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ATP-dependent transport of amphiphilic cations across the hepatocyte canalicular membrane mediated by *mdr1* P-glycoprotein

Michael Müller**, Rosmarie Mayer, Ulrike Hero, Dietrich Keppler*

Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

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Abstract

The ATP-dependent transport of the three ^{3}H-labeled, amphiphilic cations quinidine, *N*-(*n*-pentyl)-quinidinium, and *N*-(4′,4′-azo-*n*-pentyl)-21-deoxyajmalinium was studied in rat canalicular plasma membrane vesicles. N-Alkylation of quinidine with an n-pentyl residue resulted in a permanently charged cationic substrate for ATP-dependent transport which exhibited a 10-fold higher transport rate relative to quinidine. The K_m value was 0.4 μM for *N*-(*n*-pentyl)-quinidinium and 5 μM for quinidine. The permanently cationic and photolabile derivative of ajmaline, *N*-(4′,4′-azo-*n*-pentyl)-21-deoxyajmalinium, was also an efficient substrate and served to label canalicular membrane proteins with molecular masses of 143 kDa and 108 kDa. ATP-dependent transport of the permanently charged amphiphilic cations was inhibited by the P-glycoprotein inhibitors and substrates quinidine, verapamil, and daunorubicin. The data demonstrate that N-alkylation of quinidine and ajmaline results in most efficient substrates for *mdr1* P-glycoprotein-mediated ATP-dependent transport.

Key words: Quinidine; Ajmaline; Multidrug resistance; Taurocholate; P-glycoprotein; Liver

1. Introduction

The multidrug resistance (MDR) phenomenon is often associated with the expression of multidrug resistance (*mdr*) genes encoding a family of integral membrane glycoproteins known as P-glycoproteins [1]. These transport proteins bind various hydrophobic, mostly basic drugs and chemotherapeutic agents and pump them out of the cell by an ATP-dependent process [1]. P-glycoproteins are also expressed in normal tissues such as kidney, adrenals, and liver [2–4]. In rat liver canalicular membranes the P-glycoprotein-mediated ATP-dependent transport has been detected by the accumulation of the substrates daunorubicin and vinblastine in inside-out oriented vesicles [2,5,6]. In this report we demonstrate the substrate properties for P-glycoprotein-mediated ATP-dependent transport of quinidine and the two permanently cationic quinuclidinyl compounds APD-ajmalinium and *N*-(*n*-pentyl)-quinidinium. The latter compound exhibited the highest affinity for ATP-dependent transport of amphiphilic cations known at present. These N-alkylated substrates may serve in future studies to characterize the function of *mdr*1 gene products in normal and malignant cells.

2. Materials and methods

2.1. Materials

ATP (Tris-salt), 5′-AMP (free acid), adenylyl(α,β-methylene)-diphosphonate (AMPPCP), potassium creatine phosphate, taurocholate, verapamil, daunorubicin, quinidine, and sodium vanadate were purchased from Sigma Chemical Co., St. Louis, MO, USA. 1-Iodopentane, 9-fluorenone, and potassium *tert*-butylate in tetrahydrofuran were from Aldrich, Steinheim, Germany. Creatine kinase was from Boehringer Mannheim, Mannheim, Germany. [^{3}H]Taurocholate (77.7 GBq/mmol) and sodium borohydride (503.2 GBq/mmol) were from DuPont-New England Nuclear, Boston, MA, USA. 1-Pentyl quinuclidinium iodide was synthesized by alkylation of quinidine with 1-iodopentane in oxygen-free acetone according to [7]. N-(4′,4′-azo-*n*-pentyl)-21-deoxy-[21-^{3}H]ajmalinium [8] (46 GBq/mmol) was synthesized as described [9–12] and kindly provided by Prof. G. Kurz, University of Freiburg, Germany. Nitrocellulose filters (pore size 0.2 μm) were from Schleicher and Schüll, Dassel, Germany. Glass microfibre filters (type GF/F; pore size ≥ 0.7 μm) were purchased from Whatman International Ltd., Maidstone, UK. Scintillation fluid (Filter Count) was from Canberra Packard, Warrenville, IL, USA. All other chemicals were of analytical grade.

2.2. Animals

For preparations enriched in canalicular membranes, male Wistar rats (200–250 g), from the Zentralinstitut für Versuchstierzucht, Hannover, Germany, were employed. Mutant male Wistar rats with hepatobiliary transport defect (TR$^-$ or GY) [13,14] were from Dr. F. Kuipers (University of Groningen, The Netherlands). Animals were maintained on a standard diet with free access to food and water.

*Corresponding author. Fax: (49) (6221) 422 402.

***Present address:* Division of Gastroenterology and Hepatology, Academic Hospital, NL-9713 EZ Groningen, The Netherlands.

Abbreviations: APD-ajmalinium, *N*-(4′,4′-azo-*n*-pentyl)-21-deoxy-[21-^{3}H]ajmalinium; BSEC, bile salt export carrier; CMV, canalicular membrane vesicles; LTEC, leukotriene export carrier; MDEC, multidrug export carrier; MDR, multidrug resistance; pentyl quinidinium, *N*-(*n*-pentyl)-quinidinium; RP, reversed phase.

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2.3. Preparation and characterization of membranes

Membrane fractions enriched in the hepatocyte canalicular membrane domain were prepared from rat liver and characterized as described [15,16].

2.4. Synthesis of 1-(n-pentyl)-9-[^{3}H]quinidinium

Quinidine was oxidized to quinidinone (9-oxo-quinidine) using potassium *tert*-butylate and 9-fluorenone in tetrahydrofuran according to [17]. The product was purified using thin-layer chromatography plates silica gel 60 (E. Merck, Darmstadt, Germany) with methanol/ethyl acetate/cyclohexane/acetic acid (40:40:16:2, v/v/v/v) as solvent. The relative retention of quinidinone was 0.14 compared to 0.36 for quinidine. The compound with a characteristic UV-absorption at 360 nm was extracted from the silica gel with methanol and subsequently reduced by sodium [^{3}H]borohydride (503 GBq/mmol) in methanol/0.01 M NaOH (50:50, v/v). Purification of 9-[^{3}H]quinidine was performed by RP-HPLC on a C18 Hypersil column (4.6 mm × 250 mm, 5 μm particles; Shandon, Runcorn, UK) with a C18 precolumn (Waters, Milford, MA, USA) using acetonitril/methanol/water/acetic acid (54:14:28:1, v/v/v/v; pH 5.4, adjusted with ammonium hydroxide) as the mobile-phase system. The flow rate was 0.75 ml/min. Alkylation of [9-^{3}H]quinidine with 1-iodopentane in acetone [7] resulted in 1-(n-pentyl)-[9-^{3}H]quinidinium which was again purified by the same RP-HPLC system as described above. The identity of the compound was confirmed by comigration of the non labeled n-pentyl quinidinium iodide. The purity of the ^{3}H-labeled substrates used for the kinetic experiments was ≥ 95%.

2.5. Vesicle transport studies

Transport of [^{3}H]taurocholate was measured by a rapid filtration technique using nitrocellulose filters (0.2 μm pore size) presoaked in 250 mM sucrose and 10 mM Tris-HCl (pH 7.4). The filtration was performed at 200 mbar pressure. Transport of ^{3}H-labeled APD-ajmalinium, quinidine, and pentyl quinidinium, respectively, was measured by the same technique using glass microfibre filters (pore size ≥ 0.7 μm) presoaked in Tris-buffered saline (pH 7.4). This filtration was performed at 850 mbar pressure.

2.6. Measurement of ATP-dependent transport into canalicular membrane vesicles

Membrane vesicles were incubated in the presence of 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and the labeled substrate at a final volume of 110 μl. 20 μl samples were taken at the indicated time points and diluted in 1 ml of ice-cold Tris-buffered saline (pH 7.4). This solution was applied to the pre-soaked filters and rinsed with 5 ml of ice-cold Tris-buffered saline (pH 7.4) containing 0.05% Tween and with 10 ml of Tris-buffered saline (pH 7.4). In control experiments ATP was replaced by 5′-AMP. Transport rates were calculated by subtracting the values in the presence of 5′-AMP from those in the presence of ATP.

2.7. Photoaffinity labeling and SDS-PAGE

Canalicular membrane vesicles (50 g protein) were incubated with APD-ajmalinium (185 KBq) at 37°C for 10 min and subsequently irradiated at 350 nm for 10 min at 4°C. After irradiation labeled proteins were separated by SDS-PAGE (7.5% gel, unboiled samples). Incorporated radioactivity was determined as described [8,15].

3. Results

3.1. ATP-dependent transport of organic cations in rat liver canalicular membrane vesicles

ATP-dependent transport of three different cationic compounds was observed using highly enriched rat liver CMV. [^{3}H]Quinidine (Fig. 1), *N*-(*n*-pentyl)-[^{3}H]quinidinium (Fig. 2), and APD-[^{3}H]ajmalinium (Fig. 3) exhibited ATP-dependent linear uptake into inside-out CMV in the presence of ATP and a regenerating system. Identical

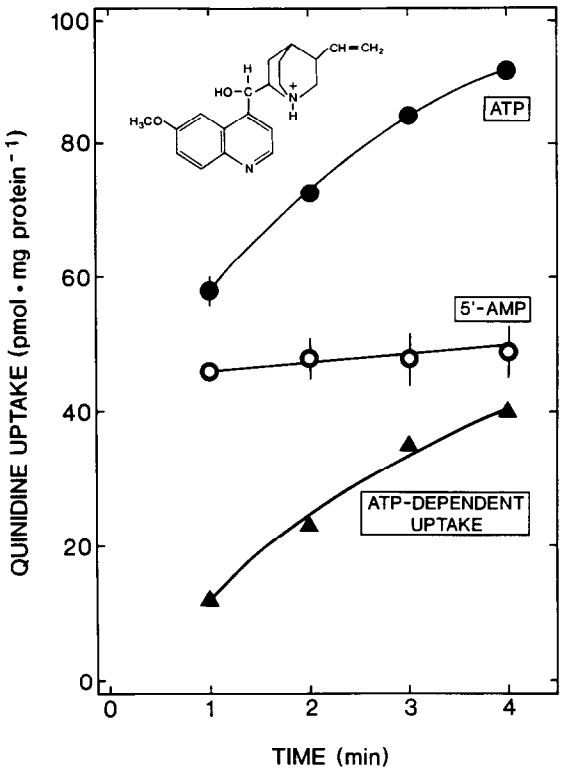

Fig. 1. ATP-dependent transport of [^{3}H]quinidine into rat liver canalicular membrane vesicles. Membrane vesicles (30 μg of protein) were incubated with ^{3}H-labeled quinidine (5 μM) at 37°C; 20 μl aliquots were removed and [^{3}H]quinidine uptake was determined by the rapid filtration technique described in section 2. Data are expressed as the mean ± S.E.M. from 4 separate experiments. ATP-dependent transport was calculated from the difference of [^{3}H]quinidine incorporated in the presence of ATP and 5′-AMP.

values for binding of *N*-(*n*-pentyl)-[^{3}H]quinidinium and APD-[^{3}H]ajmalinium were obtained in the presence of 5′-AMP or the non-hydrolyzable ATP analog adenylyl-(β,γ-methylene)-diphosphonate indicating that ATP hydrolysis is required for transport. The monoquaternary cations were more efficiently transported than the non-alkylated quinidine as evidenced by the 10-fold (for pentyl quinidinium) to 15-fold (for APD-ajmalinium) higher transport rates. The non-ATP-dependent binding was lowest for pentyl quinidinium which also exhibited the lowest K_m value of the 3 organic cations investigated (Table 1). Furthermore, the efficiency of the ATP-dependent transport of quinidine, pentyl quinidinium, and APD-ajmalinium, calculated by the V_{max}/K_m ratio (Table 1), amounted to 5, 450, and 77 (ml × mg^{-1} × min^{-1}), respectively.

Using liver CMV from transport mutant rat liver (TR$^-$ or GY), the rates of ATP-dependent transport of both cations, APD-ajmalinium (5 μM) and pentyl quinidinium (1 μM), were similar as the transport rates using CMV from normal Wistar rats (153 ± 10 pmol × min^{-1} × mg protein^{-1} (85% of normal) and

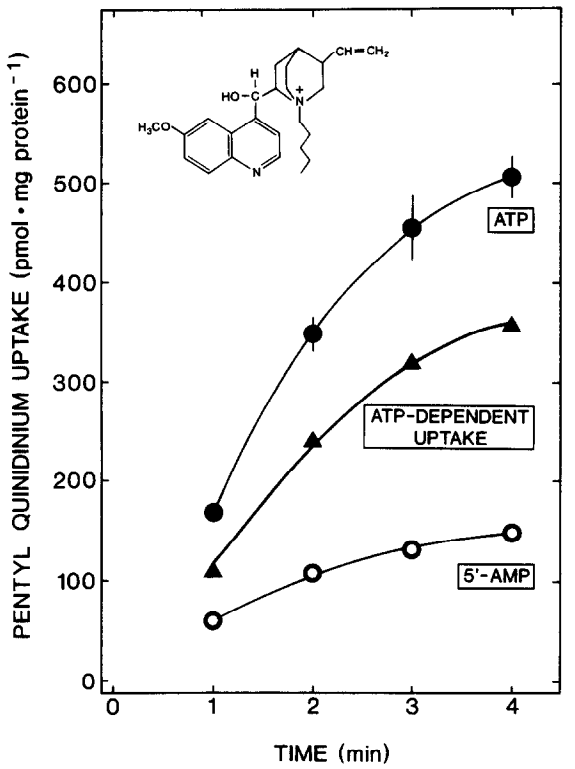

Fig. 2. ATP-dependent transport of ^{3}H-labeled *N*-(*n*-pentyl)-quinidinium (5 μM) into canalicular membrane vesicles. Incubation conditions were the same as described in the legend to Fig. 1. Each point represents the mean from 4 separate experiments.

115 ± 5 pmol × min^{-1} × mg protein^{-1} (94% of normal), respectively).

3.2. *Photoaffinity labeling of rat liver canalicular membranes*

Photoaffinity labeling of CMV from rat liver with the photolabile ajmalinium derivative revealed a predominant labeling of proteins with apparent molecular masses of 143 and 108 kDa (Fig. 4).

3.3. *Specificity of ATP-dependent transport of amphiphilic cations*

Vanadate inhibited the ATP-dependent transport of both permanently charged organic cations with a half maximal concentration of 20–30 μM. The ATP-dependent transport of pentyl quinidinium and APD-ajmalinium was inhibited to a similar extent by various organic cations known to interact with *mdr1* P-glycoprotein (Table 2). As an exception, daunorubicin inhibited the ATP-dependent APD-ajmalinium transport more efficiently than the transport of pentyl quinidinium. In contrast, no significant inhibition of ATP-dependent taurocholate transport was observed in the presence of several cationic inhibitors (Table 2).

Table 1
Kinetic parameters of the ATP-dependent transport of amphiphilic cations in hepatocyte canalicular membrane vesicles

Substrate	V_{max} (pmol · mg^{-1} · min^{-1})	K_m (μM)
Quinidine	25	5
Pentyl quinidinium	180	0.4
APD-ajmalinium	770	10

The kinetic constants were derived from double reciprocal plots according to Lineweaver and Burk. Transport studies were performed as described in section 2. Values are from quadruplicate determinations with all standard deviations below 15% of the mean.

4. Discussion

4.1. *ATP-dependent transport of amphiphilic cations into canalicular membrane vesicles mediated by* mdr1 *P-glycoprotein*

The liver plays a central role in the disposition of exogenous and endogenous compounds [18,19]. Primary-active ATP-dependent transport is the major mechanism to secrete toxic and non-toxic, exogenous as well as endogenous substances across the hepatocyte canalicular membrane into bile [4,6,15,19–23]. Four distinct ATP-dependent carriers were so far described in this membrane domain [2–6,19–24]: (i) the ATP-driven bile salt export carrier (BSEC) [15,20–22], (ii) the ATP-dependent leukotriene export carrier (LTEC) which

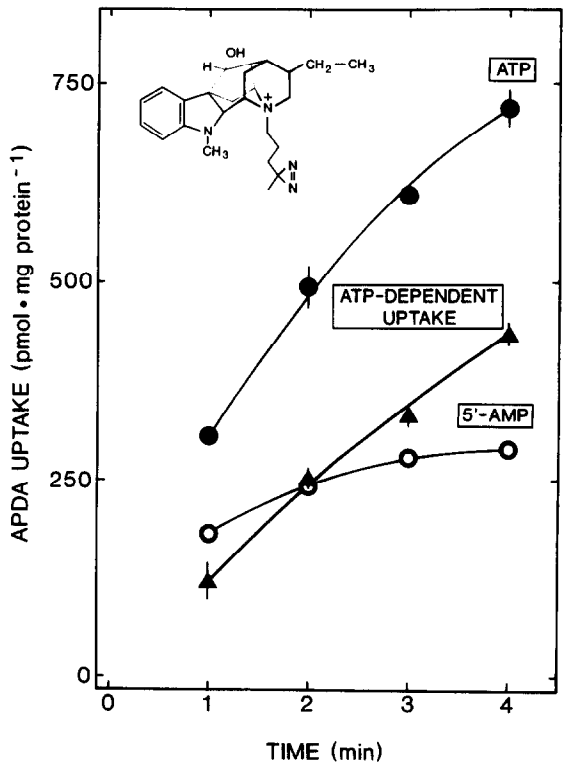

Fig. 3. ATP-dependent uptake of ^{3}H-labeled APD-ajmalinium (APDA) (5 μM) into canalicular membrane vesicles. Incubation conditions were the same as described in the legend to Fig. 1. Each point represents the mean from 4 separate experiments.

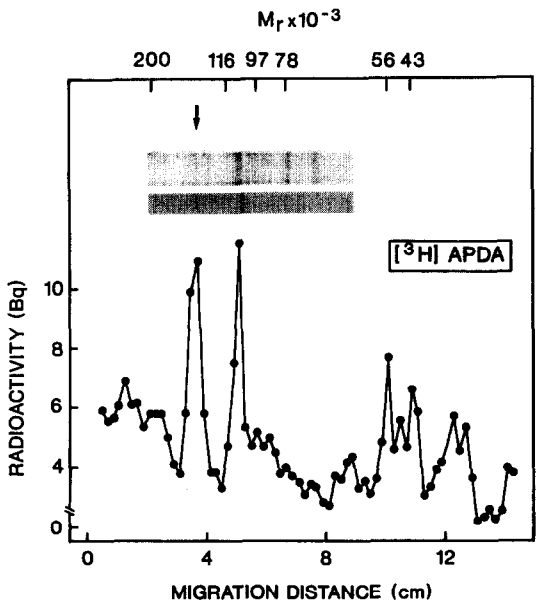

Fig. 4. Photoaffinity labeling of rat liver canalicular membranes with ^{3}H-labeled APD-ajmalinium (APDA). CMV (50 μg) were incubated with ^{3}H-labeled APD-ajmalinium (185 kBq) for 10 min. Photoaffinity labeling and SDS-PAGE (7.5% gel, unboiled samples) were performed as described in section 2. The curve shows the radioactivity in the gel slices. The lane above the curve indicates the proteins reacting with the C219 antibody in the Western blot. The upper lane shows the Coomassie-stained gel with the molecular weight standards at the top.

transports leukotriene C_4 and related conjugates of various exogenous and endogenous substances [23], (iii) the ATP-dependent daunorubicin transport mediated by *mdr1* P-glycoprotein (multidrug export carrier, MDEC) [2–6], which was established in analogy to its function in multidrug-resistant tumor cells, although endogenous substrates for this export carrier have so far not been clearly defined, and (iv) the *mdr2* P-glycoprotein which plays an essential role in the secretion of phospholipids into bile as evidenced recently by *mdr2* gene knockout in mice [24].

Substrates for *mdr1* P-glycoprotein can differ in their chemical structure and in their physicochemical properties. Many of them contain cationic charges at the pH of body fluids but are not permanently charged. The sodium channel blocker quinidine is an anti-arrhythmic agent known for a long time [25]. This drug and its stereoisomer quinine are both, like the calcium channel blocker verapamil, potent multidrug reversal agents [26–29]. Another antiarrhythmic agent, ajmaline [25], is also potent as multidrug reversal agent [28] and resembles quinidine in structural aspects. Both alkaloid molecules contain an aromatic heterocycle structure and a quinuclidinyl moiety. Moreover, both organic bases have similar molecular weights (quinidine 324; ajmaline 326). In this study we have investigated the influence of quaternization of the tertiary amine structure, resulting in the permanently cationic substrates APD-ajmalinium (M_r = 407) and pentyl quinidinium (M_r = 395), on the kinetic behavior in ATP-dependent transport across the canalicular membrane of the hepatocyte. Several lines of evidence presented in this study strongly suggest that *mdr1* P-glycoprotein in canalicular membranes transports not only quinidine but also the monoquaternary n-pentyl derivatives of ajmaline and quinidine:

1. The photolabile ajmaline derivative labeled a canalicular membrane protein in the molecular weight range of P-glycoprotein, as shown by comigration of the photoaffinity-labeled protein with the P-glycoprotein detected by the C219 antibody (Fig. 4).
2. The ATP-dependent transport of the monoquaternary cations in CMV was sensitive to inhibition by typical multidrug reversal agents or known P-glycoprotein substrates at inhibitor concentrations which did not inhibit the BSEC- or LTEC-mediated transport systems (Table 2) [21,23].
3. The uptake rates for the two permanently charged cations were similar in CMV prepared from normal Wistar rat liver and transport mutant liver (see section 3.1), which lacks functionally active LTEC but expresses P-glycoproteins [4,23].

The major portion of the P-glycoprotein detectable by the monoclonal antibody C219 in CMV from rat or mouse liver represents the *mdr2* gene product [3] indicating a relatively low expression of the *mdr1a* and *mdr1b*

Table 2
Inhibitor specificity of the ATP-dependent transport of amphiphilic cations across the canalicular membrane in comparison with the ATP-dependent bile salt (taurocholate) transport

Substrate	Inhibitor (20 μM)	Rate (pmol · mg^{-1} · min^{-1})	% of control
APD-ajmalinium (5 μM)	control	180 ± 18	100
	vanadate	90 ± 18	49 ± 10
	quinidine	25 ± 9	14 ± 5
	pentyl quinidinium	9 ± 7	5 ± 4
	verapamil	27 ± 2	15 ± 1
	daunorubicin	20 ± 9	11 ± 5
	taurocholate	126 ± 18	70 ± 10
Pentyl quinidinium (1 μM)	control	122 ± 6	100
	vanadate	73 ± 5	60 ± 4
	quinidine	21 ± 7	17 ± 6
	pentyl quinidinium	6 ± 5	5 ± 4
	verapamil	12 ± 4	10 ± 3
	daunorubicin	87 ± 9	71 ± 8
	taurocholate	110 ± 2	90 ± 2
Taurocholate (5 μM)	control	125 ± 6	100
	quinidine	129 ± 1	103 ± 9
	pentyl quinidinium	114 ± 3	91 ± 2
	verapamil	134 ± 6	107 ± 5

Transport studies were performed as described in section 2. Values are expressed as the mean ± S.E.M. from quadruplicate determinations.

genes in normal liver. However, highly efficient organic cation transport was demonstrated in this study by use of the amphiphilic quinuclidinium and ajmalinium compounds (Table 1). The efficiency of the ATP-dependent transport of pentyl quinidinium, calculated by the V_{max}/K_m ratio, was 450 and thus in accordance with the ratios obtained for the physiological BSEC substrate taurocholate (180), and for leukotriene C_4 (425) as the best substrate for LTEC, in contrast to the established P-glycoprotein substrate daunorubicin with a ratio of 14 [6]. Pentyl quinidinium may, therefore, serve as a highly specific substrate in future studies defining physiological *mdr1* P-glycoprotein substrates.

4.2. *Implications for the mechanism of action of the multidrug export carrier*

Most P-glycoprotein substrates or inhibitors are weak bases or lipophilic uncharged molecules such as steroid hormones [1,29]. Passive diffusion of the uncharged basic substrate is faster than transport of the protonated form. The pH gradient across the membrane influences, therefore, drug accumulation. APD-ajmalinium, as a permanently charged cation, is not taken up by cells through passive diffusion but only by carrier-mediated transport [12,30]. As pointed out by Gottesman and Pastan [1] *mdr1* P-glycoprotein may mediate both reduced influx of drugs into the cytosol and increased efflux out of the cell. A model has been proposed suggesting the removal of hydrophobic drugs directly out of the plasma membrane in a process comparable to the action of a 'hydrophobic vacuum cleaner' [1,31]. One may speculate that the *mdr1* gene product functions not only as a 'hydrophobic vacuum cleaner' for hydrophobic basic drugs concentrated in the plasma membrane lipid bilayer, but also as a pump for more hydrophilic monoquaternary cations such as pentyl quinidinium or APD-ajmalinium.

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