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CYTOSOLIC FREE Ca^{2+} IN DAUNORUBICIN AND VINCRISTINE RESISTANT EHRLICH ASCITES TUMOR CELLS

DRUG ACCUMULATION IS INDEPENDENT OF INTRACELLULAR Ca^{2+} CHANGES

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Abstract—The possible role of intracellular calcium on daunorubicin (DNR) accumulation in wild-type (EHR2) and multi-drug resistant (MDR) Ehrlich ascites tumor cell subline was investigated. DNR accumulation was not enhanced either by increasing the concentration of cellular calcium with the calcium ionophore ionomycin nor by chelating the cytosolic free Ca^{2+} by the membrane permeable Ca^{2+}-buffering agents BAPTA or MAPTAM. No effect was observed in the presence of extremely low extracellular calcium concentration that prevent transmembrane calcium influx or when the cells were calcium depleted using EGTA and ionomycin. Using the fluorescent Ca^{2+} indicator fura-2 it is further shown that both drug-resistant daunorubicin (EHR2/DNR+) and vincristine (EHR/VCR+) sublines had lower (50–80 nM) concentration of cytosolic free calcium ($[Ca^{2+}]_i$) compared to their corresponding wild-type parenteral tumors (140–180 nM). In calcium free medium, however, no significant difference was found, all cell lines having a $[Ca^{2+}]_i$ of 60–80 nM. Furthermore, the total amount of Ca^{2+} released to the cytosol with 10 μM ionomycin and 5 mM EGTA was 3–4-fold higher in EHR2 than in EHR2/DNR+ or EHR2/VCR+. Mobilization of Ca^{2+} with 1 μM ionomycin was almost identical in the presence and absence of Ca^{2+} in the extracellular medium in EHR2 as well as in EHR2/DNR+ suggesting that the increase in $[Ca^{2+}]_i$ is mainly due to discharge of Ca^{2+} from intracellular stores. Furthermore, the total cell calcium $[Ca^{2+}]_t$ concentration was slightly higher in EHR2/DNR+ and EHR2/VCR+ cells compared to EHR2. Incubation of the cells with the Ca^{2+}-channel blocker verapamil or the intracellular Ca^{2+}-antagonist TMB-8 causes depression of the Ca^{2+}-response in terms of rise in $[Ca^{2+}]_i$ caused by ionomycin. Sorcin, a major calcium-binding protein (M_r 22 kDa), is shown to be overproduced in EHR2/DNR+ cells. The overproduction of this protein in resistant cells may be related to the difference in the intracellular calcium observed in this study. Thus, though handling of Ca^{2+} is different in wild-type and MDR cell lines, our data suggest that calcium is not involved directly in drug transport processes and the level of Ca^{2+} *per se* have no influence on drug accumulation.

The emergence of resistance is a major problem in cancer chemotherapy. Acquired resistance to anthracyclines is invariably associated with cross resistance to the structurally unrelated vinca alkaloids and vice versa (MDR). The MDR phenotype is commonly associated with reduced drug accumulation, considered to be due, in part, to an energy dependent efflux mechanism in resistant cells [1]. Since the original observation by Tsuruo *et al.* [2, 3] that the calcium channel blocker verapamil was able to both modulate resistance and inhibit active drug efflux in resistant cells, there have been numerous confirmatory reports on the abilities of both calcium channel blockers and calmodulin inhibitors to reverse MDR. Both verapamil and trifluoperazine have been shown to be specific inhibitors of voltage-dependent calcium channel and calmodulin-dependent processes in mammalian cells, respectively [4, 5]. However, whether intracellular calcium is involved in the ability of these drugs to circumvent multi-drug resistance is still not fully known.

It seems that there is a general difference between wild-type and resistant tumor cells in their handling of intracellular calcium. Thus Tsuruo *et al.* [6], using ^{45}Ca, reported an increased calcium content in several resistant sublines compared to wild-type cells, while Nair *et al.* [7] found that wild-type cells had more free calcium and similar total calcium per unit protein compared to resistant cells. Furthermore, the few studies on the role of calcium in relation to MDR have yielded contradictory results. Thus Cano-Gauci and Riordan [8] have shown that verapamil was unable to alter $^{45}Ca^{2+}$ accumulation in wild-type or resistant cells [8], suggesting that these agents act in a calcium-independent manner. Kessel and Wilberling [9] have shown that while several calcium antagonists inhibited outward transport of DNR and modified the drug resistance pattern, these agents failed to alter calcium fluxes in P388/S and P388/ADR. Also, the ability to circumvent MDR did not correlate well with the effect on voltage-gated

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calcium channels [3, 10, 11]. These studies, however, should not be considered to have excluded the involvement of *intracellular calcium* in drug transport processes. Changes of intracellular free Ca^{2+} concentration control many cellular processes and drives the secretory mechanism of a wide variety of cells [12, 13]. Whether there is any relationship between changes in intracellular free Ca^{2+} levels and the ability of tumor cells to expel cytotoxic drugs into the medium is not well investigated. To our knowledge no study comparing drug accumulation and intracellular calcium concentrations in tumor cells has been performed. Therefore, in this study we have investigated the influence of intracellular calcium on daunorubicin accumulation in Ehrlich ascites tumor cells while inducing important changes in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Materials

DNR was obtained from Farmitalia (Milan, Italy) vincristine from Eli Lilly (U.S.A.) and verapamil from Meda A/S (Copenhagen). Fura-2/AM (acetoxymethylester), MAP-TAM/AM, BAPTA/AM and TPEN (Tetrakis (2-pyridylmethyl)ethylenediamine) were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). EGTA (ethyleneglycol-bis-(*b*-aminotehylether)-*N,N,N,N*-tetraacetic acid) and DTPA (diethylenetriamine-pentaacetic acid) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Chelex-100 resin columns were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Ionomycin and TMB-8 were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). All chemicals were of analytical grade.

Polyclonal chicken antibody against pure "denatured" sorcin was a generous gift from Marian B. Meyers, Memorial Sloan-Kettering Cancer Center (NY, U.S.A.). Peroxidase conjugated rabbit anti-chicken antibody was purchased from Zymed Laboratories (San Francisco, CA, U.S.A.).

Buffers

Standard medium was a phosphate buffer (pH adjusted to 7.45) containing (mM): 57 NaCl, 5 KCl, 1.3 $MgSO_4$, 9 NaH_2PO_4, 51 Na_2HPO_4 and 10 glucose. Calf serum (2–5%, v/v) was added to the medium in all trials. In suspensions containing Ca^{2+} the medium consisted of a Tris buffer (pH adjusted to 7.45) containing (mM): 50 Tris–HCl, 5 KCl, 92 NaCl and 1.3 $MgSO_4$.

For fluorescence measurement of Ca^{2+} a *modified medium* was used in mM: 145 NaCl, 5 KCl, 1 Na_2HPO_4, 1 $CaCl_2$, 0.5 $MgSO_4$, 5 glucose and 20 HEPES buffer. The medium was adjusted to pH 7.4 at 37° with NaOH. The water was distilled and Ca^{2+} decontaminated through Chelex-100 columns.

Cells and cell culture

The wild-type EHR2 and the DNR (EHR2/DNR+) and VCR (EHR2/VDR+) resistant sublines have previously been described in detail [14], and exhibit all the characteristics of the MDR phenotype including P-glycoprotein [15]. All sublines were maintained as ascitic tumors in first generation hybrids of female Swiss mice and male inbred DBA/2 mice by weekly transplantation of the tumor. Ascites fluid was removed 6–8 days after inoculation of the tumor. No DNR or VCR was administered in the last passage before *in vitro* experiments.

Viability test with trypan blue, performed before each experiment, showed that more than 90% of the tumor cells were intact. Preparation of cells, incubation, sampling and washing procedures were performed as described previously [1].

Drug accumulation studies

Drug accumulation in cells was determined by measuring the total drug fluorescence extracted from the drained pellet with 0.3 N HCl: 50% ethanol [1]. Fluorescence of the extractions was determined in an Aminco-Bowman spectrofluorometer (excitation, 470 nm; emission, 585 nm), and the drug concentration was determined by comparison with spectrophotometrically adjusted standards [16].

Measurement of intracellular calcium

Particular care was exercised to avoid possible pitfalls of Ca^{2+} measurements with fluorescent dyes, especially dye leakage, incomplete hydrolysis of the dye etc, as it is described in more detail elsewhere [17].

Cell loading. Fura-2/AM was kept in stock solution of dimethylsulfoxide and diluted in standard medium immediately before use. In order to improve dye loading this fura-2/AM colloidal solution was briefly sonicated to obtain fine emulsions and an equal volume of the non-ionic detergent Pluronic F-127 (25%, w/v) was added. Pluronic F-127 was used in order to improve dye loading [18].

Ehrlich ascites tumor cells (5×10^6 cells/mL) were loaded by incubation for 15–30 min with 1 μM of the above fura-2/AM solution in standard buffer containing 2% bovine serum albumin. And finally, only those preparations where 340/380 ratios were >10 were used suggesting that cell loading was successful.

Checking of fura-2/AM hydrolysis. As previously reported for granulocytes [19], the presence of unhydrolysed esters of fura-2 in the cells may result in an underestimation of the $[Ca^{2+}]_i$. To check the confidency of our data we performed several manipulations. One way is to check that the excitation peak of the loaded dye corresponds to that of fura-2 (excitation spectrum of fura-2/AM is different from that of fura-2). The ratio value should also correspond to that of fura-2. In other experiments we plotted the $[Ca^{2+}]_i$ of our measurements as the function of intracellular fura-2 concentration. There was no correlation between the two values (otherwise, the presence of unhydrolysed dye will give a false increase in $[Ca^{2+}]_i$ because increase in the intracellular concentration of fura-2 should cause an increase in the amount of this insensitive form of fura-2). Finally, the fact that fura-2 quenches completely upon addition of Mn^{2+} but fura-2/AM does not quench was also used to investigate the degree of fura-2 hydrolysis. After loading a cell sample was centrifuged and resuspended in modified medium where Ca^{2+} were changed with equimolar concentration of Mn^{2+} (1 mM) and containing 1 μM

Table 1.

	Cytosolic free Ca^{2+} (nM)		Total calcium (mM)
	$+Ca^{2+}$	$-Ca^{2+}$	
EHR2	160 ± 20	70 ± 10	3.15 ± 0.15
EHR2/DNR+	65 ± 15	70 ± 10	5.54 ± 0.21
EHR2/VCR+	65 ± 15	70 ± 10	4.62 ± 0.27

inomycin. In all our experiments this resulted in total quenching of fluorescence (this means that fura-2/AM is hydrolysed and converted into fura-2).

Leakage. Fura-2 seems to leak out of some cells. To estimate the rate of leakage we used two simple methods. In the first method we simply measured the fluorescence of the medium every 15 min after pelleting the cells by means of centrifugation. In the other method 50 μL of a 20 M EGTA solution was added to different samples every 10 min. The abrupt decrease in fluorescence signal was taken as the amount of extracellular dye. The rate of leakage varied between 10–20% per hr at 37° but this varied from cell preparation to cell preparation and was temperature dependent. However, incubation at room temperature reduced this leakage to 5–8% per hr. In some preparations we made use of probenecid to reduce dye leakage [20].

Presence of heavy metals. It has been reported recently that the presence of heavy metals in Ehrlich ascites tumor cells might disturb $[Ca^{2+}]_i$ values [21]. Therefore, all $[Ca^{2+}]_i$ were corrected for heavy metal interference using the heavy metal chelator TPEN [21].

Instrumentation

Time course of changes in fluorescence was measured by single- or dual-excitation wavelength spectrometry (to obtain full advantage of the capabilities of the dye) as previously described for epithelial cells [18] and fibroblasts [22]. This technique is more cumbersome and was therefore only used in those experiments to determine the absolute concentration of intracellular Ca^{2+}. The advantages of the dual wavelength method have already been described [23]. Autofluorescence values were obtained from matching suspensions of unloaded cells from the same batch. The dissociation constant of the Ca^{2+}-fura-2/AM complex was assumed to be 224 nM [24]. None of the experimental agents used altered the autofluorescence of the cells at the concentrations used.

Total calcium was measured using a modification of a previously described method [25]. Briefly, Ehrlich tumor cells were transferred into several 75 μL hematocrit tubes containing 10 μL EGTA on top of 20 μL of 2:1 (v/v) mixture of butylphthalate and dinonylphthalate (BDH Chemicals, Poole, U.K.). The tubes were centrifuged in a hematocrit centrifuge for 15 min. The length of the tubes was measured (1 mm = 1 μL). The bottom parts of the tubes containing the cells was amputated and the content was put into 10 mmol/L NaCl and 3 mM La^{3+} to lyse and to liberate the bound calcium, respectively. The calcium content of the solution was determined by atomic absorption spectrometry (Perkin–Elmer 403). The concentrations were expressed as the mean value of two to three different hematocrit tubes sized above 10 μL.

Immunological detection of sorcin

Eluted proteins were measured by Bio-Rad protein assay.

Western transfer procedure. Cells washed with PBS (150 mM NaCl, 50 mM phosphate, pH 7.2) were spun down at 150 *g*. The cell pellets were then lysed (v/v, 1:3) in 1% Triton X-100 in 50 mM Tris (pH 7.2) as described by Meyers *et al.* [26]. After centrifugation at 1400 *g*, aliquots of the supernatants containing 500–2000 μg of protein were loaded onto a 16% (w/w) SDS–PAGE with 0.1% (w/w) SDS according to the method described by Laemmli [27]. Proteins were blotted onto nitrocellulose paper after electrophoresis. The paper was washed in a 150 mM NaCl, 50 mM Tris (pH 7.4) buffer containing 3% (w/w) bovine serum albumin, and 0.1% (v/v) Tween 20, and probed overnight at 4° with the sorcin antibody at 1:50 dilution. Peroxidase conjugated rabbit anti-chicken antibody was used as the secondary antibody at a dilution of 1:250 applied for 2 hr. The blot was developed using 3-amino-9-ethyl carbazole as chromogen. Controls were performed by omission of the primary antibody.

RESULTS

Measurement of basal calcium levels

The level of $[Ca^{2+}]_i$ in resting EHR2 cells as measured with dual excitation was significantly higher (160 ± 20 nM, mean ± SE, N = 50) than EHR2/DNR+ or EHR2/VCR+ (65 ± 15 nM, mean ± SE, N = 47), ($P < 0.01$). However, in the absence of calcium and in the presence of 0.2 mM EGTA in the extracellular medium, the cytosolic free calcium concentration was to 60–80 nM in all three cell lines (Table 1).

In contrast, total calcium in resistant cells was lower than in the sensitive line (Table 1).

Manipulations of intracellular calcium levels and DNR uptake

Since the cytosolic free Ca^{2+} concentration is low in the resting state, the drug extrusion mechanisms must involve either a release of Ca^{2+} from intracellular stores or an entry of Ca^{2+} from the

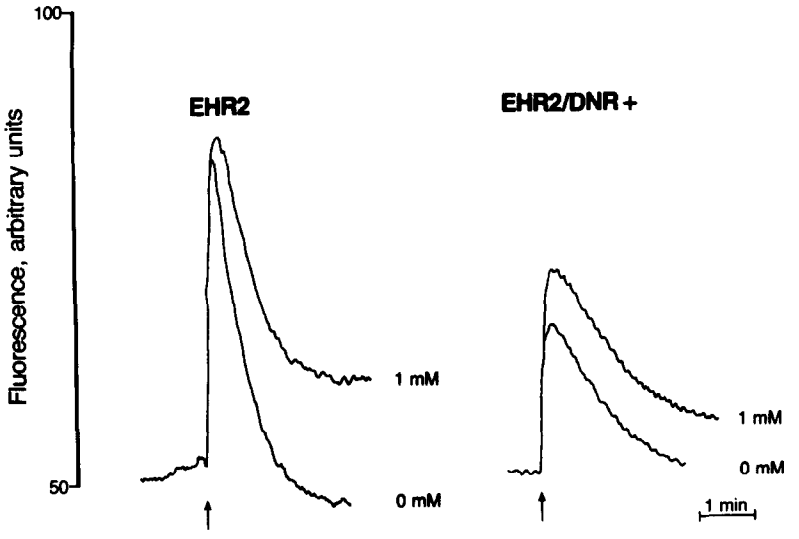

Fig. 1.Time course of ionophore-induced Ca^{2+} changes in wild-type (EHR2) and drug resistant (EHR2/DNR+) Ehrlich ascites tumor cells incubated with (1 mM) or without Ca^{2+} (0 mM). Ca^{2+} changes were monitored by fura-2 fluorescence using 340 and 380 nm as excitation and emission, respectively. The scale of fluorescence is arbitrary. Cells (5×10^6 cells/mL) were loaded with fura-2/AM as described in Materials and Methods. At the time indicated (arrows) 1 μM ionomycin was added. Each panel represents a typical trace of 48 different experiments.

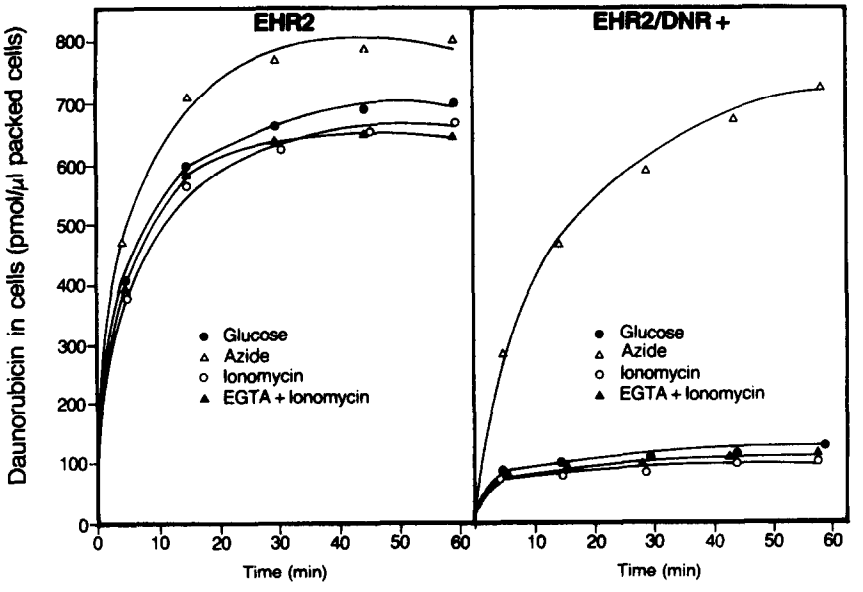

Fig. 2. Influence on time course uptake of DNR following depletion of intracellular Ca^{2+} pools with 5 mM EGTA and 1 μM ionomycin. The effect of ionomycin and sodium azide on DNR uptake are also shown. Glucose trace is used as control. A typical trace of three experiments.

external medium. One way to investigate this matter is to use a calcium ionophore which selectively affects Ca^{2+} movements and which experimentally induce changes in the free Ca^{2+} concentration in cells. In the present study we used the non-fluorescent Ca^{2+}-ionophore ionomycin. Figure 1 shows time course changes of fura-2 fluorescence, i.e. in $[Ca^{2+}]_i$, following exposure to ionomycin in *in vivo* Ehrlich ascites tumor cells incubated in the presence of 1 mM extracellular calcium. A maximum 10-fold and 6-fold increase in $[Ca^{2+}]_i$ was obtained with 1 μM ionomycin in EHR2 and EHR2/DNR+, respectively. The effect on $[Ca^{2+}]_i$ occurred promptly upon addition of the calcium ionophore, and $[Ca^{2+}]_i$ returned to normal values within 3 min. Ionomycin at concentrations (10–100 μM) several times higher than used in physiological studies, caused only a transient increase in the cytosolic free Ca^{2+} concentration.

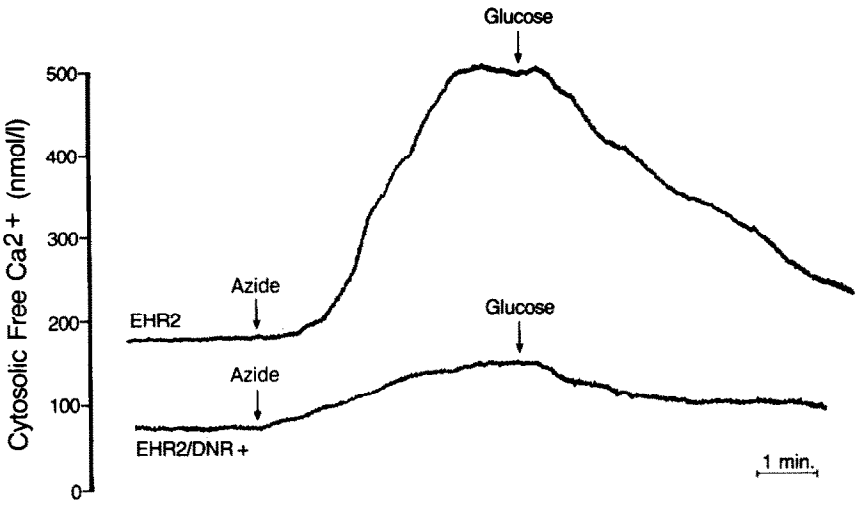

Fig. 3. Effect of sodium azide and glucose on $[Ca^{2}]_i$ in wild-type (EHR2) and drug resistant (EHR2/DNR+) tumor cells. Conditions as in Fig. 1. At the time indicated by the arrows the additions were 10 μM sodium azide and 10 mM glucose. A typical trace of seven experiments.

To investigate to which extent Ca^{2+} entry and Ca^{2+} release from intracellular pools contributed to the ionomycin-induced rise in $[Ca^{2+}]_i$, EHR2 and EHR2/DNR+ were suspended in medium without Ca^{2+} and containing 0.2 mM EGTA (Fig. 1, 0 mM). As can be seen from the figure this treatment resulted in $[Ca^{2+}]_i$ increase similar in magnitude and duration to those observed in the presence of 1 mM calcium suggesting that ionomycin mobilizes calcium to the cytosol preferentially from intracellular pools.

The time course of DNR uptake in sensitive and resistant whole cells using ionomycin is shown in Fig. 2. The data demonstrate that adding 10 μM ionomycin to resistant cells does not increase the uptake of DNR to the levels of sensitive cells. In another experiment we depleted the intracellular calcium stores by pretreating wild-type and resistant tumor cells with 1 μM ionomycin in the presence of 5 mM EGTA. This treatment lowered the cytosolic concentration of free Ca^{2+} to extremely low levels (<10 nM) (data not shown). Measurement of DNR-uptake in EHR2 and EHR2/DNR+ cells under this condition is shown in Fig. 2. This treatment also failed to enhance DNR uptake.

In the next experiment the effect of energy depletion on $[Ca^{2+}]_i$ was investigated. To prevent any active outward transport process, energy metabolism was abolished by omission of glucose for 10 min before the cells were challenged with sodium azide or ionomycin. As can be seen from Fig. 3, addition of azide caused a rapid increase in $[Ca^{2+}]_i$ in EHR2. However, in EHR2/DNR+ cells the Ca^{2+} increase was much lower. Upon addition of glucose to the poisoned cells, the $[Ca^{2+}]_i$ rapidly decreases and reaches a new steady state slightly higher than the initial values. However, addition of glucose later than 15–20 min after sodium azide, a continuous increase in fluorescence (i.e. in $[Ca^{2+}]_i$) was seen in both cell lines (data not shown). The initial cytosolic Ca^{2+} rise originated from the intracellular stores since we observed a similar Ca^{2+} increase when cells were incubated in calcium free medium. Parallel experiments showing the influence of azide on DNR uptake time course were performed (Fig. 2). These data show that there is no correlation between $[Ca^{2+}]_i$ changes and DNR uptake.

To investigate whether release of Ca^{2+} from intracellular pools is involved in the mechanism of drug accumulation experiments were performed using the intracellular Ca^{2+}-buffering agents BAPTA and MAPTAM. These can be accumulated in the cytoplasm of intact cells and thus buffer the release of calcium from intracellular stores. Wild-type and resistant tumor cells were preloaded with BAPTA or MAPTAM and DNR uptake experiments were carried out as described in Materials and Methods. The experiments were performed in the presence (1 mM) as well as in the absence of calcium. The buffering capacity of these agents was ensured by the lack of ionomycin to increase $[Ca^{2+}]_i$ in BAPTA or MAPTAM loaded cells. Figure 4 shows the influence of BAPTA (upper trace) and MAPTAM (lower trace) on the time course level of DNR in whole cells from wild-type and resistant lines. It is seen that neither BAPTA, at 5 and 30 μM, nor MAPTAM, at concentration 50 μM increases the steady-state uptake of DNR in resistant cells. However, MAPTAM at 300 μM increased slightly, but significantly, the uptake of DNR in sensitive as well as in resistant cells. This slight increase in DNR accumulation was probably due to a toxic effect of the high concentration of the used chelator.

Taken together, these data support that DNR uptake is not dependent on intracellular calcium release or influx of extracellular Ca^{2+}.

Cytotoxicity of DNR in combination with TMB-8

TMB-8 has been reported to be an intracellular Ca^{2+} antagonist [28] and has been shown only recently to enhance chloroquine accumulation in

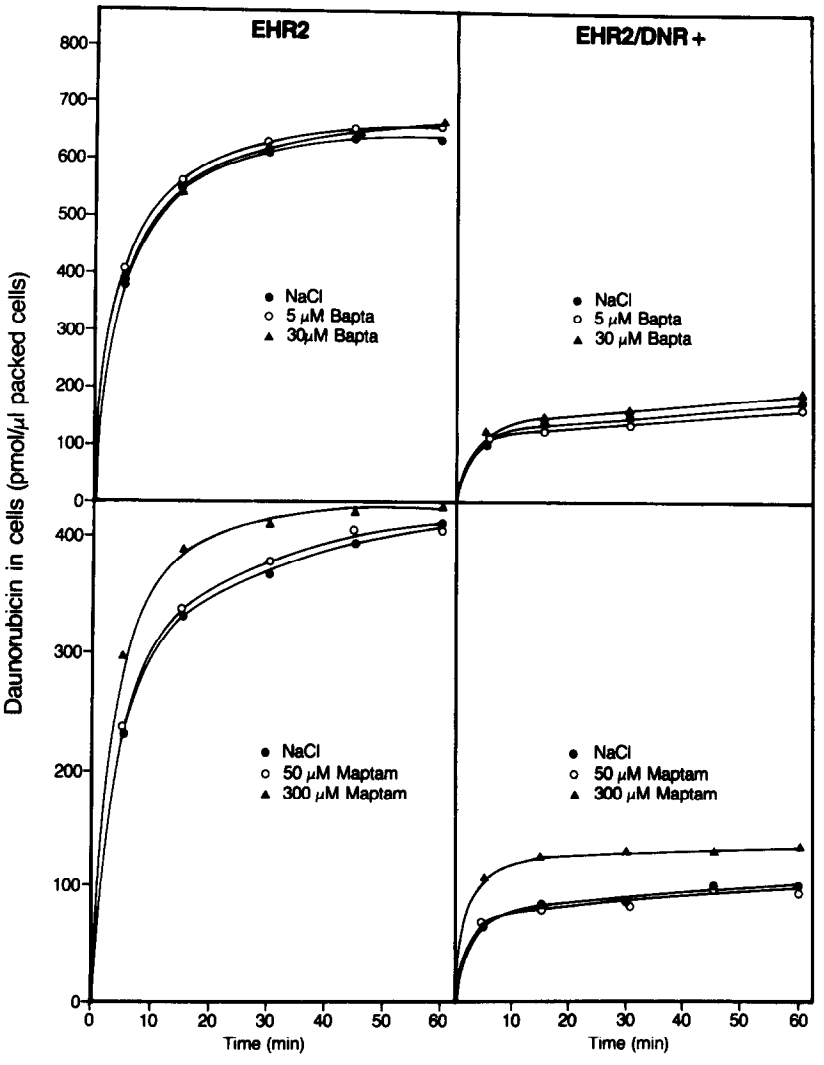

Fig. 4. Effect of BAPTA (upper trace) and MAPTAM (lower trace) on time course of DNR uptake in whole cells. BAPTA or MAPTAM loaded wild-type Ehrlich tumor cells and its resistant subline (5 μL packed-cells/mL) were incubated in standard medium, pH 7.45 at 37°. DNR (5 μM) was added at time zero. At the time indicated the cellular content of DNR was determined by measuring the total drug fluorescence extracted from the cells as described in Materials and Methods. Trace with NaCl is used as control. Typical trace of five experiments.

resistant *Plasmodium falciparum* [29]. Due to its dual effect as a modulator of MDR and calcium TMB-8 was used to study whether it has any effect on drug accumulation in tumor cells and whether this was related to intracellular Ca^{2+} release from cellular Ca^{2+}-pools. Figure 5 shows the dose–response curves to DNR combined with TMB-8 or verapamil in relation to simultaneously performed dose–response curves to TMB-8 alone in resistant *in vitro* cell lines. Cytotoxicity was assayed by enumeration of surviving cells. As can be seen from the figure the dose–response curve for DNR/TMB-8 is very close to and parallel to the DNR/verapamil curve indicating that TMB-8 may be a potential drug resistance modulation agent.

To analyse the effects of verapamil and TMB-8 on Ca^{2+}-movements wild-type tumor cells were incubated for 60 min, in medium containing 1 mM calcium together with either 5 μM verapamil or 50 μM TMB-8. Figure 6A shows that in EHR2 cells the fura-2 fluorescence increase induced by 1 μM ionomycin is partially blocked by verapamil and TMB-8. The per cent inhibition caused by verapamil and TMB-8 was almost similar, 37 ± 7% at 5 μM (50 μM for TMB-8) and 82 ± 6% at 25 μM (100 μM for TMB-8). Similar results were obtained with EHR2/DNR+ (data not shown). To understand the mechanism of reduction of the Ca^{2+}-transient by verapamil and TMB-8 we performed the same experiments in the absence of calcium and in the presence of 0.5 mM EGTA in the external medium. In this condition, possible Ca^{2+} entry from the extracellular medium is abolished, so that the $[Ca^{2+}]_i$ rise is due only to its release from the cellular stores.

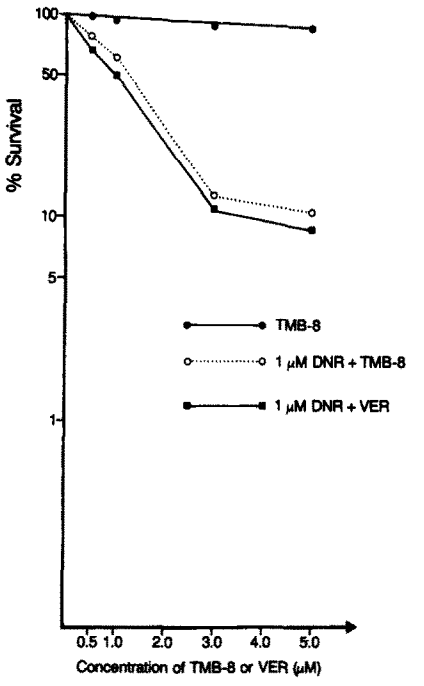

Fig. 5. Dose–survival curves for EHR2/DNR+/VT incubated for 3 days with either TMB-8 alone (●—●) or 1 μM DNR and TMB-8 (○····○) or 1 μM DNR and VER (■—■). Survival with 1 μM DNR alone was 100% (data not shown). Survival with VER alone was 100% for all tested concentrations.

The results from Fig. 6B shows that nearly the same inhibition was obtained in Ca^{2+} free medium. This indicates that both TMB-8 and verapamil inhibit calcium release from intracellular stores.

The time course of DNR uptake in sensitive and resistant whole cells is shown in Fig. 7. The data demonstrate that adding 100 μM TMB-8 to resistant cells increased the uptake of DNR (from 180 to 350 pmol, at 60 min) but not to the levels of sensitive cells. However, compared to the energy depletion experiment (Fig. 2) with azide DNR accumulation was only about 50%. In EHR2 cells this effect was less pronounced. Thus the enhanced cytotoxicity seen when DNR and TMB-8 are combined (Fig. 5) agrees with the enhanced uptake of DNR in EHR2/DNR+ cells (Fig. 7).

In a previous study [30], we have demonstrated the voltage-dependent calcium channel blocker verapamil, was shown to overcome DNR resistance in Ehrlich ascites tumor cells. To investigate whether Ehrlich ascites tumor cells possess voltage sensitive calcium channels the external K^+ concentration was increased by adding 50–100 mM KCl to the cuvettes with the cells in order to depolarize the plasma membrane while monitoring $[Ca^{2+}]_i$. This had no effect on $[Ca^{2+}]_i$ (data not shown). The lack of effect of depolarization on $[Ca^{2+}]_i$ argues against the presence of voltage-activated Ca^{2+} channels in wild-type and resistant ascites tumor cells. This finding indicates that the effect of verapamil cannot be ascribed to the action of verapamil on voltage sensitive Ca^{2+}-channels. The effect of verapamil on the membrane transport of DNR could be a result of facilitation of influx, of reduction of active outward transport, or of both mechanisms [30].

Presence of sorcin

Sorcin, a calcium-binding protein, is reported to be overproduced in several MDR cell lines [26]. To investigate whether there was any relationship between the lower $[Ca^{2+}]_i$ in EHR2/DNR+ observed and sorcin content in our cell preparations we performed Western blot detection of sorcin studies with antisorcin antibody on EHR2 and EHR2/DNR+. The results are shown in Fig. 8. A broad band of molecular mass 20 kDa, present in resistant but absent in wild-type Ehrlich ascites tumor cells, is readily detected (Fig. 8, lane B).

DISCUSSION

There have been relatively few and partly contradictory reports in the literature on the role of calcium in multi-drug resistance (MDR). Therefore, an attempt has been made to analyse the effect of increasing or buffering intracellular free Ca^{2+}, or of inhibiting its release from Ca^{2+}-pools on DNR accumulation.

In the present study, we have determined that drug transport processes are neither associated with nor require calcium. Using Ehrlich ascites tumor cells we showed that no correlation exists between drug accumulation and the level of $[Ca^{2+}]_i$. Neither increase in intracellular Ca^{2+}, absence of external Ca^{2+}, inhibition of Ca^{2+} release from intracellular pools nor buffering of cytosolic free calcium were sufficient to change the accumulatoon of DNR in daunorubicin and vincristine resistant Ehrlich ascites tumor cells. Thus, our results do not confirm those of Murray *et al.* [31], who reported that extracellular calcium modifies the accumulation and retention of daunorubicin by Ehrlich ascites carcinoma cells. Comparison of time course uptake of DNR (Fig. 2) is shown to be inversely proportional to Ca^{2+} increase (Fig. 3) in energy depleted wild-type and resistant cells.

Furthermore, we have determined that the $[Ca^{2+}]_i$ in wild-type Ehrlich ascites tumor cells is approximately 2–3-fold higher than in both examined MDR lines. However, when suspended in Ca^{2+} free media, the cytosolic free calcium in EHR2 and EHR2/DNR+ was similar. Also, the total calcium content was about 1–2-fold higher in MDR cells. Finally, the total exchangeable Ca^{2+} mobilized from the intracellular pools by ionomycin in medium containing EGTA was 3–4-fold higher in EHR2 than in EHR2/DNR+ cells. This indicates that in the wild-type cells Ca^{2+} is more readily exchangeable. In agreement with other studies our results indicate that the intracellular calcium homeostasis is altered in the resistant tumor cells. Using murine leukemic cells (P388) Nair *et al.* [7], found that sensitive P388 had higher free calcium than resistant P388 cells. Tsuruo *et al.* [6], demonstrated that the total calcium content of VCR-resistant P388 leukemic cells was 1–2-fold higher than the parent sensitive cells, especially in the form of EGTA-removable surface

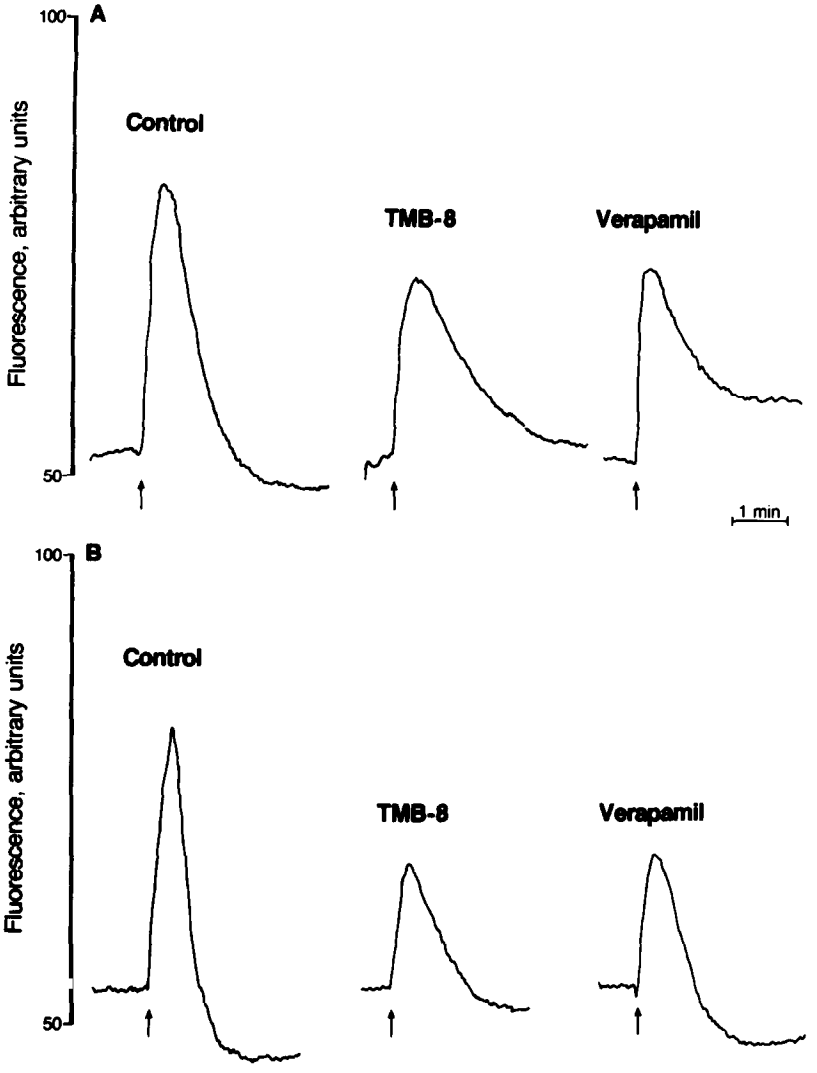

Fig. 6. Effect of TMB-8 and verapamil on fura-2 fluorescence (i.e. on $[Ca^{2+}]_i$) in wild-type ascites tumor cells incubated for 1 hr with 5 μM verapamil or 50 μM TMB-8, respectively, in medium with (A) or without Ca^{2+} (B). Immediately before fluorescence measurements 0.5 mM EGTA was added to experiment B. At the time indicated (arrows) 1 μM ionomycin was added. Typical traces of at least 10 experiments. For experimental details see Materials and Methods.

bound. The reason for this is not clear. This could be due to changes in the concentration of Ca^{2+}-binding protein, in the permeability of the cell membrane to Ca^{2+} or to changes in the activity of Ca^{2+}-ATPases. Increased levels of calmodulin have been observed in tumor cells [32] but Tsuruo *et al.* [6] found that the calmodulin content of resistant is almost the same as wild-type tumor cells. However, other calcium-binding proteins may also play a role. Recently, Meyers *et al.* [26] found that sorcin, a calcium-binding protein, is overproduced in several multi-drug-resistant cell lines. The lower cytosolic Ca^{2+} level and the higher total calcium content in resistant cells could be explained by a greater amount of sorcin found in resistant tumor cells in this study. Calcium might be effectively removed by sorcin. Meyers *et al.* [26] have proposed that MDR cells may utilize calcium in a different manner than in wild-type malignant cells, and sorcin may be part of that altered calcium pattern. It would be interesting to test a resistant cell line with the same background as EHR2/DNR+ but which did not overproduce sorcin to compare calcium characteristics. The weaker Ca^{2+}-mobilizing effect of ionomycin in resistant cells could be explained by changes in the permeability of the cell membrane to calcium. This could be due to differences in plasma membrane lipids between drug-sensitive and -resistant tumor cells [33]. The known abnormally overall high levels of cholesterol in tumor cell plasma membrane resulting in a decreased membrane fluidity might be one responsible factor for the inability of the ionomycin molecule itself to move Ca^{2+} across the plasma membrane [34]. Furthermore, it is also shown that the effects of the ionophore is more pronounced in endomembranes than in the plasma membrane.

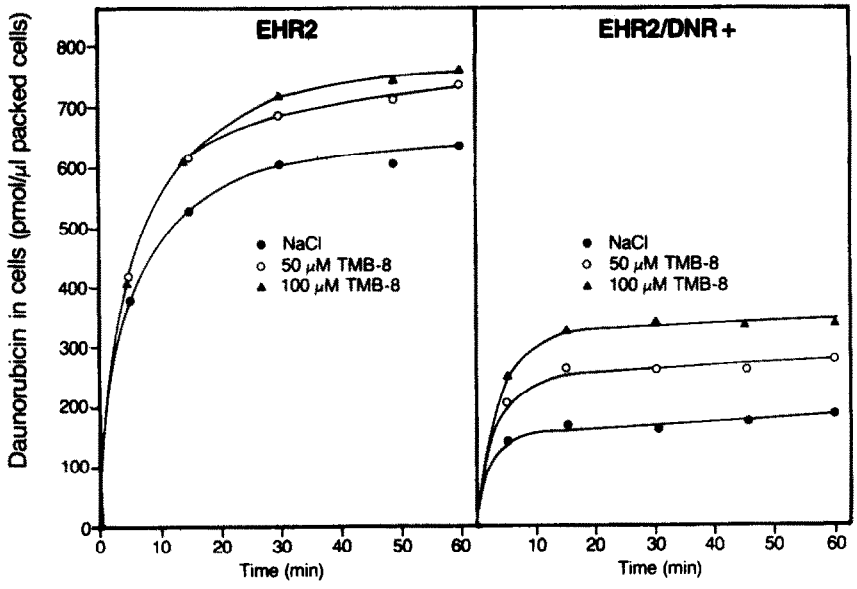

Fig. 7. Effect of increasing concentrations of TMB-8 on the time course uptake of DNR in wild-type and resistant cells. For experimental details see the legend to Fig. 4.

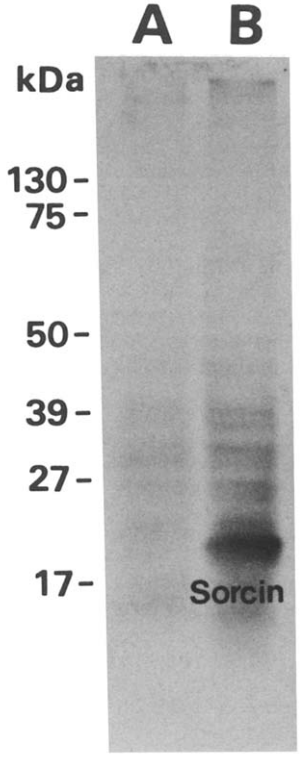

Fig. 8. Western blot detection of sorcin with antisorcin antibody on EHR2 cells (lane A) and EHR2/DNR+ cells (lane B) with protein loads of 1000 µg.

Similar effect has been demonstrated in GH4C1 rat pituitary cells [35].

It is well established that calcium channel blockers, such as verapamil, are able to modulate MDR by increasing drug accumulation [4, 5]. However, the exact mechanism by which verapamil increase drug accumulation in resistant cells is still not known. A tempting hypothesis is that verapamil by interfering with calcium fluxes via voltage-dependent Ca^{2+}-channels would affect drug efflux. However, the insensitivity of $[Ca^{2+}]_i$ to membrane depolarization by KCl argues against the presence of voltage-activated Ca^{2+}-channels in both Ehrlich ascites tumor cell lines. Our study confirms, therefore, the findings by Huet and Robert, who recently reported that the reversal of resistance by verapamil is not due to a direct effect on voltage sensitive calcium channels [36].

Calcium channel blockers like verapamil bind to the plasma membrane glycoprotein (Pgp) in MDR cells suggesting a competitive inhibition of the energy dependent efflux mechanism [37, 38]. However, verapamil [39] and trifluoperazone [40] accumulate equally in wild-type cells and MDR cells, indicating that these modulators do not serve as substrate for the outward transport system. Probably these agents can interact with P-glycoprotein and thus inhibit the drug efflux function of the protein [41]. Cellular factors regulating the activity of P-glycoprotein are not known. On the other hand, divalent cation(s) are essential for the ATPase activity of the P-glycoprotein [42].

The Ca^{2+} antagonist TMB-8 has been shown to enhance the accumulation of chloroquine by resistant *Plasmodium falciparum* [29]. But, its effect on tumor cells has never been reported. TMB-8 has been shown to inhibit Ca^{2+} release from the sarcoplasmic reticulum of smooth and skeletal muscles [28] and

inositol triphosphate induced Ca^{2+} release from membranes vesicles of oat roots [43]. In adrenal glomerulosa cells [44] and in neutrophils [45], however, TMB-8 inhibits calcium uptake. In the present study, we show that TMB-8 inhibits Ca^{2+} release from intracellular stores.

The lower concentration of intracellular free calcium observed in MDR cells compared to wild-type cells could explain the decrease in the overall activity of these cells, i.e. MDR cells are often more differentiated cells [46] and have a slower rate of proliferation [11]. Probably, changes toward tumor resistance is accompanied by a decrease in cytoplasmic free Ca^{2+} and increase in total calcium. One may postulate that with increasing resistance, as with aging [47], total cell calcium content increases but calcium availability (i.e. cytosolic free Ca^{2+}) is diminished.

In conclusion, the circumvention of resistance by verapamil cannot be explained by its effect on $[Ca^{2+}]_i$ but rather by its effect on P-glycoprotein on the plasma membrane.

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