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The multidrug-resistance-reverser verapamil interferes with cellular P-glycoprotein-mediated pumping of daunorubicin as a non-competing substrate

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We examined P-glycoprotein-mediated verapamil transport, using two drug-sensitive and multidrug resistant cell-line couples, i.e. A2780, 2780AD and SW-1573, SW-1573/1R500. The interaction of ³H-labeled verapamil with cells was measured using a flow-through system. The verapamil-containing medium was pumped over the cells and monitored on-line for radioactivity. In the P-glycoprotein-expressing cells, verapamil accumulation was increased by vinblastine and some known multidrug resistant (MDR) modifiers. Subsequent removal of these modifiers caused release of verapamil into the medium against a verapamil concentration gradient. In this manner, we obtained evidence that verapamil is actively transported by the MDR-related P-glycoprotein.

Using the flow-through system, we also exposed the cells to flowing culture medium containing daunorubicin, and measured the inhibition of daunorubicin efflux by verapamil. We found that, although the active efflux of daunorubicin was maximally blocked by verapamil short-term, longer-term active efflux of daunorubicin resumed. At a daunorubicin concentration in the flowing medium of 5 μM, increasing the verapamil concentration resulted in the same short-term effects, but in a significantly longer period of a maximal inhibition of daunorubicin efflux from the cells. At a daunorubicin concentration of 20 μM, increasing the verapamil concentration affected neither the short-term nor the long-term effects. These and other observations are in agreement with a model in which daunorubicin and verapamil are non-competing substrates for P-glycoprotein.

In conclusion, we obtained evidence that verapamil is actively transported by the MDR-related P-glycoprotein and that verapamil and daunorubicin are non-competing substrates for P-glycoprotein. Consequently, the effectiveness of verapamil as an MDR antagonist may be compromised because it is extruded by P-glycoprotein.

A major problem in the treatment of cancer patients is the development of resistance against natural-product anticancer drugs, such as anthracyclines and vinca alkaloids. In vitro cells have also been made resistant to such anticancer drugs. This resistance is called multidrug resistance (MDR). It is usually associated with a high-molecular-mass P-glycoprotein in the cellular plasma membrane [1, 2]. A common assumption has been that this protein acts as an energy-dependent efflux pump, which maintains a low intracellular drug concentration in MDR cancer cells [2, 3]. In 1981 Tsuruo et al. [4] reported that verapamil potentiated the cytotoxic activity of vincristine in the MDR murine leukemia cell line P388. Since that time, many compounds with different structural features have been found which reverse P-glycoprotein associated MDR [5, 6]. At a high concentration, verapamil is one of the compounds with the strongest in vitro effect on the vincristine or doxorubicin cytotoxicity in resistant mouse and human tumor cells [7, 8]. The main problem for clinical use of MDR modulators is the inability to show clear cytotoxic effects at the relatively low concentrations of the modulator that can be achieved clinically [9]. Another problem is that even relatively high concentrations of the MDR modulator usually do not completely reverse MDR; in particular, they do not restore the accumulation of the MDR-related drugs daunorubicin or doxorubicin [10]. The aim of our study was to examine the possible mechanisms underlying these two problems, with special emphasis on the cellular plasma membrane transport of verapamil into intact cells.

In photoaffinity-labeling studies, competition of verapamil with a vinblastine photoactive analogue for binding P-glycoprotein has been shown [11, 12]. Binding of photoactive analogues of verapamil was totally or partly inhibited by some known MDR modifiers (Cremophor EL, 100%) or MDR-related drugs (doxorubicin or vincristine in 5000-fold excess medium concentration, reduced the amount of radio-labeling by 67% or 78%, respectively) [13, 14]. If verapamil is a competing inhibitor of the pumping of the anticancer drug employed, the effectiveness of verapamil might be compromised at high (or lower) concentrations of daunorubicin because of the increase in intracellular drug concentration ensuing from partial P-glycoprotein inhibition. Consequently,
it is of interest to examine whether verapamil is a competing or a non-competing MDR modifier.

Verapamil increased cellular ATP consumption and lactate formation specifically in P-glycoprotein-containing MDR cells [15]. One possible explanation for these observations was that this resistance modifier might be transported by an ATP-hydrolysis dependent, P-glycoprotein-mediated, mechanism [15]. P-glycoprotein was indeed identified as a specific acceptor for photoaffinity analogues of verapamil in a Chinese hamster lung cell line resistant to vincristine [16] as well as in a human myelogenous leukemia cell line [13]. If verapamil is removed from the cell by P-glycoprotein, its effectiveness as a reversing agent might decrease. Decreased verapamil accumulation and increased efflux of verapamil have been reported for MDR cells [3, 13, 17–20]. However, in the drug-sensitive and MDR cell lines EHR2 and EHR2/ DNR ++, Sehested et al. [21] found no MDR-related reduction in verapamil accumulation, nor did they find an increased verapamil accumulation after energy depletion. Although some studies indicate a P-glycoprotein-mediated export of verapamil [3, 13, 17–20], which may account for the low effectiveness of the modifier, to date, it has not yet been proven that such a mechanism exists.

Here we examine these two possible explanations for the problems, using verapamil as an MDR antagonist in vivo. It was essential that this examination was performed in the physiological context of the intact cell. To this purpose, we employed the flow-through system [22–24]. We studied the transport of verapamil in two highly resistant cell lines, the human ovarian cancer cell line 2780Ad and the human lung cancer cell line SW-1573/1R500, and in their drug-sensitive counterparts A2780 and SW-1573. Cremophor EL, bepridil, vinblastine, daunorubicin, and amiodarone were used as known MDR modifiers or MDR-related drugs [14, 25–28]. Interpretation of the results obtained with intact cells was complicated because more than one process is involved in determining drug accumulation. To facilitate the interpretation, we developed a quantitative model of a cell with a passive membrane permeability parallel to an active pump for two substances.

Our results suggest that verapamil is a substrate for the P-glycoprotein but as such it does not compete with daunorubicin. Consequently, the low effectiveness by which verapamil reverses the MDR phenotype may not be caused by its competition with a certain anticancer drug, but by removal of verapamil by P-glycoprotein from the cell.

**MATERIALS AND METHODS**

**Chemicals**

Verapamil HCl and 2-deoxy-D-glucose were obtained from Sigma Chemical Co. Daunorubicin HCl was purchased from Specia. [N-3H-methyl]verapamil hydrochloride (specific activity 70 Ci/mmol) was obtained from New England Nuclear. Cremophor EL was a gift from Sandoz. Bepridil monohydrochloride monohydrate, β-l-(2-methyl-propoxy)-methyl]-N-phenyl-N-(phenylmethyl)-1-(pyrrolidin)e-ethanamine (ORG 5730) was obtained from Organon International BV. Sodium azide and doxorubicin were obtained from Baker Chemicals and Laboratoire Roger Bellon, respectively. Vinblastine sulphate was obtained from Eli Lilly and progesterone was obtained from Sigma.

**Cells**

The source and culture conditions of the wild-type human ovarian carcinoma cell line A2780, its resistant subline 2780Ad and of the wild-type human non-small-cell lung cancer cell line SW-1573 and its resistant subline SW-1573/1R500, have been described before [28–31]. The cells were checked for mycoplasma by the mycoplasma T. C. rapid-detection system with a 3H-labeled DNA probe from Gen-Probe Inc. They were found to be negative.

**Determination of changes of verapamil accumulation**

Verapamil accumulation was determined in various cell lines, using a flow-through system and a flow-through-radioactivity detector. The flow-through system has been described in detail before [22, 24, 32]. Basically, a monolayer of approximately 10⁷ cells present on the bottom of a chamber (surface 50 cm², height 0.1 mm) was perfused with drug-containing medium A (Dulbecco's modified minimal essential medium without bicarbonate and phenol red, but containing 20 mM Hepes, 5% fetal bovine serum, 5.6 mM glucose and 4 mM glutamine. The perfusion medium (pH 7.3) was passed over the cells at a constant flow rate of 200 µl/min, at 37°C. Then 3H-labeled verapamil was added to the perfusion medium (0.1 µM verapamil, containing 0.05 nM 3H-labeled verapamil) and cells accumulated verapamil until the steady-state was reached, i.e. when the concentration of verapamil at the output of the flow-through system attained the same level as the input concentration. The extracellular concentrations of verapamil were measured on-line at the output of the flow-through system by measuring the radioactivity every 6 s with a window of 0–20 kV, in a radioactivity flow detector (Flo-oneβ, Radiomatic Instruments & Chemicals Co.). Using a flow cell of 0.5 ml, Pico-aqua scintillation liquid (Packard Instruments) at a flow rate of 800 µl/min, the data were collected and processed with the software programme A250-1.6 (Radiomatic Instruments & Chemicals Co.). The verapamil input concentration in the perfusion medium was checked by measuring radioactivity in the same manner as described above, but the cell-containing flow-through system in between was omitted.

**Effects of various modifiers or drugs on the accumulation of verapamil**

When extracellular steady-state concentrations of verapamil had been reached, pulse injections (15 µl) of the modifying agent were introduced into the flowing medium via an HPLC injection valve. This caused a band of modifier-containing medium to move over the cells (each pulse diluted into a volume of 50–200 µl). A series of pulse injections of modifier was administered at time intervals of 60 s in order to approach a one-step rise in the concentration of modifier and a high concentration of modifier was used in order to obtain maximal effects. Average concentrations of modifying agents or drugs in the perfusion medium to which the cells were exposed were 0.4–1.4 mg/ml for Cremophor EL, 30–100 µM for daunorubicin, 20–70 µM for bepridil, 15–60 µM for vinblastine and 20–70 µM for amiodarone. The increased cellular verapamil accumulation was represented by a decrease of the level of radioactivity in the medium at the outlet of the system. At the end of the experiments, the same series of modifier pulses were repeated as a control for each cell line used. No differences were found compared to
the effects of the same modifiers observed at an earlier stage of the experiment. Then the cells were harvested and counted in a hemocytometer. The viability of the cells (routinely > 90%) was determined by the trypan blue exclusion test.

**Effects of verapamil on the cellular accumulation of daunorubicin**

Using the flow-through system, daunorubicin was added to the perfusion medium instead of verapamil. Daunorubicin concentrations were determined by on-line measurement of fluorescence or light absorption as described elsewhere [22]. When the daunorubicin concentration in the effluent became constant, various concentrations of verapamil were added to the daunorubicin-containing perfusion medium. We used an energy-depleting medium C i.e. medium A without glucose but supplemented with 10 mM sodium azide, 6.1 mM deoxyglucose and 5% fetal bovine serum that had been dialyzed for 24 h against 1 mM KH₂PO₄, pH 7.4, using a Visking-30 dialysis tube, 1:200 by vol. (Radiometer). This caused a decrease of the cytosolic pH of about 0.5 [23]. The drop in pH could be compensated by the addition of 40 mM methylamine to medium C (unpublished results). Therefore, in order to measure the effects of energy depletion without altering intracellular cytosolic pH values, we used medium C supplemented with 40 mM methylamine. The increased rate of daunorubicin accumulation in the cells, caused by the verapamil addition or a shift to medium C, was reflected by a decrease in the daunorubicin concentration in the eluent at the outlet of the flow-through system. From the short-term maximal decrease of the daunorubicin concentration, the net inward accumulation rate was calculated according to:

\[ u_{\text{in},x} = \text{Flux} \cdot (D^{\text{in}} - D^{\text{out}}), \]

where \( u_{\text{in},x} \), \( F \), \( D^{\text{in}} \), \( D^{\text{out}} \) and \( N \) represent the short-term maximal net inward accumulation rate of daunorubicin (mol·min⁻¹·cell⁻¹), the flow rate (l/min), the medium concentration of daunorubicin at the input of the flow-through system (mol/l), the minimum extracellular daunorubicin concentration at the output of the flow-through system attained after verapamil addition or medium substitution (mol/l), and the number of cells, respectively. Previously, we demonstrated the specificity of the verapamil effects for P-glycoprotein in several human wild-type colon cell lines, i.e. HT29, SW1116 and COLO 320, the murine colon cell line C-26 and, for the drug-sensitive/MDR human ovarian cancer cell line couple A2780/2780Ad [22]. Therefore, the disturbed equilibrium between inward and outward transport of daunorubicin after addition of verapamil is most likely a result of the inhibition of the P-glycoprotein-mediated efflux pump. If sufficient verapamil was added to completely inhibit the pump, then

\[ u_{\text{in},x} = -u_{\text{pump},x}, \]

where \( u_{\text{pump},x} \) refers to the pump flux in the steady-state situation before addition of verapamil.

**Modelling the transport of daunorubicin after addition of verapamil**

Our ultimate model featured non-competitive inhibition of daunorubicin transport by verapamil. The overall transport of daunorubicin across the cell membrane was considered to result from (a) passive transport of the uncharged form of the drug and (b) the active efflux of the drug. The net inward flux of daunorubicin was described as

\[ u_{\text{in}} = k \cdot D^{\text{in}} - k \cdot X^{\text{in}} - u_{\text{pump}}, \]

where \( u_{\text{in}}, k, D^{\text{in}}, X^{\text{in}} \) and \( u_{\text{pump}} \) represent the net inward cellular accumulation rate of daunorubicin (mol·min⁻¹·cell⁻¹), passive permeation coefficient (l·min⁻¹·cell⁻¹), the extracellular uncharged daunorubicin concentration (mol/l), the intracellular free cytosolic uncharged daunorubicin concentration (mol/l), and the active pump efflux rate (mol·min⁻¹·cell⁻¹), respectively. The terms \( k \cdot D^{\text{in}} \) and \( k \cdot X^{\text{in}} \) represent the passive influx of daunorubicin (mol·min⁻¹·cell⁻¹) and the passive efflux of daunorubicin (mol·min⁻¹·cell⁻¹), respectively. We calculated the concentration of the internal free cytosolic daunorubicin before addition of the inhibitor, using this kinetic model for daunorubicin transport. The assumptions underlying this equation, have been discussed elsewhere [23].

We examined the case in which there is no competition between daunorubicin and verapamil for P-glycoprotein-mediated pumping, but where verapamil inhibits the pumping of daunorubicin. In the rapid-binding case (taking the simplest case), one derives for the rate at which daunorubicin should be pumped [23, 33, 34]:

\[ u_{\text{pump},x} = \frac{[V_{\text{max},x} \cdot (X/K_{m,x})^p] - [V_{\text{max},d} \cdot (D/K_{d,x})^p]}{[1 + (X/K_{m,x})^p + (D/K_{d,x})^p] \cdot [1 + KL/K_{m,y} + KL_{m,1}}. \]

For the rate at which verapamil is pumped,

\[ u_{\text{pump},y} = \frac{(V_{\text{max},y} \cdot (K_{m,y})^p - (V_{\text{max},d} \cdot (I/K_{m,1}))}{1 + KL/K_{m,y} + KL_{m,1}}. \]

Eqn (5) purports that daunorubicin does not affect the pumping of verapamil. In Eqn (4), \( u_{\text{pump},x} \) and \( V_{\text{max},x} \) have been defined as the P-glycoprotein-mediated active pump efflux rate for daunorubicin (mol·min⁻¹·cell⁻¹), and the maximal pump rate for daunorubicin across the plasma membrane from the cytosol into the external medium (mol·min⁻¹·cell⁻¹), respectively. \( X \) and \( K_{m,x} \) correspond to the cytosolic concentration of free daunorubicin (mol/l) and the Michaelis-Menten constant for the P-glycoprotein-mediated efflux of daunorubicin (mol/l), respectively. The superscript h corresponds to a co-operativity index for daunorubicin according to T. L. Hill [35]. \( V_{\text{max},d} \), \( D \) and \( K_{m,d} \) are the maximal P-glycoprotein-mediated influx rate of daunorubicin from the external medium into the cytosol (mol·min⁻¹·cell⁻¹), the external concentration of daunorubicin (mol/l) and the corresponding Michaelis-Menten constant for the P-glycoprotein-mediated influx of daunorubicin (mol/l), respectively. In Eqn (5), \( u_{\text{pump},y} \) \( V_{\text{max},y} \) and \( V_{\text{max},d} \) represent the P-glycoprotein-mediated active pump efflux rate for verapamil (mol·min⁻¹·cell⁻¹), the maximal pump rate for verapamil across the plasma membrane from the cytosol into the external medium (mol·min⁻¹·cell⁻¹) and the maximal P-glycoprotein-mediated influx rate for verapamil from the external medium into the cytosol (mol·min⁻¹·cell⁻¹), respectively. In the alternative model where there is competition between daunorubicin and verapamil, the denominators of Eqn (4) and Eqn (5) were replaced by \( 1 + (X/K_{m,x})^p + (D/K_{m,d})^p + Y\cdot K_{m,y} + IL/K_{m,1} \) [33].
In order to simulate the time-dependent change of the total extracellular daunorubicin concentration, or the change of the net accumulation rate of daunorubicin after addition of verapamil, we integrated a set of coupled differential equations. These equations were derived from Eqn 3, in which \( D^0 \) and \( X_{\text{on}} \) were replaced by the total, i.e. protonated plus unprotonated concentrations of the extracellular daunorubicin and intracellular daunorubicin, respectively, in order to simplify the equation. This replacement was justified by using the passive leakage constants for the total daunorubicin concentrations at a known pH.

\[
\begin{align*}
\frac{dX}{dt} &= \frac{\nu_{\text{in},D}}{V_D} \left( k \cdot D - k \cdot X - \nu_{\text{pump}, X} \right) \\
\frac{dY}{dt} &= \frac{\nu_{\text{in},I}}{V_I} \left( k' \cdot I - k' \cdot Y - \nu_{\text{pump}, Y} \right).
\end{align*}
\]

(6) (7)

In these equations, \( \nu_{\text{in},D} \), \( \nu_{\text{in},I} \), \( k' \), \( V_D \) and \( V_I \) represent the net inward accumulation rate of daunorubicin (mol \cdot min\(^{-1}\) \cdot cell\(^{-1}\)), the net inward accumulation rate of verapamil (mol \cdot min\(^{-1}\) \cdot cell\(^{-1}\)), the passive permeation coefficient for verapamil (1 \cdot min\(^{-1}\) \cdot cell\(^{-1}\)), the apparent intracellular, daunorubicin-accessible volume of a cell (l/cell), and the apparent intracellular, verapamil-accessible volume of a cell (l/cell), respectively. Most of the intracellular drug is not dissolved in the intracellular aqueous volume, but is bound to intracellular structures. Assuming that this binding is nonsaturated and in rapid equilibrium, it can be accounted for by attributing an extended apparent volume to the cell. Effects of changes in extracellular daunorubicin or verapamil concentrations as a result of cellular activities were neglected. After replacing the terms \( \nu_{\text{pump}, X} \) and \( \nu_{\text{pump}, Y} \) using Eqs 4 and 5, this set of coupled differential equations was numerically integrated, using the Mathematical Modelling System 'MLAB'.

**RESULTS**

Verapamil accumulation in parent A2780 and SW-1573 cells and their resistant counterparts

Using the flow-through system, the interaction between verapamil and cells which grow in monolayer was monitored on-line. In Fig. 1a and b, the verapamil concentration in the output medium is shown as a function of time, after adding verapamil to the influx medium. Fig. 1a gives the differences between the time-dependent verapamil concentrations obtained for the drug-sensitive human lung cancer cell line SW-1573 and its MDR counterpart SW-1573/1R500. In Fig. 1b, the differences between the graph obtained for the human ovarian cancer cell line A2780 and that for its resistant counterpart SW-1573/1R500 are shown. We interpret these data in terms of MDR specific differences in total verapamil accumulation. Steady-state verapamil concentrations were obtained after approximately 15 min using the MDR cell lines 2780\(^{10}\) or SW-1573/1R500, and after 60–90 min using their drug-sensitive counterparts A2780 or SW-1573. From these data, the total verapamil accumulation/cell was obtained (see also Eqn 1) as:

\[
Q = \frac{F}{N} \int_{t=0}^{t=t_a} (P - P') \, dt.
\]

(8)

Here \( Q \), \( t = 0 \), \( t = t_a \), \( P \) and \( P' \) represent the total verapamil accumulation/cell (mol/cell), the time required to pass the void volume after adding verapamil to the flowing medium, the time at which the steady-state was (virtually) reached, the medium concentration of verapamil at the inlet of the flow-through system (mol/l), and the time-dependent medium concentration of verapamil (mol/l) at the outlet of the flow-through system, respectively. The input concentration of verapamil was determined by taking a time average of the steady-state output concentration of verapamil. At an extracellular verapamil concentration of 0.1 \( \mu \)M, the accumulation of verapamil in the drug-sensitive cell lines A2780 and SW-1573 was significantly higher when compared to the accumulation of verapamil in the resistant cell lines 2780\(^{10}\) and SW-1573/1R-500 (Student \( t \)-test \( P < 0.01 \)). The total verapamil accumulation in the parent cell lines SW-1573 and A2780, corrected for the delay curve in the flow-through system without cells (volume of cells in the monolayer was less than 5% of the total volume of the flow-through system and, therefore, could be neglected), was 28 ± 5 pmol · (10\(^6\) cells\(^{-1}\)) and 73 ± 14 pmol · (10\(^6\) cells\(^{-1}\)), respectively (means ± SD from three and two independent experiments, respectively). No significant difference was found when we compared the uptake curve for the MDR cell lines SW-1573/1R500 or 2780\(^{10}\) with the curve for the flow-through system without cells; at most 10 pmol · (10\(^6\) cells\(^{-1}\)) verapamil accumulated.
were washed out, an increase of the verapamil medium concentration above the steady-state concentration was observed. This indicates an efflux of the verapamil which had been taken up additionally during the presence of Cremophor EL. The effects of a series of pulses of daunorubicin, bepridil, vinblastine or amiodarone were examined for the MDR 2780AD cell line (not shown). A significant decrease of the steady-state verapamil concentrations was obtained using vinblastine or amiodarone (20–25%) and only a small decrease was obtained, using bepridil (10–15%). In these experiments, daunorubicin caused no decrease in the steady-state verapamil concentrations. When drug-sensitive A2780 cells were used, daunorubicin, bepridil, vinblastine or amiodarone only increased the extracellular verapamil concentrations, possibly by competition for binding sites which are not MDR specific, or had no effect at all (not shown).

The dynamic response of the extracellular daunorubicin concentration to various verapamil medium concentrations

To examine whether verapamil is a competing or a non-competing substrate for P-glycoprotein-mediated daunorubicin transport, we studied the effects which variation in verapamil medium concentrations had on the daunorubicin uptake by the cells. We measured the fluorescence or light absorbance of daunorubicin instead of the radioactivity of verapamil. We described how the cellular daunorubicin accumulation rate can be calculated from disturbance of the steady-state between inward and outward transport of daunorubicin, induced by the MDR modifiers (Eqn 1). This was presumably caused by an inhibition of the P-glycoprotein-mediated efflux by these modifiers [22].

In Fig. 3a, adding 8 μM verapamil to the daunorubicin-containing flowing medium resulted in a decrease of the extracellular daunorubicin concentration of the medium above the cell line 2780AD. This effect was not found for its drug-sensitive counterpart A2780 (not shown). This is an indication of MDR specificity (see also [22, 24]). We changed the concentration of verapamil in the flowing medium, which contained 5 μM daunorubicin and found that the maximal initial accumulation rate of daunorubicin (maximal depth of the dip) after adding 32 μM verapamil differed only 11% from the rate obtained with 16 μM verapamil (Fig. 4). Increasing the verapamil concentrations over 30–100 μM caused the maximal initial accumulation rate of daunorubicin to increase by only 4% (Fig. 3b). We measured the rise of the verapamil concentration at 5 μM daunorubicin, using 3H-labeled verapamil as mentioned above (not shown). When the depth of the daunorubicin signal was at its maximum, the verapamil concentration had attained at least 70% of its maximum, at concentrations of verapamil of 4 μM or higher (Fig. 3a and b).

We compared the effects of 50 μM verapamil and the effects after complete inhibition of P-glycoprotein by energypedition without changing the intracellular pH (addition of medium C supplemented with 40 mM methylamine; see Materials and Methods) and we found the same maximal initial accumulation rates of daunorubicin (Fig. 3c). These results suggest that the P-glycoprotein-mediated initial efflux is virtually completely blocked by 30 μM verapamil.

In contrast, at a daunorubicin concentration of 5 μM in the flowing medium, the accumulation rate of daunorubicin 30 min after 30 μM verapamil had been added, differed significantly from the accumulation rate 30 min after 100 μM

Effects of various MDR modifiers or MDR-related drugs on the cellular verapamil accumulation

Changes of cellular verapamil accumulation, caused by adding various MDR modifiers or MDR-related drugs to the verapamil-containing flowing medium, were measured after the steady-state had been reached. These changes could be observed as changes in the verapamil medium concentration, which were measured at the outlet of the flow-through system. Fig. 2 shows that a series of pulse injections of the MDR modifier Cremophor EL [25] resulted in a decrease of the extracellular verapamil concentration for the cell lines 2780AD and SW-1573/1R500. The extracellular verapamil concentration was only slightly affected for A2780 and SW-1573 cells. This indicates that the decrease depends on the presence of P-glycoprotein. When the Cremophor EL pulses

in these MDR cells. The ratio of verapamil accumulation in the drug-sensitive cells and the verapamil accumulation in their MDR counterparts proved to be at least 2.6 ± 0.2 and 7 ± 2 for SW-1573/SW-1573/1R500 and A2780/2780AD cells, respectively.

Effects of various MDR modifiers or MDR-related drugs on the cellular verapamil accumulation

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Fig. 3. Effects of various verapamil concentrations or energy depletion on the extracellular daunorubicin concentration, using 2780AD cells. Conditions: see Fig. 1; extracellular daunorubicin concentration ([DN]), 5 µM; verapamil (Vp) was added to the drug-containing perfusion medium at the indicated time point. (a) The effects of 8 µM verapamil on the extracellular daunorubicin concentration. (b) The effects of 30 µM and 100 µM verapamil on the extracellular daunorubicin concentration. Change of daunorubicin containing medium supplemented with various concentrations of verapamil was performed after allowing the cells to efflux the extra accumulated daunorubicin, using medium without verapamil. Two graphs are projected in one time axis. (c) The effects of 50 µM verapamil and subsequently 375 µM verapamil on the extracellular daunorubicin concentration (-----) or the effects of energy depleting medium (medium C), containing 40 mM methylamine on the extracellular daunorubicin concentration (--.--.--). Extracellular daunorubicin concentration, 10 µM; change of daunorubicin-containing medium supplemented with various concentrations of verapamil or using daunorubicin-containing energy-depleting medium C was performed after allowing the cells to efflux the extra accumulated daunorubicin, using medium A without verapamil. Two graphs are projected in one time axis. (d) See Fig. 3b, extracellular daunorubicin concentration, 20 µM.
verapamil had been added (Fig. 3b). Moreover, energy deple-
tion at constant intracellular pH resulted in a different long-
term dynamic response than the effect of a verapamil concen-
tration of 50 μM (Fig. 3c). Increasing the extracellular ve-
rapamil concentration to 375 μM after extra daunorubicin
had been accumulated for 30 min caused an increase in the
daunorubicin influx rate. This demonstrated the fact that,
although shortly after its addition, 50 μM verapamil sufficed
to block the active efflux of daunorubicin, higher concentra-
tions of verapamil or energy-depleting medium were needed
in order to block this active efflux permanently. These ex-
periments employed extracellular daunorubicin concentra-
tions of 10 μM (Fig. 3c).

When we used a daunorubicin concentration of 20 μM in
the flowing medium, only small differences between the
graphs with 30 μM and with 100 μM verapamil were ob-
served (Fig. 3d). After 15, 25, 35 and 45 min, the differences
daunorubicin concentrations in the output were 0.52, 0.54,
0.56 and 0.52 μM, respectively.

Modelling the daunorubicin transport
after addition of verapamil

Various processes require attention when verapamil is
added to cells which are in steady-state with an extracellular
concentration of daunorubicin. These processes include the
pumping and leakage of daunorubicin before adding verap-
amil, which set the intracellular daunorubicin concentration,
as well as the pumping and the leakage of both daunorubicin
and verapamil after the latter has been added. These pro-
cesses change with time as do the intracellular concentra-
tions of daunorubicin and verapamil. In order to facilitate under-
standing the experiments of Figs 3 and 4, we constructed a
kinetic elaboration of a simple but realistic model for these
experiments. In short (for details, refer to Materials and
Methods), this model contains a nonsaturable leakage of
daunorubicin and verapamil across the membrane, and a
pump which actively extrudes the daunorubicin and verap-
amil molecules from the cells. In our non-competition model
(see Eeqns 4 and 5), we assumed that daunorubicin and verap-
amil are pumped independently and that verapamil func-
tions as a non-competing inhibitor of the daunorubicin pump-
ing. We also analyzed the implications of a competition model
where daunorubicin and verapamil are presumably pumped by the same pump and will compete for this pump-
ing.

We did not fit kinetic parameters to our experimental
data. We preferred to use values for the kinetic parameters
which were available in the literature or seemed to be 'best
estimations', as follows.

We used a value of 0.01 nl · min⁻¹ · cell⁻¹ for k,
the passive permeation constant for total daunorubicin. The pas-
itive permeation coefficient for total daunorubicin was mea-
sured to be 0.01 nl · min⁻¹ · cell⁻¹ for 2780AD cells [23].

We used a value of 1.0 nmol · min⁻¹·(10⁶ cells)⁻¹ for
Vₘₐₓ,X, the maximal pump rate in the 2780AD cells for pump-
ding daunorubicin from the cytosol into the medium. This
value could not be measured, because at an extracellular me-
dium concentration of 50 μM, the relationship between the
net accumulation rate of daunorubicin and the medium con-
centration did not show saturation [22]. The value we used
is about twice the highest estimated maximal pump rate
which we measured before [22].

For the Kᵢₐₙ,X, the Michaelis-Menten constant for cytosolic
free daunorubicin, we used a value of 1 μM. This value was
estimated at 1.4 ± 0.7 μM as described elsewhere [23].

For h, the Hill coefficient of the pump for daunorubicin
molecules, we used the value of 1.5. This assumes co-opera-
tivity between two daunorubicin molecules for binding to the
P-glycoprotein [35]. No data for this Hill coefficient are known for 2780AD cells. The average estimated Hill coeffi-
cient for P-glycoprotein for five other human cancer cell
lines, i.e. the epidermoid carcinoma cell line KB3-1 and its
MDR sublines KB8 and KB8-5 and the colon carcinoma cell
lines SW1116 and COLO320 was 1.5 ± 0.2 [23].

The value for the ratio of (Kᵢₐₙ,D)ₚₐ₉/Vₘₐₓ,D the Michaelis-
Menten constant for P-glycoprotein-mediated back flux di-
vided by the maximal P-glycoprotein-mediated influx rate
for daunorubicin, was chosen to be 10⁻⁵ mol⁻¹ · min · cell
⁻¹. Allowing for some slippage of the pump [37], we took
Vₘₐₓ,X , (Kᵢₐₙ,D)ₚₐ₉/Vₘₐₓ,D (Kᵢₐₙ,X)ᵃ to equal 10⁷ and
(Kᵢₐₙ,D)ₚₐ₉/Vₘₐₓ,D to equal 10⁻⁰³.

Kᵢₐₙ,D, the Kᵢₐₙ for P-glycoprotein-mediated back flux of
daunorubicin was assumed to be 1000 · D⁻¹. This implies
virtually no back flux of the pump from outside.

Kᵢₐₙ, Y the Michaelis-Menten constant for P-glycoprotein-
mediated efflux of verapamil, was assumed to be 0.35 μM.

Vᵯ and Vᵣ, the apparent intracellular, drug-accessible vol-
ume of a cell for daunorubicin and verapamil, respectively,
was taken as 430 pl for daunorubicin and 730 pl for verap-
amil. The apparent cellular drug-accessible distribution vol-

Fig. 4. Initial net influx rates of daunorubicin as function of various medium concentrations of verapamil ([Vp]), using 2780AD cells.
(a) Experimental data, using the flow-through system (■). Conditions, see Fig. 3b. (b) Theoretical curve as calculated using Eqns 4-7 and
using the parameters as described in the Results (—).
ume for the two drugs may be calculated from the total cellular drug amount in the steady-state for wild-type cells, divided by the used extracellular drug concentration, which should equal the cytosolic free drug concentration. In this manner, $V_p$ was calculated to be 430 pl/cell [17] and $V_i$ was 730 pl/cell (this study).

The passive permeation coefficient for verapamil, $k'$, was assumed to be 0.5 nl \cdot min^{-1} \cdot cell^{-1}. No conclusive experimental data on the permeation coefficient of verapamil are known. In our laboratory, we observed that the transport of verapamil is rapid at 37°C (half-life of efflux was less than 1 min; unpublished results) whereas transport of daunorubicin via passive efflux is much slower (half-life of efflux was more than 15 min) [23]. This may be explained by a lower apparent intracellular drug-accessible volume for verapamil, or a higher value of the passive permeation coefficient for verapamil, comparing verapamil and daunorubicin.

Since we found an apparent intracellular drug-accessible volume of a cell which was higher for verapamil than for daunorubicin (see above), our choice of a passive permeation coefficient for verapamil which is fifty-times higher than the passive permeation coefficient for daunorubicin may be justified.

We assumed a value of 1.75 nmol \cdot min^{-1} \cdot (10^6 cells)^{-1} for $V_{max,y}$, the maximal pump rate in the 2780AD cells for transporting verapamil from the cytosolic side into the medium.

The value for the ratio of $K_m, J/V_{max, y}$, the Michaelis-Menten constant for P-glycoprotein-mediated back inhibition divided by the maximal $P$-glycoprotein-mediated influx rate for verapamil, was chosen to be $2 \times 10^{-12} \text{ min} \cdot \text{cell}^{-1}$.

The $K_m, y$ was assumed to be 1000 \text{ \mu M}, which implies virtually no back inhibition of $P$-glycoprotein by extracellular verapamil.

At $t = 0$, the verapamil medium concentration was brought from 0 to 1.

The kinetic and differential equations which were used to calculate the implications of these parameter values, are described in Materials and Methods (Eqns 4–7). We calculated the rate of the net cellular daunorubicin accumulation as a function of time after administration of verapamil. It was assumed that the extracellular daunorubicin and verapamil were present at a constant concentration throughout the experiment (due to limitations in the experimental set-up this is not strictly true, but changes in the extracellular concentration resulting from movement across the cellular plasma membranes are much smaller than the changes in intracellular concentration). Before verapamil was added, we assumed that a steady-state had been reached. Our calculations focused on the following questions, concerning the dynamic response of the extracellular daunorubicin concentration after the addition of verapamil.

(a) How should we interpret the phenomenon (Figs 3b–d) that, in the range of verapamil concentrations where immediately after verapamil has been added it completely inhibits the pump, the net influx of daunorubicin decreases with time after verapamil has been added?

(b) Why is this decrease of the net influx of daunorubicin, after verapamil has been added, slower at higher verapamil concentrations, when a concentration of 5 \text{ \mu M} or 10 \text{ \mu M} daunorubicin is used in the flowing medium (Figs 3b and c)?

(c) Why is the decrease with time of the net influx of daunorubicin after verapamil has been added, the same for different verapamil concentrations, when a concentration of 20 \text{ \mu M} daunorubicin is used in the flowing medium (Fig. 3d)?

(d) Do these phenomena reflect competition by daunorubicin for the verapamil-binding-site on the pump, thereby causing a reduction of the inhibitory effects of verapamil, or do they reflect a non-competing transport of verapamil and daunorubicin?

When the pump was completely and continuously inhibited, our simulations showed that the net daunorubicin influx decreased with time after the inhibitor had been added (results not shown). However, this occurred more slowly than in simulations of inhibition by verapamil (Fig. 5a). When we increased the apparent intracellular volume by a factor of 10 (i.e. by reducing the intracellular drug concentrations), this decrease with time in the uptake rate was also considerably slower (simulation results; not shown). These simulation results suggest the following interpretation: the decrease with time of the net daunorubicin influx after verapamil was added is partly due to a decrease with time of the net leakage into the cells and partly due to the slight activity of the pump which remains and increases with time. The change with time of the two processes is caused by the decrease with time of the intracellular daunorubicin concentration. The increase in its turn is caused by the net influx, which is initiated by adding verapamil. This addresses question (a).

The increase in pump flux will be proportional to the remaining activity of the pump. This leads to the phenomenon that when the pump is inhibited by 97% rather than by 99%, the net daunorubicin influx may decrease up to three times more quickly compared to that after the pump has become completely and continuously inhibited. These mechanisms which are operative in the simulation may account for the divergence of the experimental data of Fig. 3b, answering question (b) above.

When the experiment of Fig. 3b was simulated with models of non-competing (Fig. 5a) and competing (Fig. 5b) interference of verapamil with daunorubicin pumping at extracellular daunorubicin concentrations of 5 \text{ \mu M}, the observation (Fig. 3b) that the data for the two concentrations of verapamil diverged was reaffirmed; we compared the calculated net efflux rates for the two graphs in the non-competition model (Fig. 5a) and found a difference of 1.8 pmol \cdot min^{-1} \cdot (10^6 cells)^{-1} and 4.5 pmol \cdot min^{-1} \cdot (10^6 cells)^{-1}, at 1 min and 15 min, respectively, after verapamil had been added. For the competition model (Fig. 5b), these differences were 2.0 pmol \cdot min^{-1} \cdot (10^6 cells)^{-1} and 9.7 pmol \cdot min^{-1} \cdot (10^6 cells)^{-1} for 1 min and 15 min, respectively. A model with irreversible inhibition, where the inhibitor was taken immediately to equilibrate across the plasma membrane, also produced the divergence. This confirmed our assumption that the observed divergence between the two graphs in Fig. 3b does not discriminate between the competitive and non-competitive interaction of verapamil with daunorubicin.

In the above argumentation, it was supposed that the pumping rate would increase with an increase in the intracellular concentration of daunorubicin. If the pump was saturated by daunorubicin shortly after the influx had started, then the change with time of the net influx rate might not depend on the verapamil concentration (in the concentration range where verapamil virtually completely inhibits the pump). We confirmed this by simulation for the non-competition model; for $K_m$ values of daunorubicin which were below 0.5 \text{ \mu M} and when the extracellular daunorubicin concentration was 5 \text{ \mu M}, the graphs for 30 \text{ \mu M} and 100 \text{ \mu M} verap-
Fig. 5. Theoretical curves, calculating the net efflux rate of daunorubicin after addition of verapamil to the daunorubicin-containing perfusion medium. We used Eqns 4−7 and the parameters which have been listed in the results section. (a) The theoretical curve of the net efflux rate of daunorubicin, using the non-competition model, comparing the effects of different medium concentrations of verapamil. The concentration of daunorubicin in the perfusion medium was set to 5 μM, the concentration of verapamil was set to 30 μM (−) or 100 μM (−−−−). Two graphs have been projected in one time axis. (b) See Fig. 5a, using the competition model. (c) See Fig. 5a, the concentration of daunorubicin in the perfusion medium was set to 20 μM. (d) See Fig. 5c, using the competition model.

A competitive inhibitor effectively increases the \( K_m \) of the substrate. Therefore, if verapamil interfered competitively with the daunorubicin being pumped, saturation of the pump with daunorubicin would occur at higher daunorubicin concentrations in the medium than for the non-competitive model. Consequently, the graphs for 30 μM verapamil and 100 μM verapamil might continue to diverge at high concentrations of daunorubicin. This suggestion is confirmed by the simulation represented in Fig. 5d (confer Fig. 5c). Since it is known that the \( K_m \) of the pump for daunorubicin lies around 1.4 μM [23], these considerations and simulation results (Figs 5c and d) suggest that Fig. 3c cannot be accounted for by the model in which verapamil and daunorubicin compete. On the contrary, it can be considered to be evidence for non-competing interference of verapamil with pumping of daunorubicin. Consequently, we answer question (d) as follows: verapamil and daunorubicin are non-competing substrates for the daunorubicin pump.

DISCUSSION

The cell lines 2780AD and SW-1573/IR500 have high levels of P-glycoprotein [22, 34], whereas their drug-sensitive counterparts have no detectable levels of P-glycoprotein and showed only low levels (SW-1573) or non-detectable levels (A2780) of mRNA encoding for P-glycoprotein [22, 38]. By means of a flow-through system, the present paper demonstrated increased verapamil accumulation in the parent cell lines when compared to their resistant counterparts. This MDR-specific decrease of verapamil accumulation could well result from pumping of verapamil by P-glycoprotein [3, 13, 17−20].

Sehested et al. [21] found similar accumulation of verapamil comparing wild-type EHR2 to MDR EHR2/DNR+ cells, which was energy dependent. Perhaps the rapid passive membrane transport of verapamil occluded the MDR effect on the accumulation in their studies. The high passive permeability may also have caused underestimations of verapamil accumulation in our earlier centrifugation experiments [17]. When the flow-through system was used, no wash steps were needed to determine the verapamil accumulation. Therefore, the method used in this paper may be optimal for detecting the pumping of substrates with high passive membrane permeability.
After changing to medium containing 30 μM verapamil, an almost complete inhibition of active daunorubicin efflux was obtained shortly after passage of the void volume, using P-glycoprotein containing SW1573/1R500 cells (our observations) and 2780 cells (Fig. 3b). A number of MDR cell lines has been described that lack P-glycoprotein [38–41]. Under similar measuring conditions, less than 20% of the inhibition was observed using the non-P-glycoprotein MDR cell lines SW1573/2R120 [38] and GLC4/ADR [39], even with 50–100 μM verapamil in the flowing medium. The stronger effect of 30 μM verapamil shows that virtually all daunorubicin transport, measured in this study, resulted from P-glycoprotein activity.

This paper demonstrated increased accumulation of verapamil when agents known to modify the P-glycoprotein-mediated pumping of MDR-related drugs (i.e. amiodarone, bepridil, Cremophor EL or vinblastine) were added to the MDR cells. These results can be considered as further evidence that the MDR dependence of verapamil accumulation is mediated by P-glycoprotein. Most important for the interpretation that verapamil may be actively pumped was that upon removal of Cremophor EL (Fig. 2), amiodarone or vinblastine (not shown), the cells released verapamil against the concentration gradient (at this time the extracellular verapamil concentration exceeded the intracellular concentration of verapamil, as demonstrated by the influx just preceding Cremophor EL removal).

Daunorubicin did not appear to inhibit verapamil transport when we used steady-state concentrations of 0.1 μM verapamil (not shown). However, small differences in the steady-state levels of radioactivity may have been masked by the background variations of radioactivity detection. Fig. 3d did provide more substantial evidence for the non-competitive model for the interference of verapamil with daunorubicin (Fig. 3d). As shown by the simulations in Figs 5a–d, in the competition model, the two lines in Fig. 3d would have diverged, in contrast with the experimental result. The results of the simulation study (Fig. 4) corresponded well with the experimental results (Fig. 4).

Our simulation studies further demonstrated that the experimental graphs, as illustrated in Figs 3 and 4, can be explained as follows. The inhibition of the pump causes an increase in the intracellular substrate concentration, which increases passive efflux and reactivates the pump for this substrate (Fig. 3). In order to inhibit P-glycoprotein effectively, one should use much higher verapamil concentrations than concentrations around the $K_v$, for verapamil (Fig. 4), because verapamil itself is pumped and therefore the intracellular free concentration of verapamil is lower than the extracellular concentration of verapamil. Simulations without co-operativity of daunorubicin being pumped (i.e. $h = 1$), or with co-operativity between daunorubicin and verapamil being pumped, revealed only non-competition models that would match our experimental data (Figs 3 and 4; simulations not shown).

The simulations in Fig. 4 and Figs 5a–d could also be obtained in a model where verapamil functioned as an inhibitor rather than a substrate for pumping (simulations; not shown). Consequently, Figs 3 and 4 do not provide evidence for the contention that verapamil is pumped by MDR cells. The evidence for this pumping is shown in Figs 1 and 2. We consider it most likely that the pumping is mediated by P-glycoprotein. First, both the cell lines that pumped verapamil expressed P-glycoprotein. Second, we have no indications for the presence of non-P-glycoprotein MDR in the cell lines used. Third, various MDR modifiers, known to inhibit P-glycoprotein-mediated pumping of various MDR-related drugs, interfered with verapamil pumping by these cells. If both verapamil and daunorubicin are pumped by P-glycoprotein, then, in the simplest model which is consistent with our experimental results, they function as non-competing substrates for that pump.

In conclusion, we found MDR-specific active verapamil transport against a concentration gradient in two drug-sensitive MDR cell line couples, i.e. A2780, 2780 and SW-1573, SW-1573/1R500, which indicates that verapamil itself is a substrate for P-glycoprotein and evidence that verapamil and daunorubicin are non-competing substrates for P-glycoprotein-mediated transport from MDR cells. This implies that the effectiveness of verapamil is not compromised by the presence of daunorubicin.

The interpretation which best matches the data presently available implies that P-glycoprotein contains two binding sites, both of which may bind daunorubicin [22, 42, 43]. Verapamil binds to the site which has the lowest affinity for daunorubicin and is then pumped. In this case, daunorubicin is not expected to interfere with the binding of verapamil to this second daunorubicin binding site (non-competition model). Consequently, only at high daunorubicin concentrations may verapamil begin to inhibit competitively.

The question whether potential MDR reversors, such as verapamil, are competing or non-competing inhibitors of P-glycoprotein and whether the P-glycoprotein substrate (the anticancer drug) is applied above or below the $K_v$ of the pump for this substrate, is of more than academic interest. For example, we consider the case where an anticancer drug is applied such that its intracellular concentration is not far below the $K_v$ of the P-glycoprotein. Two MDR reversors may be available, one a competing, the other a non-competing inhibitor. We apply these reversors at a concentration where they each cause 99% of the maximal influx of the drug into the MDR cell. Our results imply that this 99% inhibition may not be sufficient because some time after the addition of the reversor, the pump may have regained activity and that the non-competing inhibitor is the better choice because the increase with time in pumping of the drug by the pump will be smaller. Anticancer agents are to be preferred for which the P-glycoprotein, at the same pump activity, has a low $K_v$, such that saturation of the pump occurs. In that case, an increase in the dosage of the drug or addition of a non-competing inhibitor of P-glycoprotein, will be most effective.

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