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REDUCED OUABAIN-SENSITIVE POTASSIUM ENTRY AS A POSSIBLE MECHANISM OF MULTIDRUG-RESISTANCE IN P388 CELLS

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Abstract—Multidrug-resistant P388 cells were found to be resistant also to a variety of ammonium, phosphonium and arsonium compounds. As previously shown for anthracyclines and vinca alkaloids, the resistance to the permanently charged lipophilic cationic compounds could be circumvented by verapamil. Relative to drug-sensitive cells, K^+ uptake and plasma membrane Mg-ATPase activity in multidrug-resistant cells are ouabain resistant. The intracellular K^+ concentration in drug-resistant cells is maintained at a normal level by increased activity of the furosemide sensitive transport system. It is suggested that the reduced activity of the electrogenic Na^+–K^+ pump in multidrug-resistant, cells could result in a lower transmembrane potential and therefore reduced accumulation of cationic lipophilic compounds.

Tumor cells that acquire resistance to Adriamycin® (ADR†) frequently show also cross-resistance to other anthracyclines, vinca alkaloids, colchicine, actinomycin D and some other compounds with seemingly unrelated structure or mechanism of action [1, 2]. This phenomenon was termed multidrug-resistance (MDR).

Dano [3] confirmed earlier studies where, in comparison to wild-type cells, the accumulation of daunomycin in MDR cells was found to be reduced and he suggested that this reduction was due to an active extrusion mechanism. It has been proposed that a 170 kDa membrane glycoprotein (P-glycoprotein), found in many [4, 5] but not all [6–8] MDR cell lines, is the drug-efflux system. While at present this extrusion mechanism remains ill-defined, most investigators seem to agree that ADR, as well as the other drugs mentioned above, enter drug-sensitive and MDR cells by passive diffusion processes.

Skovsgaard [9] noted that the cellular uptake of ADR was pH dependent and suggested that like many drugs, ADR is passively transported into the cell in its unionized form.

Mayer *et al.* [10] have demonstrated that ADR and vinblastine, as some other cationic lipophilic compounds, are accumulated by liposomes in response to transmembrane potential (inside negative). This finding raises the possibility that these drugs may also enter cells in their ionized form, in accordance with the cell's trans-membrane potential. A potential gradient across the cell membrane might explain why *N*-trimethyladriamycin, a permanently charged analogue of ADR, was found to be an almost as active as ADR against ADR-sensitive cells [2]. According to this hypothesis, cells with reduced membrane potential would be resistant to ADR and vinblastine as well as to permanently charged lipophilic cationic compounds. In fact, resistance of MDR cells to three such compounds: triphenylmethylphosphonium bromide, ethidium bromide and *N*-trimethyladriamycin has been already reported [1, 2, 11]. Recently, it was found that the uptake of tetraphenylphosphonium in MDR KB cells was significantly lower than that measured in drug-sensitive KB cells [12]. Attempts to measure the membrane potential in drug-sensitive and MDR cells were carried out by an indirect approach. A reduced accumulation of 3,3′-dipentyloxacarbocyanide in MDR cells was interpreted as indicating that these cells had significantly lower membrane potential than drug-sensitive cells [13]. However, it was argued that the reduced accumulation of the dye in MDR cells may have resulted from its extrusion by the drug-efflux mechanism [12]. It seems that a definite answer will have to await a direct measurement of the cell membrane potential.

However, if these indirect measurements reflect a real difference in the magnitude of the membrane potential, between drug-sensitive and MDR cells, then one could expect: (a) that MDR cells will also display resistance toward other permanently charged lipophilic cationic compounds; (b) that verapamil, which was found to restore drug sensitivity in MDR cells should also increase the sensitivity of this cell line toward the lipophilic cations; (c) although not the sole determinant, the potassium entry processes have substantial influence on the magnitude of the membrane potential, therefore, the extent or nature of K^+ influx in MDR cells may differ from those occurring in drug-sensitive cells.

MATERIALS AND METHODS

Tricaprylylmethylammonium chloride (Aliquat 336), tetraoctylammonium bromide, cetrimonium bromide, cetylpyridinium chloride, quinaldine red, tetraphenylarsonium chloride, tetraphenylphosphonium chloride, methyltriphenylphosphonium

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† Abbreviations: ADR, Adriamycin; MDR, multidrug-resistance; P388/ADR, multidrug-resistant P388 cells; ED_{50}, concentration inhibiting the growth rate by 50%.

bromide, ethidium bromide, 3,8-diamino-6-phenylphenanthridine, dimidium bromide, neutral red, phenosafranin and safranine O, were purchased from Sigma-Aldrich Israel Ltd (Petach-Tikva, Israel). NSC 9404-C/2 was obtained from the Pharmaceutical Resources Branch, The National Cancer Institute (Bethesda, MD, U.S.A.).

ADR-sensitive and -resistant P388 murine leukemia cells were grown in culture as described previously [14]. The sensitivity of both cell lines to a compound was assessed as follows. Cells (1×10^5 per mL) were cultured in the presence of various compound concentrations and the cell density was measured in the next 4 days. The slope of the log cell density versus time plot was calculated by linear regression analysis. The growth rate at each concentration was expressed as the percentage of the control growth rate. Dose–effect curves were thus produced and were use to determine the compound's concentration inhibiting the growth rate by 50% (ED_{50}). Cell viability was determined by trypan blue exclusion.

The measurements of ^{86}Rb influx in both cell lines were carried out as follows: 80×10^6 viable cells were washed in solution A, composed of (mM): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 25 Na-HEPES and 5 glucose (pH 7.4). Then the cells were resuspended in solution A (or in solution A without $CaCl_2$) and preincubated for 20 min at 37° with and without ouabain (Sigma Chemical Co., St Louis, MO, U.S.A.), furosemide (Teva Pharmaceutical Industries, Petach-Tikva, Israel), or both drugs. The cell mixture volume was 4 mL. The reaction was initiated by adding 2 μCi ^{86}Rb (^{86}Rubidium 1 mCi/mL, sp. act. 1 mCi/mg, Amersham, Buckinghamshire, U.K.). In preliminary experiments the ^{86}Rb uptake was linear for at least 20 min, therefore in the following experiments 0.6 mL samples (in duplicates) were withdrawn at 1, 5 and 10 min, and layered on 0.2 mL silicon–oil mixture (550 fluid: 200 fluid 4:1 v/v, Dow Corning Co., Midland, MI, U.S.A.) in Eppendorf tubes. The tubes were centrifuged in a 5412 Eppendorf centrifuge (Eppendorf Geratebau, Netheler & Hinz GmbH, Hamburg, Germany) for 2 min. The upper and part of the oil layers were aspirated and the bottom of the tube containing the cell pellet was clipped into scintillation vials. After adding scintillation fluid the radioactivity was counted in a Beta scintillation spectrometer.

For measuring ^{86}Rb efflux, 1×10^8 viable cells were incubated at 37° for 2 hr in complete growth medium with 10 μCi ^{86}Rb. Then the cells were washed twice with 10 mL of cold solution A. The cells were resuspended in 10 mL of solution A preheated to 37° and 0.6 mL samples (in duplicates) were withdrawn at 1, 3, 5, 7, 10, 15 and 20 min and processed as described in the ^{86}Rb influx studies. For measuring the extracellular ^{86}Rb concentration, after the centrifugation and prior to the aspiration of the upper layer, 0.2 mL of that layer was transferred into scintillation vials for counting.

For measuring the cellular potassium and sodium content, choline chloride (150 mM) washed cells were resuspended in 1 mL of distillated water, and then homogenized in a Dounce tissue grinder. After 30 min centrifugation at 15,000 rpm the supernatant was collected. The potassium concentration was determined with an ion selective electrode for potassium in a Synchron CX3 Clinical System Instrument (Beckman Instruments Inc., San Ramon, CA, U.S.A.). Sodium concentration was determined at 295 nm with an atomic absorption spectrophotometer (model 403, Perkin–Elmer, Norwalk, CT, U.S.A.).

For measurements of plasma membrane Mg-ATPase activity, cells of both lines were disrupted by nitrogen cavitation and the plasma membranes were isolated as described previously [15]. The plasma membranes were resuspended in 50 mM Tris–HCl containing 1 mM EDTA (pH 7.4). Total and ouabain-resistant Mg-ATPase activities were determined as described by Lichtstein and Samuelov [16]. In brief, the membrane ability to hydrolyse ATP was determined in a reaction mixture containing 50 mM Tris-Cl, 100 mM NaCl, 5 mM KCl and 4 mM $MgCl_2$ with or without 1 mM ouabain. After 20 min preincubation, the reaction was started by adding ATP (final concentration 2 mM). After 0, 5, 10, 20 and 40 min the reaction was stopped by adding cold trichloroacetic acid (final concentration 5%) and cooling to 4°. After 5 min of centrifugation the supernatants were separated and the inorganic phosphate concentration was determined by the method of Itaya and Ui [17]. Protein concentration was determined with the Coomassie protein assay reagent (Pierce, Rockford, IL, U.S.A.).

RESULTS

As shown in Table 1, in a series of phenazine compounds phenyl substitution of a ring nitrogen results in a modest increase in the growth inhibitory effect in drug-sensitive P388 cells and in a marked decrease in the growth inhibitory activity in MDR P388 cells. Similar changes in growth inhibitory activity are obtained also by N-methylation of 3,8-diamino-6-phenylphenanthridine (dimidium). Thereafter, we have tested the growth inhibitory activities of a variety of ammonium, arsonium and phosphonium organic cations in drug-sensitive and MDR P388 cells (Table 2). The ED_{50} values obtained for these compounds in MDR cells were 6–222-fold higher than those obtained in drug-sensitive P388 cells. Verapamil, at a concentration that does not affect the growth rate of cells from both lines (10 μM), increased the sensitivity of MDR P388 cells to these cationic compounds 5–50-fold while in drug-sensitive cells the sensitivity was increased only 1.5–4-fold. There was no correlation between the degree of increase in sensitivity induced by verapamil in P388/ADR cells to that obtained in drug-sensitive P388 cells. The intensity of the verapamil effect was also independent of the relative resistance of MDR cells to these compounds.

The rate of K^+ influx in both cell lines was calculated from the measurements of the uptake of its analogue ^{86}Rb, in six experiments conducted on separate batches of cells at different dates. It was found that in P388 and P388/ADR cells these rates were 513 ± 51 and 722 ± 42 pmol $K^+/1 \times 10^6$ cells/min (mean ± SE) respectively. On a t-test for paired

Table 1. Growth inhibition of drug-sensitive and MDR P388 cells by phenazines and 6-phenylphenatridines

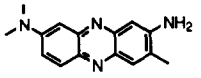
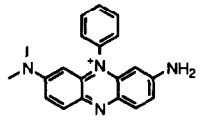
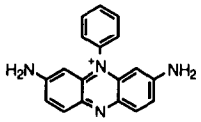
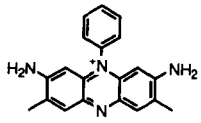
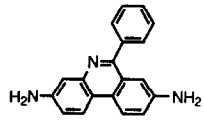
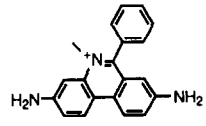
Compound		ED_{50} (μM) P388	P388/ADR
Neutral red		4.5	4.5
NSC 9404-C/2		3	200
Phenosafranin		2	100
Safranin O		2	60
3,8-Diamino-6-phenylphenanthridine		20	20
Dimidium		2	200

Table 2. Growth inhibition of P388 cells and their MDR subline by lipophilic cations in the absence and presence of 10 μM verapamil

	ED_{50} (μM) P388	P388 +verapamil	P388/ADR	P388/ADR +verapamil
Tetraoctylammonium	0.045	—	0.8	—
Aliquat 336	0.2	—	1.2	—
Cetylpyridinium	0.45	—	6.0	—
Quinaldine red	0.4	0.1	10	0.4
Cetrimonium	0.45	0.2	20	1.0
Tetraphenylarsonium	2.0	0.8	60	8.0
Ethidium	0.45	0.3	100	2.0
Tetraphenylphosphonium	3.0	2.0	100	20
Methyltriphenylphosphonium	40	20	1000	40

data (by date), the difference between these means was significant ($P < 0.01$).

As shown in Fig. 1, in ADR-sensitive P388 cells ouabain inhibited the ^{86}Rb uptake in a concentration dependent manner. The plateau level of inhibition obtained at 1.5–2 mM ouabain apparently represents the fraction of the K^+ that enters the cells via the Na^+–K^+ pump. In two separate experiments carried out with cells harvested on different dates, the fraction of ^{86}Rb uptake inhibited by 2 mM ouabain was >80% in drug-sensitive P388 cells and <35 in P388/ADR cells. When the cells were incubated in medium containing 2 mM CaCl (four separate experiments carried out with cells harvested on different dates), the effect of ouabain on the ^{86}Rb influx in these cell lines was qualitatively similar to that observed in the absence of calcium ions, but quantitatively the inhibition obtained was lower (60% in P388 and 10% in P388/ADR cells).

While relatively insensitive to ouabain, the ^{86}Rb

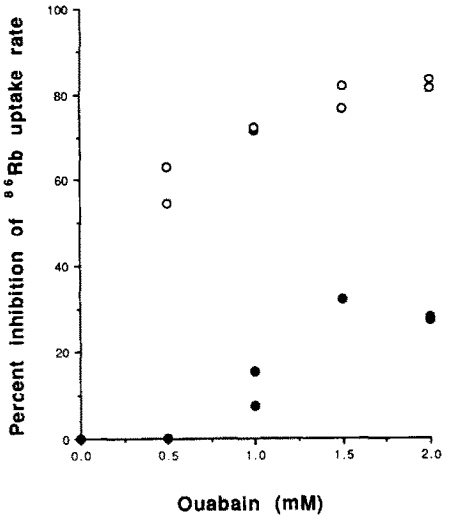


Fig. 1. The effect of ouabain on the ^{86}Rb uptake rate in drug-sensitive (○) and MDR (●) P388 cells. The cells were incubated in medium without $CaCl_2$.

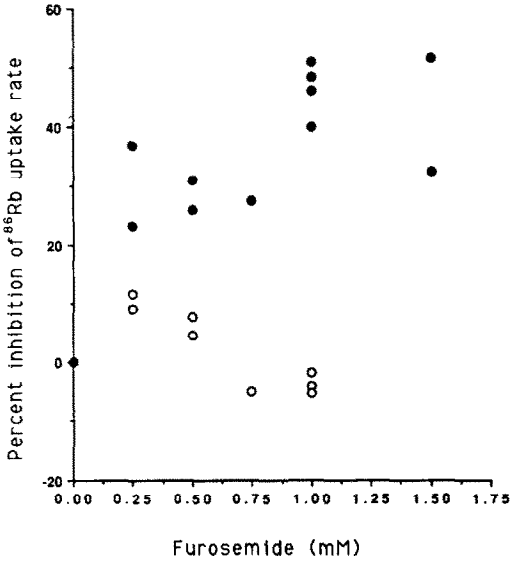


Fig. 2. The effect of furosemide on the ^{86}Rb uptake rate in drug-sensitive (○) and MDR (●) P388 cells.

influx in P388/ADR cells showed marked sensitivity to furosemide (Fig. 2). At a concentration of 0.25 mM, furosemide inhibited 23–37% of this uptake and maximal inhibition (40-52%) was obtained with 1 mM concentration of the drug. The effect of furosemide on the ^{86}Rb influx rate in P388 cells was much less prominent. At a concentration of 0.25 mM, furosemide lowered the influx by only 9–12% and at higher concentrations the inhibition observed was even smaller. In the presence of 2 mM ouabain and 1 mM furosemide the residual fraction of the ^{86}Rb influx rate in P388 and P388/ADR cells was 13.8–17.0% and 7.9–12.5%, respectively.

Table 3. Ouabain-sensitive Mg-ATPase activity in plasma membrane of P388 cells and of their MDR subline

	Mg-ATPase activity (nmol P_i released/mg protein/min)	
	Total	Ouabain (1 mM)
P388	10.20 ± 0.72	6.44 ± 0.36
P388/ADR	8.57 ± 0.33	7.21 ± 0.30

Values are means ± SD.

^{86}Rb efflux rate was determined in four experiments carried out on different batches of cells on separate dates. At the beginning of the efflux experiments the intracellular concentration of ^{86}Rb was >1000 higher than its extracellular concentration and the extracellular ^{86}Rb was <25% of the total ^{86}Rb in the cell suspension. Over the next 20 min, in both cell lines ^{86}Rb was released into the medium in an exponential manner. However, there was no significant difference in the ^{86}Rb efflux rate between these cell lines (data not shown).

The potassium contents of drug-sensitive P388 and P388/ADR cells were 25.51 ± 2.11 and 26.52 ± 1.03 nEq/1 × 10^6 viable cells, respectively (mean ± SD). The sodium contents of drug-sensitive P388 and P388/ADR cells were 12.8 ± 0.1 and 12.6 ± 0.3 nEq/1 × 10^6 viable cells respectively. As the cellular free water volume of drug-sensitive P388 cell cell is not significantly different from that of MDR P388 cell [18], the intracellular K^+ and Na^+ concentrations are also similar.

Under the conditions studied, the Mg-ATPase activity of plasma membranes obtained from these cell lines was linear for at least 40 min and <10% of the ATP present was consumed. As shown in Table 3, while in plasma membranes of drug-sensitive P388 cells, 3.76 P_i nmol/mg protein/min (37%) of the Mg-ATPase activity was inhibited by ouabain, only 1.36 nmol P_i/mg protein/min (16%) of this enzyme activity was inhibited by the drug in plasma membranes of P388/ADR cells.

DISCUSSION

The growth inhibition of drug-sensitive P388 cells by Neutral red or 3,8-diamino-6-phenylphenanthridine is similar to their effect in MDR P388 cells (Table 1). Substitution on a phenazine ring nitrogen by a phenyl group or N-methylation of the phenanthridine, results in permanently charged cationic compounds which are somewhat stronger inhibitors of the growth of drug-sensitive P388 cells and are markedly weaker inhibitors of the growth of MDR P388 cells. As shown in Table 2, P388/ADR cells are also resistant to a variety of lipophilic ammonium, arsonium and phosphonium compounds. These results suggest that MDR is in fact resistance toward compounds that are lipophilic cations. One of the most persistent features of MDR cells is that in the presence of certain agents, such as verapamil, the level of resistance is reduced greatly [19]. As shown in Table 2, whenever tested, verapamil also

markedly reduced the resistance of P388/ADR cells toward these lipophilic cationic compounds. As found previously with ADR, the effect of verapamil on the sensitivity of drug-sensitive P388 cells to these compounds was only marginal.

Lipophilic cationic compounds could be viewed also as cationic detergents, and their growth inhibitory activity might therefore be related to some interaction with the cell plasma membrane. According to this view, the relative resistance of MDR cells to these compounds suggests that the membrane lipid composition in these cells may differ from that of drug-sensitive cells. A differential cytotoxicity of non-ionic detergents, from the Triton X series, toward drug-sensitive and MDR Chinese hamster ovary cells was also reported [1]. In fact, the lipid composition of MDR P388 cells has already been reported to be different from that of drug-sensitive cells [15, 20].

Furthermore, the reversal of the resistance to these cationic compounds in MDR P388 cells by verapamil may be related to changes in lipid composition induced in these cells by the drug [21.

However, if these cationic compounds inhibit cell growth by acting at an intracellular site, their entry into cells with smaller transmembrane potential would be reduced and these cells would, therefore, be relatively resistant to such compounds. It was, therefore, important to explore in drug-sensitive and MDR cells the nature of the K^+ entry and exit.

Potassium can be taken up by cells via the well known ouabain sensitive Na^+–K^+ pump that exchanges 3 Na^+ for 2 K^+. This inequality in ion movement may generate a membrane potential (inside negative). Potassium could also enter cells by one or more of several types of K^+ channels [22, 23]. As previously reported for L1210 cells [24], more than 80% of the K^+ entry in drug-sensitive P388 cells could be inhibited by ouabain. Ouabain (1 mM) also inhibited 37% of the Mg-ATPase activity in these cells. In comparison to drug-sensitive P388 cells, the K^+ entry in MDR P388 cells and its Mg-ATPase activity were resistant to ouabain. Peterson and Biedler [25] have previously reported that in plasma membranes of MDR Chinese hamster cells the activity of Na^+–K^+-ATPase was lower than that measured in drug-sensitive cells. It could therefore be concluded that while in drug-sensitive P388 cells a considerable fraction of the K^+ entry is mediated by the Na^+–K^+ pump, this K^+ entry mechanism has a lesser role in maintaining the intracellular K^+ concentration in MDR P388 cells. The diminished activity of this electrogenic pump in MDR cells could result in reduced transmembrane potential and, as a consequence, reduced drug uptake. Furthermore, it is expected that the uptake of ADR in these cells would be ouabain resistant as was already reported for daunomycin in MDR Ehrlich acites cells [26]. On the other hand in HeLa cells, where a significant portion of the K^+ entry is ouabain sensitive [24], ouabain, as expected, was found to reduce the growth inhibitory effect of ADR [27].

The activity of Na^+–K^+-ATPase, a membrane lipoprotein, was reported to be dependent on interaction with certain membrane lipids. Although most of the studies indicate a requirement for negatively charged phospholipids, the enzyme activity was also reported to be supported by phosphatidylcholine (reviewed in Ref. 28). It is therefore not unreasonable to assume that the changes in lipid composition found in MDR P388 cells, in particular the decrease in phosphatidylcholine content [15, 20], may be associated with the decrease in activity of the Na^+–K^+ pump observed in these cells.

Although MDR P388 cells have diminished ouabain-sensitive potassium entry, their potassium content was found to be similar to that measured in drug-sensitive P388 cells. This suggests that the MDR cells have an alternative mechanism(s) for maintaining an intracellular/extracellular potassium gradient. As no major differences were found in the rate of K^+ efflux between these cell lines, other mechanism(s) of K^+ entry were looked for. Another mechanism for potassium entry into mammalian cells is a diuretic-sensitive K^+–Na^+–$2Cl^-$ cotransport [29, 30]. As shown in Fig. 2, a considerably larger fraction of the ^{86}Rb entry into MDR P388 cells is inhibited by furosemide than that inhibited by the drug in drug-sensitive cells. Therefore it seems that in MDR P388 cells, the reduced ability of taking up potassium by the Na^+–K^+ pump is compensated by the increased activity of the K^+–Na^+–$2Cl^-$ cotransporter. Unlike the Na^+–K^+ pump, this mechanism is electrically neutral and does not hyperpolarize the cell's plasma membrane. Increased entry of sodium and chloride into MDR P88 cells must escort the potassium entry by the cotransporter. However, as the activity of the Na^+–K^+ pump in MDR P388 cells is reduced, it is not yet clear how these cells dispose of excess of the intracellular sodium.

Recently the sequence of one of the genes responsible for cystic fibrosis was identified and its protein product and the normal counterpart were reconstructed [31]. It was suggested that the normal protein functions as a plasma membrane chloride channel [31–33]. The structure of this protein was found to be remarkably similar to the MDR P-glycoprotein [31] and we therefore tend to speculate that the P-glycoprotein, found in our MDR P388 cells, functions as the K^+–Na^+–$2Cl^-$ cotransporter. The results of the present study suggest that a decrease in transmembrane potential, due to reduced Na^+–K^+ pump activity, could result in decreased uptake of cationic lipophilic compounds whether permanently charged or mostly charged in physiological pH.

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