that prenylcysteines do interact with the two ABC transporters that have been linked to drug resistance in cancer, MDR1 and MRP. Collectively, the studies also highlight functional similarities between members of this transporter superfamily that have very limited structural homology.

We feel it should be possible to exploit the chemical properties of the novel class of prenylcysteine drug-resistance modulators to identify compounds that can reverse drug-resistant phenotypes in neoplastic cells that overexpress MDR1 and MRP and perhaps also other related transporters, such as those implicated in drug resistance in infectious diseases. Further structural manipulation of lipid-modified cysteine compounds could lead to the generation of agents that are highly specific for each transporter; such compounds could have enormous use in the prevention of drug resistance in a variety of pathological conditions.

Acknowledgements
Work from the authors' laboratory was supported by the American Cancer Society and the Bob Grubb Research Project of the Duke Comprehensive Cancer Center. L.M.H. was the recipient of a medical student fellowship from the Howard Hughes Medical Institute. We thank Lili Zhang, Julia Thissen, Becky Spence and Bill Tschantz for comments on the manuscript, John Moomaw for assistance with the figures, and Kristy Thompson for expert editorial assistance.

References
1. Morrow, C.S. & Cowan, K. (1993). Drug resistance and cancer. *Adv. Exp. Med. Biol.* **330**, 287-305.
2. Kaye, S.B. (1995). Clinical drug resistance: the role of factors other than P-glycoprotein. *Am. J. Medicine* **99**, 40S-44S.
3. Bosch, I. & Croop, J. (1996). P-glycoprotein, multidrug resistance and cancer. *Biochim. Biophys. Acta* **188**, F37-F54.
4. Gottesman, M.M. & Pastan, I. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**, 385-427.
5. Goldstein, L.J. (1995). Clinical reversal of drug resistance. *Curr. Probl. Cancer* **19**, 65-124.
6. Higgins, C.F. (1992). ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* **8**, 67-113.
7. Juliano, R.L. & Ling, V. (1976). A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* **455**, 152-162.
8. Cole, S.P.C., *et al.*, & Deeley, R.G. (1992). Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258**, 1650-1654.
9. Henderson, D.M., Sifri, C.D., Rodgers, M., Wirth, D.F., Hendrickson, N. & Ullman, B. (1992). Multidrug resistance in leishmania donovant is conferred by amplication of a gene homologous to the mammalian mdr1 gene. *Mol. Cell. Biol.* **12**, 2855-2865.
10. Foote, S.J., Thompson, J.K., Cowman, A.F. & Kemp, D.J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell* **57**, 921-930.
11. O'Brien, M.L. & Tew, K.D. (1996). Glutathione and related enzymes in multidrug resistance. *Eur. J. Cancer* **32**, 967-978.
12. Schroder, C.P., Godwin, A.K., Odwyer, P.J., Tew, K.D., Hamilton, T.C. & Ozols, R.F. (1996). Glutathione and drug resistance. *Cancer Invest.* **14**, 158-168.
13. van Helvoort, A., *et al.*, & van Meer, G. (1996). MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. *Cell* **87**, 507-517.
14. Schinkel, A.H., *et al.*, & Borst, P. (1994). Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491-502.
15. Cole, S.P.C., *et al.*, & Deeley, R.G. (1994). Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res.* **54**, 5902-5910.
16. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S.P.C., Deeley, R.G. & Keppler, D. (1994). The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J. Biol. Chem.* **269**, 27807-27810.
17. Paulusma, C.C., *et al.*, & Elferink, R.P.J. (1996). Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271**, 1126-1128.
18. Sun, H., Zhu, Q. & Center, M.S. (1996). Expression and characterization of the multidrug resistance-associated protein in insect cells infected with a recombinant baculovirus. *Oncol. Res.* **8**, 287-293.
19. Muller, M., *et al.*, & Jansen, P.L.M. (1994). Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc. Natl Acad. Sci. USA* **91**, 13033-13037.
20. Loe, D.W., Almquist, K.C., Deeley, R.G. & Cole, S.P.C. (1996). Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. *J. Biol. Chem.* **271**, 9675-9682.
21. Raymond, M., Gros, P., Whiteway, M. & Thomas, D.Y. (1992). Functional complementation of yeast step by a mammalian multidrug resistance mdr gene. *Science* **256**, 232-234.
22. Ruetz, S., *et al.*, & Gros, P. (1996). Functional expression of the multidrug resistance-associated protein in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 4154-4160.
23. Germann, U.A., Willingham, M.C., Pastan, I. & Gottesman, M.M. (1990). Expression of the human multidrug transporter in insect cells by a recombinant baculovirus. *Biochemistry* **29**, 2295-2303.
24. Sarkadi, B.E., Price, E.M., Boucher, R.C., Germann, U.A. & Scarborough, G.A. (1992). Expression of the human multidrug resistance cDNA in insect cells generate a high activity drug-stimulated membrane ATPase. *J. Biol. Chem.* **267**, 4854-4858.
25. Zhang, L., Sachs, C.W., Fine, R.L. & Casey, P.J. (1994). Interaction of prenylcysteine methyl esters with the multidrug resistance transporter. *J. Biol. Chem.* **269**, 15973-15976.
26. Zhang, L., Sachs, C.W., Fu, H., Fine, R.L. & Casey, P.J. (1995). Characterization of prenylcysteines that interact with P-glycoprotein and inhibit drug transport in tumor cells. *J. Biol. Chem.* **270**, 22859-22865.
27. Zhang, F.L. & Casey, P.J. (1996). Protein prenylation: molecular mechanisms and functional consequences. *Annu. Rev. Biochem.* **65**, 241-269.
28. Kuchler, K., Sterne, R.E. & Thorner, J. (1989). *Saccharomyces cerevisiae* STE6 gene product: a novel pathway for protein export in eukaryotic cells. *EMBO J.* **8**, 3973-3984.
29. Sapperstein, S., Berkower, C. & Michaelis, S. (1994). Nucleotide sequence of the yeast STE14 gene, which encodes farnesylcysteine carboxyl methyltransferase, and demonstration of its essential role in a-factor export. *Mol. Cell. Biol.* **14**, 1438-1449.
30. Schafer, W.R. & Rine, J. (1992). Protein prenylation: genes, enzymes, targets and functions. *Annu. Rev. Genet.* **25**, 209-238.
31. Zhang, L., Tschantz, W.R. & Casey, P.J. (1997). Isolation and characterization of a prenylcysteine lyase from bovine brain. *J. Biol. Chem.* **272**, 23354-23359.