such vesiculation at L/P ratios smaller than 12. Lee and Kim (1988) showed the formation of micelles upon interaction of apomyoglobin with dimyristoyl-PC at a L/P ratio of 60, with an I/I_0 value of 0.25. The interaction of an amphiphilic α-helical segment of apomyoglobin with PC at acidic pH was proposed to cause micelle formation. We do not yet know if such micellar formation occurs in the present peptide system.

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Lipophilic Cations: A Group of Model Substrates for the Multidrug-Resistance Transporter†

Philippe Gros,* France Talbot, David Tang-Wai, Eitan Bibi,‡ and H. Ronald Kaback‡

Department of Biochemistry, McGill University, Montreal, Canada H3G 1Y6, and Howard Hughes Medical Institute, Departments of Physiology and Microbiology and Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024-1570

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ABSTRACT: The possibility that simple lipophilic cations such as tetraphenylphosphonium (TPP^+), tetraphenylarsonium (TPA^+), triphenylmethylphosphonium ($TPMP^+$), and diphenyldimethylphosphonium (DDP^+) are substrates for the multidrug-resistance transport protein, P-glycoprotein, was tested. Hamster cells transfected with and overexpressing mouse *mdr1* or mouse *mdr3* exhibit high levels of resistance to TPP^+ and TPA^+ (20-fold) and somewhat lower levels of resistance to $TPMP^+$ and DDP^+ (3-12-fold). Transfected cell clones expressing *mdr1* or *mdr3* mutants with decreased activity against drugs of the MDR spectrum (e.g., Vinca alkaloids and anthracyclines) also show reduced resistance to lipophilic cations. Studies with radiolabeled TPP^+ and TPA^+ demonstrate that increased resistance to cytotoxic concentrations of these lipophilic cations is correlated quantitatively with a decrease in intracellular accumulation in *mdr1*- and *mdr3*-transfected cells. This decreased intracellular accumulation is shown to be strictly dependent on intact intracellular nucleotide triphosphate pools and is reversed by verapamil, a known competitive inhibitor of P-glycoprotein. Taken together, these results demonstrate that lipophilic cations are a new class of substrates for P-glycoprotein and can be used to study its mechanism of action in homologous and heterologous systems.

Multidrug resistance is the phenomenon by which tumor cells in vivo and cultured cells in vitro become simultaneously resistant to a large group of structurally and functionally unrelated cytotoxic compounds (Gerlach et al., 1986; Moscow & Cowan, 1988). The phenomenon is caused by the overexpression of a group of membrane phosphoglycoproteins termed P-glycoproteins (P-gp) that are encoded by a small family of related *mdr* genes which become amplified and/or overexpressed in multidrug-resistant cells (Endicott & Ling, 1989). P-gp has been shown to bind ATP (Cornwell et al.,

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* To whon correspondence and reprint requests should be addressed.
‡ UCLA.

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1987; Schurr et al., 1989) and drug analogues (Cornwell et al., 1986; Safa et al., 1987), has ATPase activity (Hamada & Tsuruo, 1988), and catalyzes ATP–dependent drug efflux to effectively reduce intracellular accumulation in resistant cells (Gottesman & Pastan, 1988; Endicott & Ling, 1989). Full-length cDNA clones corresponding to two human *MDR* genes (*MDR1* and *MDR3*) and three mouse *mdr* genes (*mdr1*, *mdr2*, and *mdr3*) have been isolated and the predicted amino acid sequence of the corresponding P-gp's elucidated (Chen et al., 1986; Van Der Bliek et al., 1988; Gros et al., 1986a, 1988; Devault & Gros, 1990; Hsu et al., 1990). P-gp is postulated to be composed of 12 transmembrane hydrophobic domains and two nucleotide-binding folds and is formed by two symmetrical halves that share sequence homology and common ancestral origin with a group of bacterial transport systems that require periplasmic proteins (Ames, 1986). The biological activity of the three mouse *mdr* genes has been studied in transfection experiments, and it has been shown that mouse *mdr1* and mouse *mdr3*, but not *mdr2*, can convey the multidrug-resistance phenotype to otherwise drug-sensitive cells (Gros et al., 1986b, 1988; Devault & Gros, 1990). The *mdr* gene family is part of a larger family of *mdr*-like genes encoding sequence homologous proteins sharing similar predicted secondary structures and proposed membrane-associated transport functions. These include the *pfmdr1* gene of *Plasmodium falciparum* (Foote et al., 1989), the yeast *STE-6* gene (McGrath & Varshavsky, 1989), the human *CFTR* (Riordan et al., 1989) and *HAM* genes (Monaco et al., 1990) associated with transport of chloroquine (Krogstad et al., 1987), *a* mating pheromone (Kuchler et al., 1989), halides (Anderson et al., 1991), and antigenic peptides (Monaco et al., 1990), respectively.

One unresolved aspect of multidrug resistance is the apparent capacity of P-gp to recognize and transport a large group of cytotoxic compounds sharing little or no structural or functional similarities. Most P-gp substrates are relatively small, hydrophobic, and biplanar with a basic nitrogen atom and enter the cell by passive diffusion across the lipid bilayer (Pearce et al., 1989). Although the specific protein domains and amino acid residues involved in substrate recognition have not been formally identified, genetic and biochemical evidence support the contention that putative membrane-associated domains of P-gp directly interact with substrate (Bruggeman et al., 1989; Yoshimura et al., 1990; Raviv et al., 1990; Choi et al., 1988).

The use of lipophilic ions to measure the polarity of membrane potentials in biological systems was initiated by Grinius et al. (1971), and subsequent studies with radiolabeled lipophilic cations have demonstrated that membrane potentials can be measured quantitatively in various prokaryotic and eukaryotic systems (Felle et al., 1980; Lichtshtein et al., 1979; Bakker et al., 1986; Lichtenberg et al., 1988). The ions are constructed to be sufficiently lipophilic to enter the hydrophobic core of the membrane and to be able to delocalize their charge in order to allow passive equilibration with the electrical potential across the membrane (Haydon & Hladky, 1972). Although tetraphenylphosphonium (TPP^+)[1] and tetraphenylarsonium (TPA^+) are most permeant and therefore equilibrate with the membrane potential fastest, these cations and in addition triphenylmethylphosphonium ($TPMP^+$; Schuldiner & Kaback, 1975), diphenyldimethylammonium (in the presence of tetraphenylboron; Hirata et al., 1973; Lombardi et al., 1974), triphenylmethylarsonium, and triphenylmethylammonium all appear to equilibrate with the membrane potential over a wide range of concentrations (Kaback, 1990).

Since lipophilic cations share some of the structural characteristics of typical P-gp substrates, we explored the possibility that they may be recognized and transported by P-gp. In this paper, it is shown that a series of structurally related lipophilic cations [TPP^+, TPA^+, $TPMP^+$, and diphenyldimethylphosphonium (DDP^+)] behave as P-gp substrates in multidrug-resistant cell clones transfected with either *mdr1* or *mdr3*.

Experimental Procedures

Materials

TPA^+ (chloride salt), TPP^+ (bromide salt), $TPMP^+$ (bromide salt), and DDP^+ (iodide salt) were purchased from Aldrich Chemical Co. Gramicidin D (A), gramicidin S, vinblastine, and verapamil (VRP) were from Sigma. Stock solutions of TPA^+, TPP^+, $TPMP^+$, DDP^+, and vinblastine were prepared in phosphate-buffered saline (PBS) at 1.0 mg/mL, gramicidin D and S in dimethyl sulfoxide at 1.0 mg/mL, and VRP in methanol at 1.0 mg/mL. Stock solutions were stored at −80 °C until use. [^{3}H]TPP^+ (45 Ci/mmol; 1665 GBq/mmol) was obtained from New England Nuclear; [^{3}H]TPA^+ (23.8 mCi/mmol) was synthesized by the Isotope Synthesis Group of Hoffmann-LaRoche, Inc., under the direction of Arnold Liebman.

Methods

Cell Lines and Tissue Culture. Chinese hamster LR73 ovary cells and their drug-resistant derivatives transfected with wild-type or mutant *mdr1* (clones 1S, 1F) or *mdr3* (clones 3S, 3F) cDNAs were obtained as previously described (Gros et al., 1991). All cell lines were maintained in α-MEM supplemented with 10% fetal calf serum, 3 mM glutamine, penicillin (50 units/mL), and streptomycin (50 μg/mL). Drug-resistant transfectants were grown in the same medium supplemented with vinblastine at 25 ng/mL (1F) and 50 ng/mL (1S, 3F, and 3S).

Measurements of Cell Survival. A modification of a cell survival assay (Skehan et al., 1989) based on sulforhodamine B (SRB) staining was used. Briefly, 5×10^3 cells of control and *mdr* transfected cells were plated in 96-well titer plates in increasing concentrations of a given cytotoxic compound and incubated for 72 h at 37 °C. Cells were washed once in ice-cold PBS and fixed in 17% trichloroacetic acid in PBS for 45 min at 4 °C and then washed extensively in tap water. Total cell protein was stained with 0.4% SRB in 1% acetic acid for 15 min at room temperature, followed by four washes with 1% acetic acid. The plates were dried, the stain was dissolved in 10 mM Tris (pH 9.0), and quantitation was carried out using an automated ELISA plate reader set at 490 nm. The relative plating efficiency of each clone was calculated by dividing the absorbance observed at a given drug concentration by the absorbance detected in the same clone in medium devoid of drug and is expressed as a percentage. D_{50} is the drug dose required to reduce the plating efficiency of each cell clone by 50%.

Transport Assays. Drug-sensitive control cells and *mdr*-transfected clones were grown to confluency and harvested after trypsin treatment (2 min at 37 °C). Cells were washed once with PBS and allowed to recover for 2 h at room temperature in complete α-MEM. The cell suspensions were adjusted to 8×10^6 cells/mL in PBS containing 10 mM

[1] Abbreviations: TPP^+, tetraphenylphosphonium; TPA^+, tetraphenylarsonium; $TPMP^+$, triphenylmethylphosphonium; DDP^+, diphenyldimethylphosphonium; P-gp, P-glycoprotein; VRP, verapamil; PBS, phosphate-buffered saline; SRB, sulforhodamine B; MDR, multidrug resistance.

Table I: Effect of Lipophilic Cations on Survival Characteristics of Cell Clones Stably Transfected with *mdr1* and *mdr3*[a]

	TPA^+	TPP^+	$TPMP^+$	DDP^+	Gram D	Gram S
LR	3.4 ± 1.4	5.3 ± 0.4	44 ± 6.5	170 ± 56	0.05 ± 0.01	4.7 ± 0.7
1F	3.9 ± 1.1 (1.1×)	5.8 ± 0.4 (1.1×)	56 ± 13 (1.3×)	213 ± 42 (1.3×)	0.19 ± 0.02 (3.8×)	5.4 ± 0.6
1S	28 ± 10 (8.4×)	27 ± 2 (5.1×)	259 ± 66 (5.9×)	500 ± 22 (2.9×)	2.3 ± 0.3 (46×)	4.8 ± 0.7
3F	29 ± 10 (8.5×)	27 ± 3 (5×)	141 ± 51 (3.2×)	150 ± 14 (0.9×)	5 ± 0.8 (100×)	5.5 ± 0.7
3S	88 ± 23 (26×)	108 ± 42 (20×)	552 ± 138 (12.5×)	1150 ± 50 (6.8×)	8.6 ± 1.1 (172×)	4.7 ± 0.4
	(n = 5)	(n = 5)	(n = 5)	(n = 3)	(n = 3)	(n = 3)

[a] The drug survival of LR73 drug-sensitive cells and multidrug-resistant clones transfected with either wild-type *mdr1* (1S) or *mdr3* (3S) or mutant *mdr1* (1F) or *mdr3* (3F) is expressed as the D_{50} (μg/mL), or the dose necessary to reduce the plating efficiency of the control and transfected cell clones by 50%. Abbreviations: tetraphenylarsonium (TPA^+); tetraphenylphosphonium (TPP^+); triphenylmethylphosphonium ($TPMP^+$); dimethyldiphenylphosphonium (DDP^+); gramicidin D (Gram D); gramicidin S (Gram S).

glutamine and 10 mM glucose (+ATP) at 20 °C, and transport was initiated by adding an equal volume of a given radiolabeled compound. At given times, 0.5-mL aliquots were centrifuged (10 s at 10000*g*) through a 4:1 mixture of silicon oil and mineral oil, the supernatants and oil were removed, and the cell pellets were dissolved overnight in 1.0 N NaOH. The digests were neutralized by addition of an equal volume of 1.0 N HCl, 100-μL aliquots were spotted on filter discs (Whatman GFC) and dried, and incorporated radioactivity was measured by liquid scintillation spectrometry. Total cell protein in the digests was measured using an amido-black-based commercial assay (Bio-Rad), and results were expressed as counts/(min·mg of cell protein). For ATP depletion, cells were sequentially incubated at 37 °C with rotenone (20 ng/mL) for 15 min and 2-deoxyglucose (2 mM) for an additional 15 min in PBS lacking glucose and glutamine prior to initiation of transport. The effect of VRP on TPP^+ or TPA^+ accumulation was determined by adding the compound to the reaction mixtures at a final concentration of 100 μM.

Western Blots. P-gp was detected in highly enriched membrane preparations from drug-sensitive and *mdr*-transfected cells by Western blotting using a mouse anti-P-gp monoclonal antibody (mAb) C219 (Centocor Corp.); immune complexes were revealed with goat anti-mouse antibody coupled to alkaline phosphatase as described (Devault & Gros, 1990).

Results

The structures of the lipophilic cations used in this study are shown in Figure 1. TPA^+ and TPP^+ are homologous compounds with an arsonium or phosphonium cation bonded covalently to four phenyl groups. $TPMP^+$ and DDP^+ are analogous compounds in which one or two of the phenyl groups are substituted with one or two methyl groups, respectively.

To determine whether these compounds are substrates for mammalian P-glycoprotein, we first measured their cytotoxic effect on control LR73 hamster cells and their multidrug-resistant derivatives stably transfected with and overexpressing cDNAs encoding the two biologically active mouse mdr efflux pumps, mdr1 and mdr3. The cell clones used in this study were analyzed by Western blotting with anti-P-gp mAb C219 for the presence of specific polypeptides encoded by wild-type *mdr1* (clone 1S) or *mdr3* (clone 3S) cDNAs. As shown (Figure 2), C219 mAb recognizes 180- and 160-kDa proteins overexpressed in the *mdr1* (1S) and *mdr3* (3S) transfected cells, respectively, which are not detected in the parental LR73 drug-sensitive cells.

The drug-survival characterstics of these cell clones was determined and compared for the lipophilic cations TPP^+, TPA^+, $TPMP^+$, or DDP^+. For comparison, gramicidin D was used as a known substrate for P-gp, and gramicidin S was used as a negative control. Typical cytotoxicity assays are presented in Figure 3, and the average D_{50}'s from 3–5 experiments are

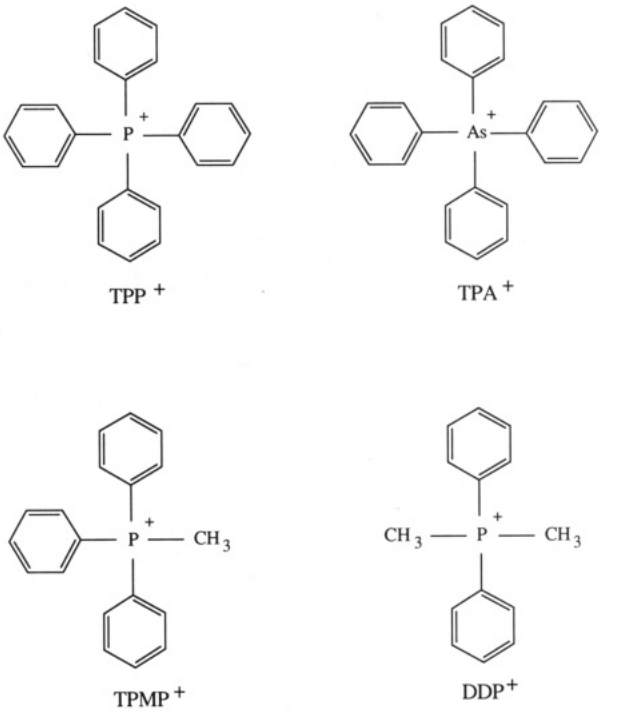

FIGURE 1: Lipophilic cations. Planar structures of each of the lipophilic cations used are shown. TPA^+, tetraphenylarsonium; TPP^+, tetraphenylphosphonium; $TPMP^+$, triphenylmethylphosphonium, DDP^+, diphenyldimethylphosphonium.

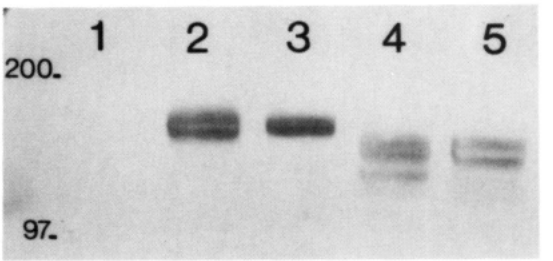

FIGURE 2: Identification of mdr1 and mdr3 proteins. Membrane fractions of drug-sensitive LR73 hamster cells (LR) and cell clones transfected with wild-type *mdr1* (1S) and *mdr3* (3S) or mutant *mdr1* (1F) and *mdr3* (3F) were analyzed by Western blotting using C219 mAb anti-Pgp. Molecular mass markers are myosin (200 kDa) and phosphorylase *b* (97 kDa).

compiled in Table I. Each of the four lipophilic cations is toxic for LR73 cells, and it appears that toxicity (i.e., D_{50}) is directly proportional to the passive permeability of planar bilayers to these compounds ($TPP^+ = TPA^+ > TPMP^+ > DDP^+$) (Haydon & Hladky, 1972). The results also suggest that each of the four lipophilic cations are substrates for P-gp, since cell clones stably expressing wild-type *mdr1* (1S) or *mdr3* (3S) exhibit D_{50}'s significantly higher than those measured for LR73 cells. Moreover, it is clear that the P-gp's encoded

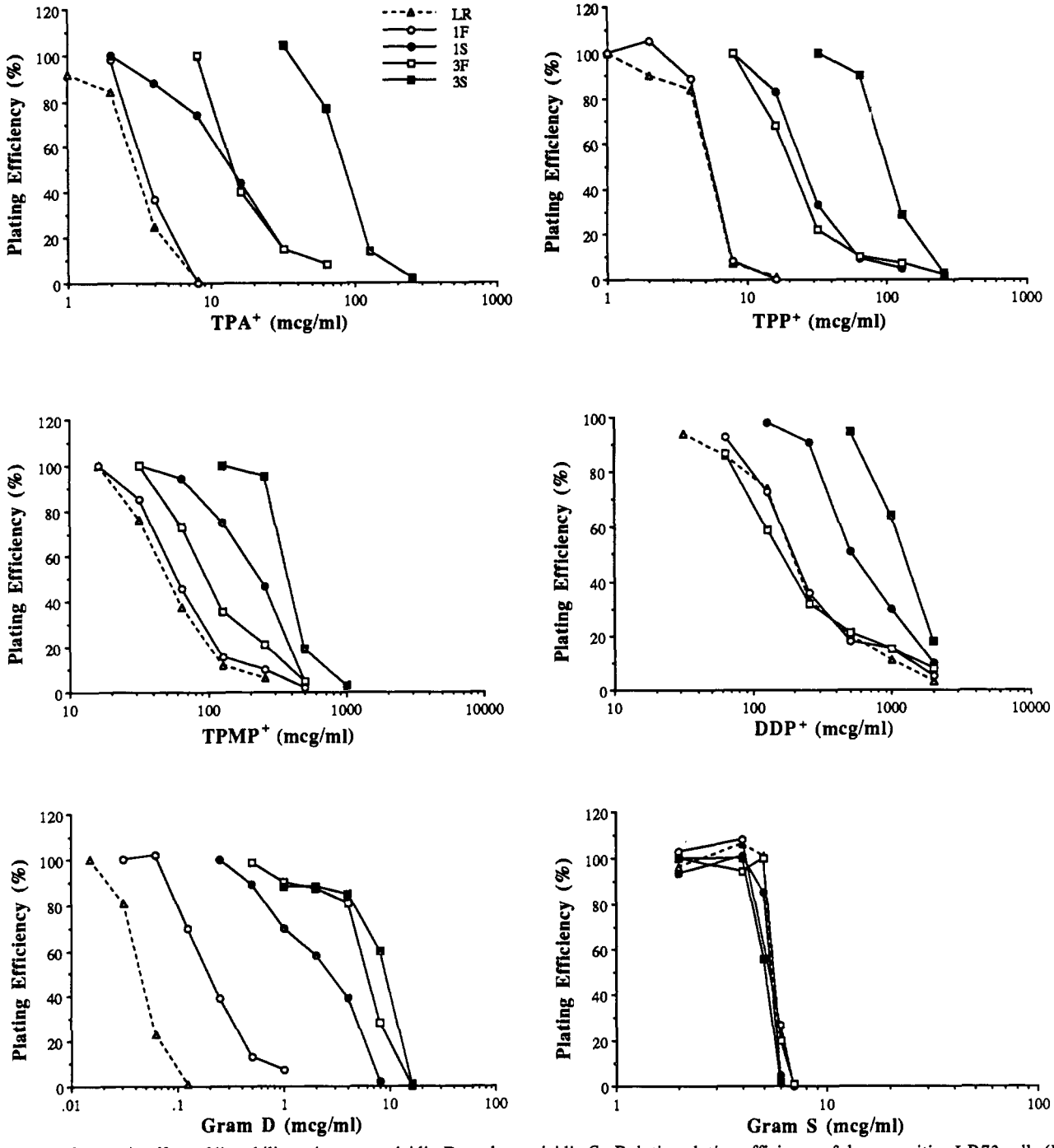

FIGURE 3: Cytotoxic effect of lipophilic cations, gramicidin D, and gramicidin S. Relative plating efficiency of drug-sensitive LR73 cells (LR; △) and multidrug-resistant cell clones transfected with wild-type *mdr1* (1S; ●) or *mdr3* (3S; ■) and mutant *mdr1* (1F; ○) or *mdr3* (3F; □) in medium containing tetraphenylarsonium (TPA^+), tetraphenylphosphonium (TPP^+), triphenylmethylphosphonium ($TPMP^+$), diphenyldimethylphosphonium (DDP^+), gramicidin D (Gram D) or gramicidin S (Gram S).

by wild-type *mdr1* and *mdr3* confer higher degrees of resistance to TPA^+ (1S, 8.4×; 3S, 26×) or TPP^+ (1S, 5.1×; 3S, 20×) than to $TPMP^+$ (1S, 5.9×; 3S, 12.5×) or DDP^+ (1S, 2.9×; 3S, 6.8×). Under the experimental conditions used, high levels of resistance to gramicidin D are detected in *mdr1* (1S, 46×) and *mdr3* (3S, 172×) transfectants, while no resistance to gramicidin S, which is not a substrate for P-glycoprotein, is observed. Finally, as shown previously for anthracyclines and Vinca alkaloids (Gros et al., 1991), levels of resistance to lipophilic cations conferred by wild-type *mdr3* are significantly higher than those conferred by *mdr1* for all drugs tested.

The cytotoxic effect of lipophilic cations was also measured on cell clones transfected with and overexpressing mutated forms of *mdr1* (1F) and *mdr3* (3F) in which Ser residues at positions 941 and 939, respectively, in putative transmembrane domain 11 (TM11) were replaced with Phe. These mutants have been shown to exhibit decreased activity and altered substrate specificity for drugs of the MDR spectrum (Gros et al., 1991). The mutant forms of *mdr1* and *mdr3* are expressed in the individual cell clones used in this study at levels comparable to those observed in cell clones overexpressing the wild-type *mdr1* (1S) and *mdr3* (3S) (Figure 2). Levels of resistance to lipophilic cations detected in the 1F clone are negligible relative to the control, suggesting that the 1F mutant cannot interact with these compounds. On the other hand, the 3F clone exhibits readily detectable levels of resistance to TPA^+ (8.5×), TPP^+ (5×), or $TPMP^+$ (3.2×), but no signif-

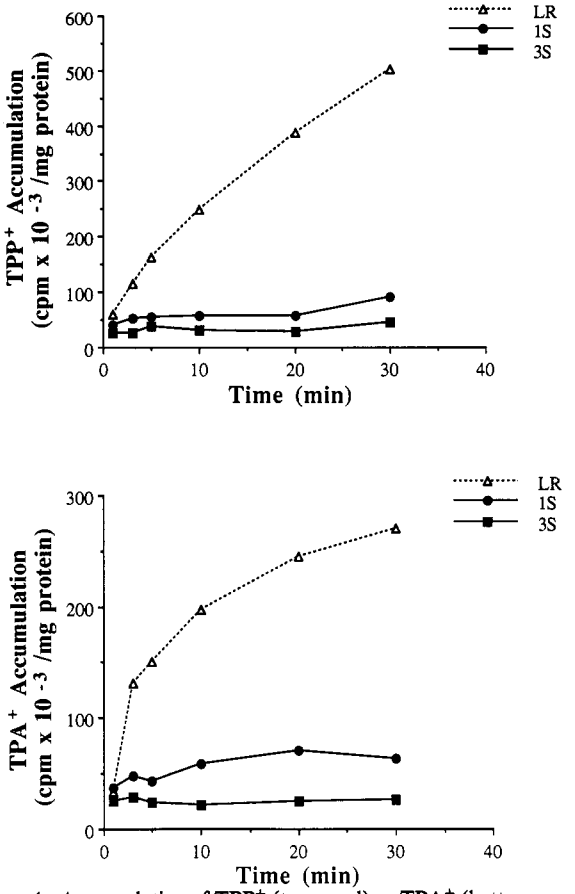

FIGURE 4: Accumulation of TPP^+ (top panel) or TPA^+ (bottom panel) in LR73 and *mdr*-transfected cells. Drug-sensitive LR73 cells (LR; △) and cell clones transfected with either wild-type *mdr1* (1S; ●) or *mdr3* (3S; ■) were incubated with either [^{3}H]TPP^+ (22.5 mCi/mmol) or [^{3}H]TPA^+ (23.8 mCi/mmol) at final concentrations of 100 μM as described under Methods. Accumulation of radioactivity was assayed at given times and is expressed as counts/(min × 10^3·mg of cell protein).

icant resistance to DDP^+ (Figure 3 and Table I). Both 1F and 3F clones express significant levels of resistance of gramicidin D (1F, 3.8×; 3F, 100×) and no resistance to gramicidin S. Finally, resistance to cytotoxic concentrations of lipophilic cations detected in 1S, 3S, and 3F was reversed by addition of VRP (5–10 μM) to the culture medium (data not shown). The results present additional genetic evidence suggesting that lipophilic cations are substrates for P-gp.

In order to further define the mechanistic basis of increased resistance to lipophilic cations detected in *mdr*-transfected cells, the pharmacokinetics of [^{3}H]TPP^+ and [^{3}H]TPA^+ accumulation were studied (Figure 4). Accumulation of TPP^+ in drug-sensitive cells increases continuously over 30 min and achieves a steady-state in approximately 60 min (not shown); TPA^+ is taken up rapidly during the first 3–5 min and begins to approach steady state after about 10–20 min. Dramatically, cells expressing wild-type *mdr1* (1S) or *mdr3* (3S) accumulate little or none of either lipophilic cation during the time course of the experiments, resulting in at least a 10-fold difference in the intracellular level of accumulation of the lipophilic cations. Therefore, reduced toxicity of these compounds for *mdr*-transfected cells is clearly correlated with reduction in intracellular accumulation mediated by *mdr1* or *mdr3*.

P-gp-mediated drug efflux in *mdr1* transfectants is known to be dependent on intracellular ATP pools (Hammond et al., 1989). Thus, to determine the effect of ATP depletion on lipophilic cation accumlation, LR73 drug-sensitive cells and their multidrug-resistant *mdr1* and *mdr3* transfectants were treated with rotenone and 2-deoxyglucose prior to exposure to [^{3}H]TPP^+ (Figure 5). While the effect of ATP depletion on TPP^+ accumulation is minimal in LR73 cells, it effectively eliminates the ability of *mdr1* or *mdr3* transfectants to reduce intracellular accumulation of the cation.

Finally, the effect of a known P-glycoprotein reversal agent, VRP, on accumulation of TPA^+ was tested on LR73 drug-sensitive cells and their multidrug-resistant *mdr1* and *mdr3* transfectants (Figure 6). VRP has little effect on accumulation in LR73 drug-sensitive cells but completely eliminates the difference normally detected between the drug-sensitive and *mdr*-transfected cells.

Discussion

The results presented in this paper demonstrate that simple lipophilic cations such as TPP^+, TPA^+, $TPMP^+$, and DDP^+ behave phenomenologically like typical cytotoxic compounds that form the multidrug-resistance spectrum (e.g., Vinca alkaloids, anthracyclines, epipodophylotoxins, and etoposides). Thus, cells overexpressing either of the two biologically active mouse *mdr* efflux pumps, *mdr1* or *mdr3*, survive higher concentrations of lipophilic cations in cytotoxicity assays than drug-sensitive parental cells. Transfected cell clones expressing *mdr1* or *mdr3* mutants with decreased activity against drugs of the MDR spectrum (e.g., Vinca alkaloids and anthracyclines) also show reduced resistance to lipophilic cations. Furthermore, increased resistance is concomitant with a reduced intracellular accumulation of these cations which is ATP-dependent and reversible by VRP, two characteristics of P-gp-mediated drug efflux (Endicott & Ling, 1989).

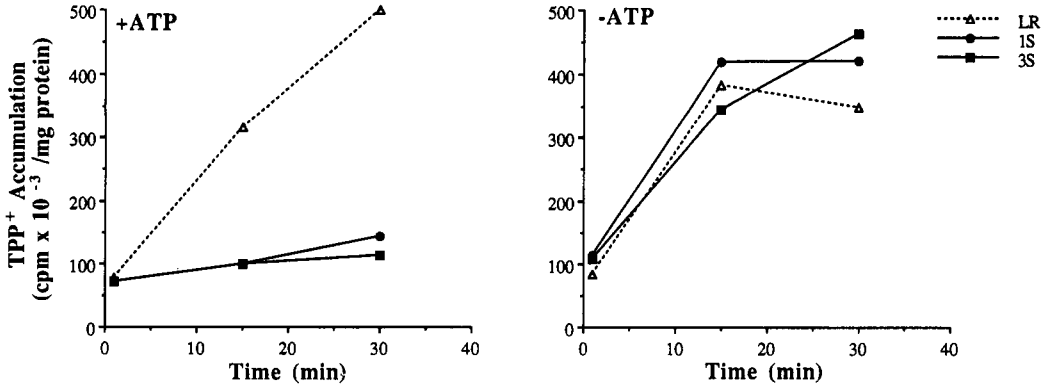

FIGURE 5: Effect of ATP depletion on accumulation of TPP^+ in LR73 and *mdr*-transfected cells. Drug-sensitive LR73 cells (LR; △) and cell clones transfected with either wild-type *mdr1* (1S; ●) or *mdr3* (3S; ■) were incubated with [^{3}H]TPP^+ (22.5 mCi/mmol) at a final concentration of 100 μM, and ATP depletion was carried out as described under Methods. Accumulation of radioactivity was assayed at given times and is expressed as counts/(min × 10^{-3}·mg of cell protein).

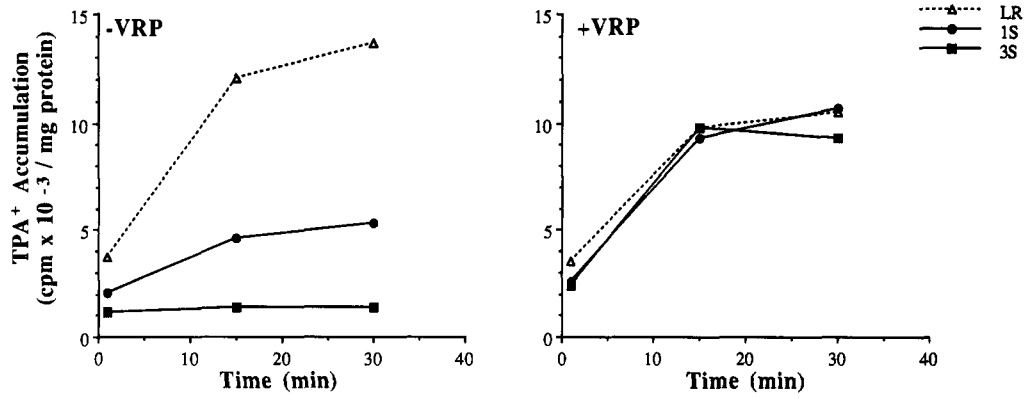

FIGURE 6: Effect of verapamil on accumulation of TPP^+ in LR73 and *mdr*-transfected cells. Drug-sensitive LR73 cells (LR; △) and cell clones transfected with either wild-type *mdr1* (1S; ●) or *mdr3* (3S; ■) were incubated with [^{3}H]TPA^+ (23.8 mCi/mmol) at a final concentration of 100 μM with (+VRP) and without (−VRP) 100 μM verapamil. Assays were carried out as described under Methods. Accumulation of radioactivity was assayed at given times and is expressed as counts/(min × 10^{-3}·mg of cell protein).

It is apparent that the toxicity of the four lipophilic cations tested varies by almost two orders of magnitude for the drug-sensitive parental cell line used (Figure 2 and Table I). TPA^+ and TPP^+ are the most toxic, exhibiting D_{50}'s of 3–5 μg/mL, followed by $TPMP^+$ (D_{50} = 44 μg/mL) and DDP^+ (D_{50} = 170 μg/mL). The relative toxicity of these compounds for LR73 drug-sensitive cells appears to be directly related to the number of phenyl groups (Figure 1), which determines their ability to delocalize charge and thus their capacity to permeate planar bilayers (Haydon & Hladky, 1972). Although the mechanism of cytotoxicity of these lipophilic cations for eukaryotic cells has not yet been elucidated, it is likely that the compounds induce cell death by dissipating the proton electrochemical gradient across the mitochondrial membrane.

Overexpression of *mdr1* or *mdr3* in transfected cell clones confers resistance to all four lipophilic cations (Figure 2 and Table I). However, the degree of resistance conferred by wild-type *mdr1* (1S) or *mdr3* (3S) is different for the four cations tested; it is greatest for TPA^+ and TPP^+, lower for $TPMP^+$, and lowest for DDP^+. These variations could reflect either differences in the affinity of P-gp for the cations or the ability of the cations to partition into the lipid bilayer where they may be more accessible to the substate-recognition site(s) of P-gp. Furthermore, *mdr1* and *mdr3* mutants (1F, 3F) harboring single amino acid substitutions within putative TM11 and showing decreased transport and altered substrate specificity also convey a lower level of resistance to lipophilic cations. Interestingly, the 1F mutant, which is known to retain the capacity to confer Vinca alkaloid resistance but not anthracycline nor colchicine resistance (Gros et al., 1991), shows no detectable level of resistance to lipophilic cations. This observation suggests that the lipophilic cations may share binding site specificity with anthracyclines and colchicine but not with the Vinca alkaloids.

The 10–20-fold increase in resistance to lipophilic cations detected in *mdr* transfectants is directly related to the 10–20-fold decrease in the intracellular accumulation of TPP^+ or TPA^+. This is in contrast to the relationship observed between intracellular accumulation of typical *mdr* drugs such as vinblastine and the level of resistance to this drug detected in *mdr1* or *mdr3* transfectants. Although *mdr1* or *mdr3* transfectants express 40- and 80-fold resistance to vinblastine, respectively, they show only 2–4-fold reduction in the intracellular accumulation of this drug (Hammond et al., 1989; data not shown). This lack of correlation has also been extensively documented for multidrug-resistant cell lines overexpressing P-gp obtained by step-wise selection protocols (Fojo et al., 1985; Horio et al., 1988). The reason for the discrepancy remains unclear. In any event, the apparent direct correlation between intracellular accumulation and toxicity observed for both TPP^+ and TPA^+ in LR73 and *mdr* transfectants appears to be unique to these compounds.

The relatively simple chemical structure of the lipophilic cations used here makes them easily amenable to extensive chemical modification, which should prove useful for characterizing interactions between P-gp and these substrates. In addition, since lipophilic cations such as TPP^+ and TPA^+ are permeant in a wide range of eukaryotic and prokaryotic membranes, they should be useful as test substrates for *mdr* expression in heterologous systems.

Added in Proof

We recently found that TPP^+ and TPA^+ could block photolabeling of P-gps by drug analogues.

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^{1}H NMR-Based Determination of the Secondary Structure of Porcine Pancreatic Spasmolytic Polypeptide: One of a New Family of "Trefoil" Motif Containing Cell Growth Factors†

Mark D. Carr
Laboratory of Molecular Structure, National Institute for Medical Research, The Ridgeway, Mill Hill, London, U.K.
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ABSTRACT: Two-dimensional ^{1}H NMR spectroscopy has been used to obtain comprehensive sequence-specific resonance assignments for the putative cell growth factor porcine pancreatic spasmolytic polypeptide, which is a 106-residue protein containing two "trefoil" domains. The patterns of sequential ($i,i+1$), medium-range ($i,i<5$), and long-range NH to NH, αCH to NH, and αCH to αCH nuclear Overhauser effects clearly show that the protein's two trefoil domains adopt essentially the same secondary structure in solution. The main feature of each domain is a seven-residue helix followed by a short antiparallel β-sheet formed from two strands of four amino acids each. This is a novel supersecondary structure, which clearly identifies the trefoil motif as a new class of growth factor associated module, distinct from other types of highly disulfide cross-linked domains, such as those found in epidermal growth factor and insulin-like growth factor I.

Porcine pancreatic spasmolytic polypeptide (PSP)[1] is a monomeric 106-residue protein (M_r = 11 700) produced in large quantities in the pancreas and is believed to act as a cell growth factor (Thim et al., 1985; Hoosein et al., 1989; Thim, 1989). The protein has been shown to inhibit both gastrointestinal motility and secretion of gastric acids (Jorgensen et al., 1982). However, recent studies of the human analogue hPSP (Tomasetto et al., 1990) suggest that the major role of the protein in vivo is to promote the healing of damaged en-

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[1] Abbreviations: 2D, two dimensional; 3D, three dimensional; DQF-COSY, double-quantum filtered correlation spectroscopy; EGF, epidermal growth factor; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; IGF-I, insulin-like growth factor I; NMR, nuclear magnetic resonance, NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PSP, porcine pancreatic spasmolytic polypeptide.

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