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P-GLYCOPROTEIN DRUG EFFLUX PUMP INVOLVED IN THE MECHANISMS OF INTRINSIC DRUG RESISTANCE IN VARIOUS COLON CANCER CELL LINES

EVIDENCE FOR A SATURATION OF ACTIVE DAUNORUBICIN TRANSPORT

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Abstract—We studied the resistance of colon tumors to anticancer agents *in vitro*. Using daunorubicin (DN), a number of cellular parameters which normally indicate acquired or multidrug resistance (MDR), were compared for several human wild-type colon cell lines, i.e. HT29, SW1116 and COLO 320, and the murine colon cell line C-26. The sensitive/MDR human ovarian cancer cell line couple A2780/2780^{AD} was used as a reference. The amount of P-glycoprotein (P-gp) was in the order HT29, A2780 ≤ SW1116 < C26 ≤ COLO 320 < 2780^{AD}. The MDR modifiers verapamil, Cremophor EL, cyclosporin A and Ro 11-2933/001 had significant effects on DN cytotoxicity, total DN accumulation and efflux, only if P-gp was present. A flow-through system was used to study the mechanism of DN transport. For the first time, evidence for saturation of an active transport of DN from the cells is reported. We discussed the possible presence of cooperative activity between at least two binding sites on the protein responsible for DN efflux, likely to be P-gp.

Cytostatic drugs have proven to be effective in the cure or palliation of several human cancers. Some cancers, such as colon cancer, are insensitive to most antitumor agents from the beginning of therapy. This is referred to as intrinsic resistance. Development of multidrug resistance (MDR†) to natural product chemotherapeutic agents, including anthracyclines, vinca alkaloids and colchicine, is thought to be due to the outgrowth of drug resistant mutant tumor cells [1, 2]. This acquired resistance has been studied intensively *in vitro*, using cell lines selected with one of the drugs [3]. Many MDR cancer cell lines showed increased levels of a glycoprotein, called P-glycoprotein (P-gp), which is believed to function as an energy-dependent drug efflux pump of broad specificity for toxic lipophilic agents [4–8]. *mdr*1 mRNA coding for P-gp has been shown to be present in several human tumors and tissues, including normal human colon tissue and some colon tumors [9]. Recently it has been reported that verapamil (Vp), which potentiates drug activity in MDR cells [10, 11], also increases drug sensitivity in some wild-type colon tumor cell lines [12]. However, the mechanisms responsible for intrinsic drug resistance and the correlation with the multidrug resistance phenotype remain to be established.

Clear potentiation effects of Vp *in vitro* in MDR or colon cancer cells were obtained only at Vp concentrations which cannot readily be achieved in patients [13, 14]. In search for agents which can potentiate drug sensitivity in resistant tumor cells at clinically achievable concentrations, bepridil (Bp) [15], cyclosporin A (CsA) [16, 17] and Ro 11-2933/001 [18, 19] were reported to be potent MDR modifiers. Moreover, it was recently found by our group that also Cremophor EL (Cr), a carrier vehicle for CsA, may prove to be potentially useful in the clinic as a resistance modifier [20].

The aim of this study was to (i) establish the relation between the presence of P-gp and the occurrence of intrinsic resistance in various wild-type colon cancer cells, (ii) get more insight into the mechanisms of the potentiation of daunorubicin (DN) accumulation in these cells by various MDR modifiers. For this purpose, we studied the effects of the two structurally dissimilar resistance modifiers Vp and Cr on DN cytotoxicity, DN accumulation and DN efflux for several wild-type colon cell lines. In some experiments we used a flow-through system [21], which allowed comparison of effects after different manipulations with the same cells. In this way we obtained evidence for a saturation of DN transport over the cell membrane of wild-type colon cancer cells, and indications that at least two binding sites for DN on the drug-transporter are involved. Part of this work has been reported in preliminary form [22].

MATERIALS AND METHODS

Drugs. Daunorubicin·HCl and verapamil·HCl

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† Abbreviations: MDR, multidrug resistant/resistance; P-gp, P-glycoprotein; Vp, verapamil; CsA, cyclosporin A; Cr, Cremophor EL; Bp, bepridil; DN, daunorubicin; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

were obtained from Specia (Paris, France) and Sigma Chemical Co. (St Louis, MO, U.S.A.), respectively. Cremophor EL and cyclosporin A were gifts from Sandoz (Basel, Switzerland). The tiapamil analog Ro 11-2933/001 was a gift from Hoffmann-La Roche B.V. (Mijdrecht, The Netherlands). Bepridil monohydrochloride monohydrate, β-[(2-methyl-propoxy)-methyl]-*N*-phenyl-*N*-(phenylmethyl)-1-pyrrolidine ethanamine (ORG 5730), was obtained from Organon International B.V. (Oss, The Netherlands). [14-^{14}C]Daunorubicin (sp. act. 45 Ci/mol) was purchased from Amersham International (Amersham, U.K.). Sodium azide was obtained from Baker Chemicals (Deventer, The Netherlands).

Cell culture. The wild-type human ovarian carcinoma cell line A2780 and its resistant subline 2780^{AD} were supplied by Dr R. F. Ozols (National Cancer Institute, Bethesda, MD, U.S.A.). The C-26 murine colon carcinoma cell line was obtained from Dr W. D. Klohs (Chemotherapy Department, Park-Davis Pharmaceutical Research Division, Ann Arbor, MI, U.S.A.) who designated the cells C-26/10. Human colon adenocarcinoma COLO 320 cells [23] were provided by Dr E. G. J. de Vries (Department of Medical Oncology, Academic Hospital, Groningen, The Netherlands). The human colon carcinoma HT29 and SW1116 were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). A2780 and 2780^{AD} cells were maintained as described elsewhere [15]. The human adenocarcinoma cell lines HT29, SW1116 [24] and COLO 320, and the murine colon cell line C-26 [12], were cultured in Dulbecco's modification of Eagle's medium (DMEM, Gibco, Paisley, U.K.) containing 20 mM HEPES (Serva, Heidelberg, F.R.G.) and supplemented with 10% fetal bovine serum (undialysed, heat inactivated, Flow Laboratories, Irvine, U.K.). The cells were checked for mycoplasma at 3-month intervals by the Hoechst stain test.

Detection of mdr*1 P-glycoprotein mRNA levels. mdr1* P-gp mRNA levels were determined using RNase protection according to Zinn *et al.* [25]. A NP40 lysis procedure [26] was used to isolate cytoplasmic RNA. The *mdr*1 probe for RNase protection was a [^{32}P]labeled 301 nucleotide *mdr*1 cDNA fragment [27]. A probe for γ-actin was included as an internal control for RNA content. Separation of RNA was performed on a acrylamide/urea gel (19:1). The protected fragments were visualized by electrophoresis using a denaturing acrylamide/urea gel (19:1) followed by autoradiography (exposure to a Kodak XS film, overnight at −70°).

Immunohistochemical staining of P-glycoprotein. Cytospins of cells were stained using three monoclonal antibodies of the IgG1 class, directed against P-glycoprotein, i.e. JSB-1 [28, 29], MRK-16 (obtained from Dr T. Tsuruo [30]) and C219 (obtained from Dr V. Ling [31]) as described elsewhere [29]. JSB-1 ascites was used in a dilution of 1:100. MRK-16 and C219 were used at final concentrations of 4 and 10 μg/mL, respectively. As a negative control antibody CT-6 ascites (1:100) was used, which is also an IgG1 [32].

Flow cytometry. Colon cell lines, A2780 or 2780^{AD} [10^6 cells/mL phosphate buffered saline (PBS)] were centrifuged for 1 min at 1000 *g*, and washed with PBS. Cells (3×10^6) were fixed by adding 100 μL fixation medium (70% methanol, 10% 10-fold concentrated PBS, 20% demineralized water) at −20° for 7 min. Then the cells were washed with 1 mL PBS, containing 1% bovine serum albumin (PBS-medium). Cells (1×10^6) were incubated for 1 hr in a volume of 200 μL, containing monoclonal antibody (JSB-1 ascites, 1:200; C219, 2.5 μg/mL or the non-relevant mouse IgG, 5 μg/mL, all diluted in PBS-medium) at 20°. The same procedure was used for unfixed cells with MRK-16 (4 μg/mL) or with the non-relevant mouse IgG (5 μg/mL). Samples were washed three times with PBS-medium, resuspended in 100 μL PBS-medium and incubated with 100 μL rabbit-antimouse-IgG-fluorescein-isothiocyanate (100 μg/mL, lot 117, Dako Immunoglobulins, Copenhagen, Denmark) in PBS-medium at 20° for 45 min. After washing the pellets (three times) the cells were resuspended in 500 μL PBS-medium and measured with a FACSTAR Plus, Becton-Dickinson Medical Systems (Sharon, MA, U.S.A.).

Effect of Vp or Cr on DN accumulation and efflux. Cells were cultured to near confluency, harvested, washed with PBS and resuspended in medium A [Dulbecco's MEM without bicarbonate and phenol-red, but containing 20 mM HEPES, 10% fetal bovine serum, glucose (5.6 mM) and glutamine (4 mM)], at cell densities of about 0.5×10^6 cells/mL. At 0°, agents were added to the cell suspensions to give a final volume of 1 mL, and concentrations of 0.4 μM for DN (including 0.1 μM [14-^{14}C]DN) with or without 10 μM Vp or 0.005% (v/v) Cr. Samples were incubated for 0 or 45 min at 37°, then rapidly cooled on ice, centrifuged, washed, and counted as described elsewhere [15]. For efflux experiments, cells were loaded with 0.4 μM DN (including [14-^{14}C]DN) at 37° for 45 min. After three washing steps with ice-cold PBS, the cells were resuspended in medium A (0.5×10^6 cells/mL) and incubated at 37° in medium A, medium A supplemented with 10 μM Vp, or medium A supplemented with 0.005% Cr. After 0, 15 or 45 min, the amount of cellular DN was determined as described above. Initial amounts of cellular DN were 80, 100, 45 and 46 pmol DN/10^6 cells for HT29, SW1116, C-26 and COLO 320, respectively. From experiments in our laboratory, it appeared that DN accumulation in suspended cells correlated well with cytotoxicity assays performed on those cells growing in monolayer. Moreover, comparing the effects of MDR modifiers on drug accumulation for those cancer cell lines in suspension or growing in monolayer, no significant differences were observed [33].

Effect of Vp or Cr on DN cytotoxicity. Effects of Vp and Cr on cellular DN toxicity were measured in a growth-inhibition assay as described elsewhere [15]. The used non-toxic concentrations of Vp and Cr were 10 μM and 0.005% (v/v), respectively. The cells were continuously exposed to the drugs. Control experiments involved exposure to one of the drugs or to PBS.

The flow-through system. This system, which has been described elsewhere [21], is represented

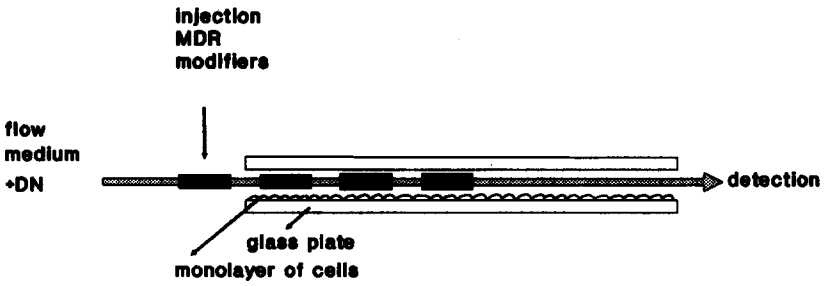

Fig. 1. Schematic representation of the flow-through system. Glass plates, in combination with a silicone glue rim, were forming a chamber of about 500 μL. A monolayer of cells, situated on the bottom of the chamber, was able to interact with the drug-containing medium, flowing over the cells. Upstream one pulse, or a series of pulses, of MDR modifiers were injected via an injection valve into the flowing medium. Downstream, the fluorescence or absorbance of the medium was measured.

schematically in Fig. 1. Cells ($5–10 \times 10^6$) were allowed to attach to the glass bottom plate of the flow-through system overnight at 37° in normal growth medium. At the day of the experiment, an HPLC pump, equipped for micro liquid chromatography, Gilson (Villiers, France) model 302, 5.S pump head, was used to pump the medium over the cells. The perfusion medium A was supplemented with 5% FCS and various concentrations of DN. The cells were allowed to equilibrate for 30 min at a flow rate of 200 μL/min. In some cases medium A without glucose but supplemented with 10 mM sodium azide was used. This medium was called medium B. When extracellular steady-state levels of DN concentrations were reached, pulse injections (15 μL) of the modifying agent were introduced into the flowing perfusion medium via an HPLC injection valve, causing a band of modifier-containing medium to move over the cells (each pulse diluted into a volume of 100–200 μL). Concentrations of modifying agents in the perfusion medium above the cells were 20 μM for Vp, CsA, Bp and Ro 11-2933/001, and 0.05% (v/v) for Cr. Series of pulse injections of modifiers were given at time intervals of 30 sec, to get maximal effects on the DN accumulation. The increased DN accumulation was represented by a dip from the steady-state fluorescence or absorption level in the medium, obtained at the outlet of the system (see below). The depth of the dip, multiplied by the medium flow rate, represents the extra accumulation rate of DN for the number of cells used. These values, can most likely be converted to the inhibited active DN efflux rate. At the outlet of the flow-through system the fluorescence intensity was measured by a fluorescence monitor (type M3000, Perkin–Elmer, Norwalk, CT, U.S.A.) at excitation/emission wavelengths of 480 nm/560 nm. At DN concentrations higher than 10 μM, light absorption at 480 nm was measured to assure linearity of the signal with the DN concentration, using a spectrophotometer (Kontron, type Uvikon 722 LC, Basel, Switzerland). At the end of the experiments, using the highest concentrations of DN used per cell line, the same series of pulses of modifiers were repeated as a control for each cell line used: no differences were observed when compared to effects of modifiers observed in an earlier stage of the experiment. Then the cells were harvested and counted in a hemocytometer. Their viability (rountinely >90%) was determined by the trypan blue exclusion test.

RESULTS

Detection of P-glycoprotein mRNA and protein levels

In an attempt to quantify the amounts of P-gp, we compared three detection methods for P-gp mRNA or protein, i.e. immunohistochemical staining, flow cytometry, both using monoclonal antibodies against P-gp, and the RNase protection assay. For the flow cytometry measurements, the MDR/sensitive cell couple 2780^{AD} and A2780 was included as a control. Using the monoclonal antibodies C219, MRK-16 or JSB-1, we found a consistently positive plasma membrane staining on cytospins of C-26 (except for MRK-16) and COLO 320 cells, a variable staining for SW1116, and no clear staining for HT29 (not shown). All cells failed to stain with CT-6, a non-relevant monoclonal antibody that was used as a negative control [29]. From the RNase detection method (Fig. 2) it appeared that HT29 and A2780 cancer cells lacked *mdr*1 mRNA. SW1116 cancer cells showed low levels of *mdr*1 mRNA, but using monoclonal antibodies against P-gp, no evidence for the presence of P-gp could be obtained with immunohistochemistry or flow cytometry (Table 1). The probe for *mdr*1 mRNA determination and the monoclonal antibody MRK-16 are both human specific and consequently for the murine C-26 cancer cells, only labeling results using JSB-1 or C219 are relevant. Positive plasma membrane staining was found for C-26 cancer cells by immunohistochemical staining on cytospins, using JSB-1 and C219. Moreover, flow cytometry showed a significantly higher fluorescence after C219 labeling with C-26 cancer cells compared to A2780, HT29 and SW1116 ($P < 0.01$). However, after JSB-1 labeling, no significant increase of fluorescence was observed, likely because of the fact that fixation as used for flow cytometry, affects the receptors for this monoclonal antibody in these cells. Labeling of C-26, compared to labeling of COLO 320, proved to be less (JSB-1) or the same (C219). High levels

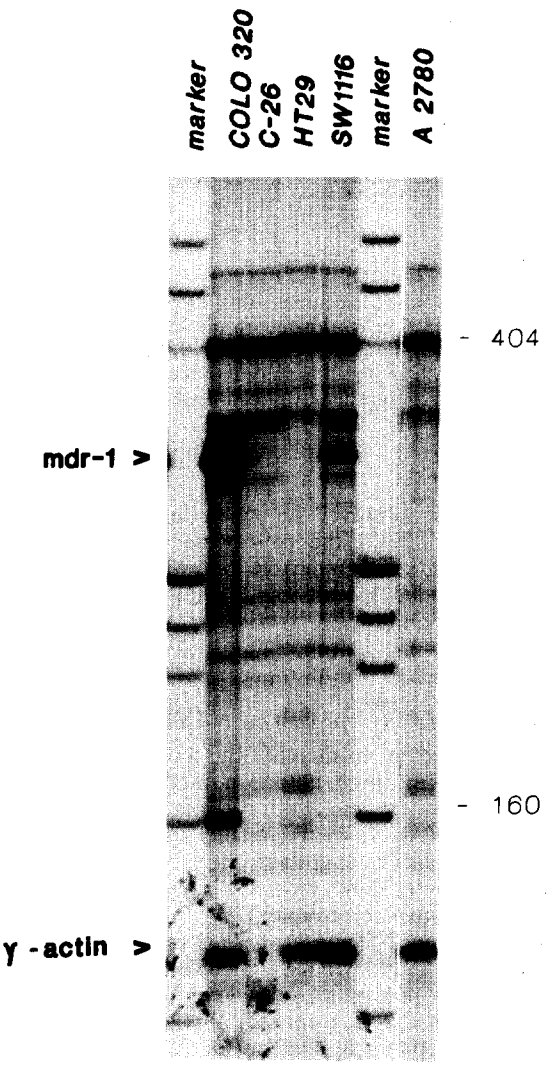

Fig. 2. *mdr*1 mRNA levels in various wild-type colon cancer cells and the sensitive ovarian A2780 cancer cells. RNase protection assay as described in Materials and Methods was done on 10 μg total cytoplasmic RNA from each cell line. The position of *mdr*1, γ actin and the size (nucleotides) of some molecular weight markers are indicated. Lanes: molecular weight markers, COLO 320 cells, C-26 cells, HT29 cells, SW1116 cells, molecular weight markers and A2780 cells.

of P-gp, using JSB-1, C219 or MRK-16, as well as high levels of *mdr*1 mRNA were found for COLO 320 cancer cells. For the highly resistant ovarian cancer cell line 2780^{AD}, labeling of P-gp proved to be highest. From the considerations as mentioned above, we estimated the amount of P-gp to be in the following order: A 2780, HT29 ≤ SW1116 < C-26 ≤ COLO 320 < 2780^{AD}.

Effect of Vp or Cr on DN accumulation and efflux

The effects of Vp and Cr on DN accumulation and efflux were studied in order to investigate whether there is a correlation with the presence of

Table 1. Staining of P-gp as measured by flow cytometry

Cell line	Antibody		
	JSB-1*	C219*	MRK-16†
A2780	1.3 ± 0.1	1.10 ± 0.02	1.1 and 1.2
2780^{AD}	5.1 ± 0.6§	4.4 ± 0.1§	33 ± 9§
HT29	1.3 ± 0.2	1.2 ± 0.1	1.0 and 1.0
SW1116	1.4 ± 0.2	1.1 ± 0.1	1.2 and 1.5
C-26	1.5 ± 0.2	1.5 ± 0.1§	NR‡
COLO 320	2.0 ± 0.2§	1.5 ± 0.1§	12 ± 3§

Values represent the channel of the mean peak (which corresponds to the mean fluorescence intensity) after incubation with a relevant monoclonal antibody, divided by the mean channel value obtained after incubation with a non-relevant antibody (mouse IgG) for each cell line. Values of two independent experiments, or means ± SD from three independent experiments are given.

* Cells were fixed and labeled as described in Materials and Methods.

† Unfixed cells were used for labeling with MRK-16 and irrelevant antibody.

‡ NR, MRK-16 is a human-specific antibody against P-gp, therefore it is not relevant for this murine cell line.

§ Significant increase of signal after labeling with JSB-1, C219 or MRK-16 when compared to the signal for A2780 using the corresponding monoclonal antibody (Student's *t*-test, $P < 0.02$).

P-gp. As can be seen in Fig. 3, Vp or Cr had no effect on DN accumulation in HT-29 cells, but increased DN accumulation in SW1116, COLO 320 and C-26 (according to the Student's *t*-test $P < 0.05$, $P < 0.02$, and $P < 0.02$, respectively). Since it has been assumed that Vp stimulates drug accumulation by inhibiting drug efflux from MDR cells (11, 34), we studied the effect of Vp or Cr on the DN efflux. In Fig. 4 representative experiments are shown. Vp and Cr inhibited the efflux of DN from COLO 320, C-26 and SW1116 cells, but only slightly affected the efflux of HT29 cells. This is in agreement with the presence of P-gp and the effects on the DN accumulation in each cell line. The effects of 10 μM Vp and 0.005% (v/v) Cr on DN accumulation and DN efflux were of the same order of magnitude.

Effect of Vp or Cr on DN cytotoxicity

The effects of 10 μM Vp and 0.005% (v/v) Cr on DN cytotoxicity are shown in Table 2. Both modifiers potentiated DN toxicity in C-26, COLO 320 and SW1116; no effect was observed in HT29 cells. This, again, is in agreement with the presence of P-gp.

Effects of MDR modifiers on the extracellular DN concentration, studied by a flow-through system

The flow-through system [21] was used as an alternative approach to find a correlation between the presence of P-gp and the effects of MDR modifiers, and to obtain more information about the mechanism of DN transport out of the cells. In our experimental set-up, a cellular DN accumulation rate can be calculated from the MDR modifier-induced disturbance of the equilibrium between inward and outward transport of DN, presumably

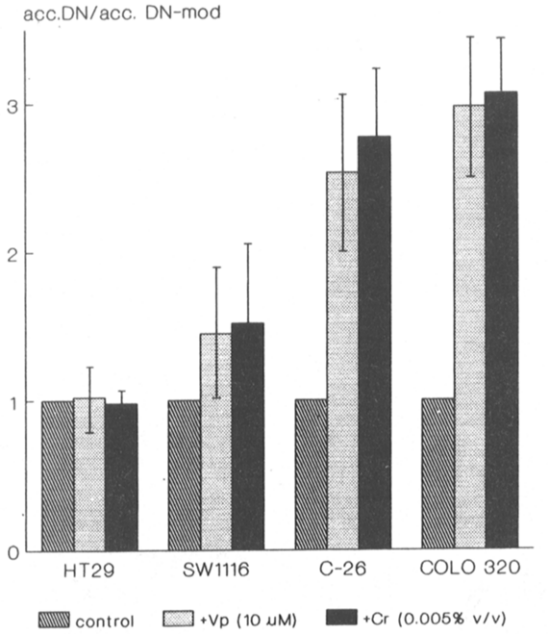

Fig. 3. Effect of Vp or Cr on the total DN accumulation in various wild-type colon cancer cell lines. Bars with standard deviations represent the total amount of accumulated DN divided by the total amount of accumulated DN in control experiments (no modifier). Values are means ± SD from 2–4 experiments. Conditions: extracellular concentration of DN, 0.4 μM; incubation, 45 min; temperature, 37°; pH 7.4; concentration of Vp, 10 μM; dilution of Cr, 0.005% (v/v).

caused by an inhibition of the DN efflux by these modifying agents. As can be seen in Fig. 5a, a pulse injection of Vp resulted in a decrease of the extracellular DN concentration in the cell lines SW1116, C-26 and COLO 320, but not in HT29. A series of pulses of modifying agents as given in Fig. 5b caused a maximal DN accumulation rate, which could not be increased using higher concentrations of modifier or more pulses in a series. The magnitude of the maximal accumulation rate was the same for Vp, CsA and Cr but was dependent on the cell line used. Extra pulses of Bp or Ro 11-2933/01, which also increased DN accumulation in the cells, could not further increase these maximal accumulation rates (not shown). The maximal accumulation rates of DN as a function of the extracellular DN concentration reached a saturation level for SW1116, C-26 and COLO 320 (Fig. 6). For the highly resistant cell line 2780^{AD}, a saturation could not be found, even at extracellular concentrations as high as 50 μM DN (Fig. 6, inset).

It has been shown for a number of cell lines that the total drug accumulation in and drug efflux from MDR cells is energy-dependent [7, 35]. Therefore we studied the net DN accumulation rate for the different colon cell lines after changing the eluent from medium A to medium B containing sodium azide, which is supposed to cause cellular energy depletion [35, 36]. Obviously the net maximal accumulation rate of DN that was caused by cellular energy depletion (Fig. 5c) was roughly two times higher than the net maximal accumulation rates obtained using modifying agents.

DISCUSSION

Using the flow-through system, we were able to monitor drug efflux by studying changes in steady-state DN medium concentrations. In this situation, DN outside the cell is in equilibrium with cytosolic free DN, which in turn is in equilibrium with nuclear or cytoplasmic bound DN. In standard efflux procedures the total cellular drug efflux from the cells is measured in drug-free medium. This implies that other efflux components, such as passive diffusion or efflux from intracellular bound DN, could interfere with an accurate determination of the P-gp-associated drug efflux. In the set-up we used, only the component of the drug efflux, that is affected by MDR modifying agents is measured, and looking to the *initial* change of the DN medium concentration after addition of Vp to the medium, the binding to other components is expected to be minimally affected.

Our findings suggest a good correlation between the presence of P-gp and the changes in DN cytotoxicity, total cellular accumulation of DN and efflux of DN, found after addition of MDR modifying agents in various wild-type colon tumor cell lines. In one cell line (SW1116) the presence of P-gp was ambiguous, probably because of its relatively low levels. This is in agreement with our findings that the Vp or Cr on DN cytotoxicity efflux and accumulation were small for SW1116, compared to the cell lines in which P-gp could be easily detected. The amount of P-gp was estimated to be in the order HT29, A2780 ≤ SW1116 < C-26 ≤ COLO 320 < 2780^{AD}. The same order was found for maximal DN transport out of the cells, that was inhibited by MDR modifiers, represented by the various saturation levels (Fig. 6); means ± SD: <5, <5, 40 ± 4, 64 ± 13, 170 ± 40, and >500 pmol DN/min/10^6 cells for A2780, HT29, SW1116, C-26, COLO 320 and 2780^{AD} cells, respectively. These findings suggest that the "maximal dip" found with the flow-through system after a series of Vp pulses, is a good indicator for P-gp activity.

For a correct interpretation of the DN accumulation rates we obtained with the flow-through system, other factors than the presence or absence of active efflux need consideration. Change of DN *binding to DNA* cannot account for the extra accumulation we found, since DN binding to isolated nuclei was similar in the presence or absence of Vp (unpublished results). We did not consider effects of Vp on *binding of DN to cell membranes*, since photoaffinity labeling experiments showed competition between Vp and DN on the protein binding sites [37–40], which shows that Vp could displace DN from these sites. Such an effect would lead to an "apparent efflux" of DN, thus to an increase of DN fluorescence in the medium instead of the decrease we found. Although it is well accepted that MDR modifying agents interact with P-gp mediated efflux, the mechanisms of potentiation of DN accumulation and cytotoxicity of MDR-related drugs by MDR modifying agents are still

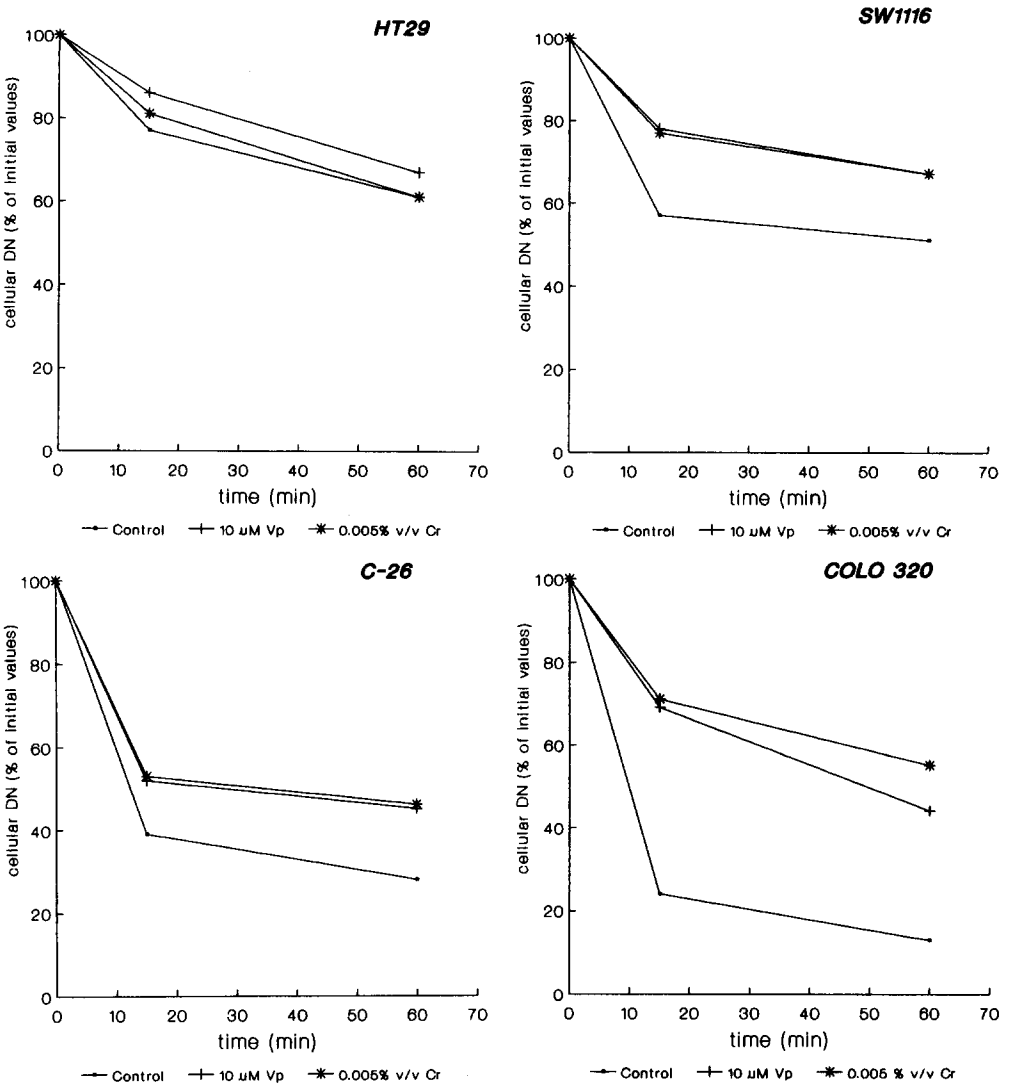

Fig. 4. Effects of Vp or Cr on DN efflux from various wild-type colon cancer cell lines. Conditions: see Fig. 2; cells were preloaded with 0.4 μM DN for 45 min and effluxed in DN free medium with Vp or Cr. Representative experiments are shown.

Table 2. Potentiation of DN cytotoxicity by Vp or Cr

Cell line*	IC_{50} (μM) Control	IC_{50} (μM) +Vp	DMF	IC_{50} (μM) +Cr	DMF
HT29	0.0215† (0.0004)	0.021 (0.001)	1.0	0.021 (0.001)	1.0
SW1116	0.024 (0.004)	0.0115 (0.0007)	2.1	0.015 (0.001)	1.6
C-26	0.11 (0.01)	0.0145 (0.0005)	7.6	0.022 (0.008)	5.0
COLO 320	0.11 (0.01)	0.024 (0.003)	4.6	0.024 (0.002)	4.6

* Cells were grown for 4 days at 37° in the presence of DN ± modifying agent.

† Values are means ± SD from 2–3 independent experiments, each performed in duplicate. SD is given between parenthesis.

DMF, dose modifying factor for 10 μM Vp or 0.005% (v/v) Cr; values are IC_{50} values for DN without modifying agent divided by the IC_{50} for DN with modifying agent.

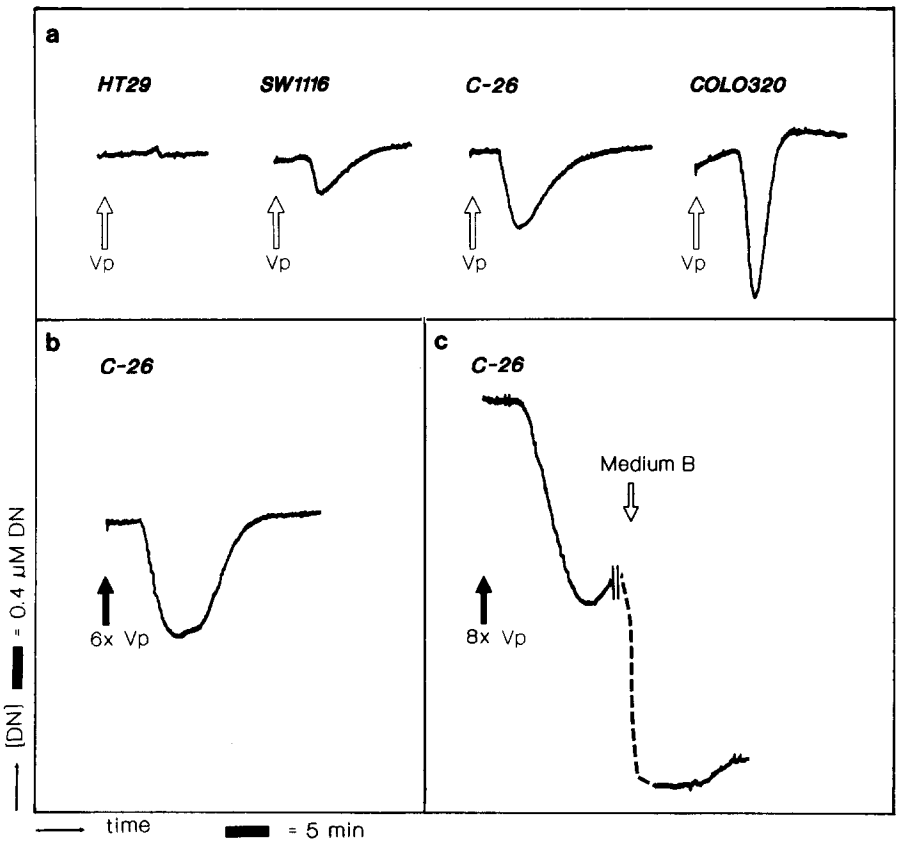

Fig. 5. Effects of MDR modifiers on the extracellular DN concentration, using the flow-through system. Conditions: number of cells, approximately 5×10^6; cell chamber volume, 500 μL; flow rate, 200 μL/min; perfusion medium, medium A; input DN concentration, 5 μM; pH 7.4; temperature, 37°. (a) Comparison of various colon cancer cell lines. (b) Maximal effects of series of pulse injections of Vp in C-26 cells. Effect of six (15 μL) pulse injections of Vp with time intervals of 30 sec, using C-26 cells; concentration of Vp above the cells was 20 μM. (c) Effect of cellular energy depletion. Perfusion medium was medium B, containing sodium azide.

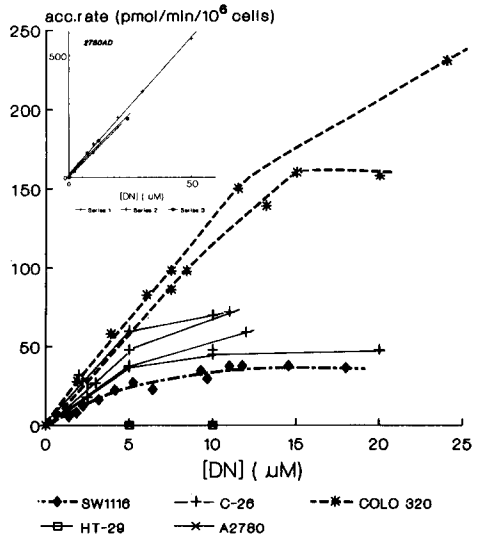

Fig. 6. Maximal net accumulation rates of DN obtained after pulse injections of Vp as a function of DN concentrations in various cell lines. Conditions, see Fig. 5, using six pulse injections of Vp with time intervals of 30 sec as a standard; initial input concentrations of DN varied stepwise, starting at the lowest concentration. Inset: results of three independent experiments with 2780^{AD} cells.

subject to many investigations. We cannot fully exclude a contribution of Vp induced changes in drug distribution by changes in pH as an explanation for the dip we found. However, we measured no change of cellular pH after addition of Vp (unpublished results). We have examined several sensitive and resistant cell line couples in the flow-through system and only in the case of P-gp containing resistant cell lines, a modulation of the steady-state situation was observed and no effects were obtained using the sensitive counterparts (human ovarian cancer A2780/2780^{AD} [41] cells, human squamous lung cancer SW1573/SW1573-500 cells, human breast cancer MCF-7/MCF-7Adr^R cells, unpublished results). Altogether, our results indicate that the dip must be explained by a resistance related factor, most likely to be the P-gp mediated efflux pump.

The maximal DN accumulation rates, obtained after pulse injections of Vp, Cr, CsA and combinations with Bp or Ro 11-2933/001 are of the same order of magnitude. This finding indicates that similar mechanisms may be responsible for the action of modifiers on the DN efflux. In photoaffinity labeling experiments it was shown that the modifying agents Vp and CsA, and probably also DN, bind to

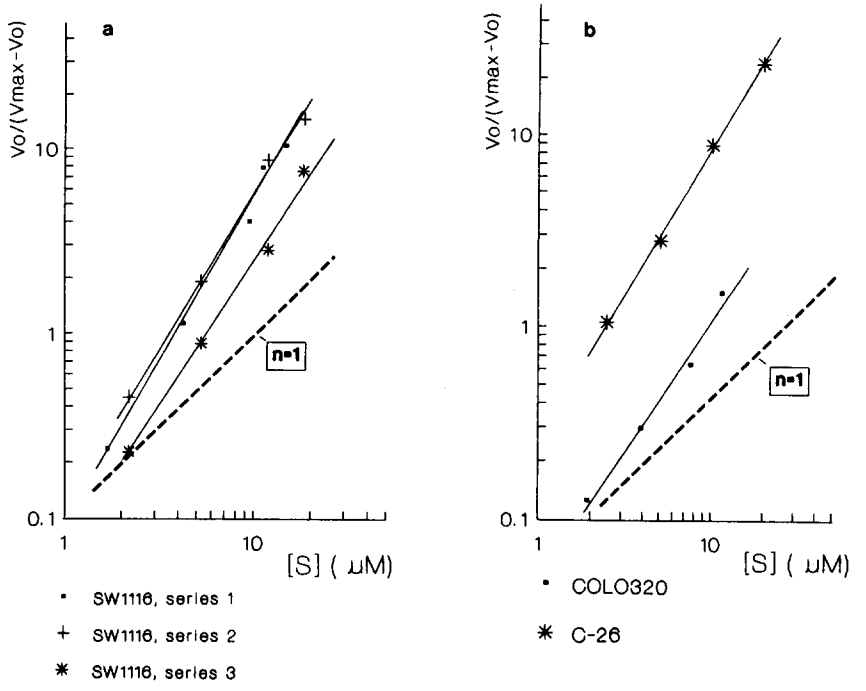

Fig. 7. Modelling cellular DN efflux of DN as a protein-mediated process. Values used are obtained from Fig. 6. Only experiments with more than three data in a series are included; V_o, the accumulation rate of DN, which represents the initial velocity, V_{max}, the maximal accumulation rates, which represent the maximal velocity, S, the extracellular substrate concentration (μM). (a) Hill plot for SW1116 cells, $V_o/(V_{max} - V_o)$ has been plotted against S, both plotted at logarithmic scales. In this way, the slope represents the Hill coefficient according to Eqn 2 (see text). (b) Hill plot for COLO 320 or C-26 cells [see (a)].

P-gp in MDR cell lines [37–40]. Therefore, we conclude that also in colon tumor cell lines with intrinsic resistance, potentiation of DN toxicity by MDR modifying agents is at least in part caused by interaction of these agents with P-gp.

Because the dips we measured in our flow-through system reflect at least partly the DN exported by the P-gp efflux pump, we tried to get more information about this transport of DN by studying the cellular pharmacokinetics of DN. Assuming that the P-gp associated drug pumping rate is proportional to the cytosol free drug concentration, saturation can be expected to occur at high cytosol free drug concentrations. Only for vincristine a saturable drug efflux process from MDR cells has been reported [42, 43]. As discussed by Ramu *et al.* [44], the lack of saturation kinetics is generally considered as evidence against an active drug efflux. One of the requirements to get a high cytosol free drug concentration, at which saturation phenomena may occur, is a low drug efflux rate. Attempts to find a saturation of the P-gp mediated efflux pump have been carried out on cells with high levels of (acquired) resistance [45]. In such cell lines however, it is likely that saturation is difficult to show, as may also appear from our results with highly resistant 2780^{AD} cells. For the wild type colon cancer cell lines however, we found a saturation of the maximal effects of modifying agents as a function of the extracellular DN concentration. A relatively low amount of cellular P-gp thus seems to be a requirement for the detection of saturation of the drug efflux. The influx of DN was not likely to be the rate limiting step, because energy depletion caused a much larger DN accumulation rate into the cells (Fig. 5c). As far as we know, this is the first time that saturation of P-gp associated DN efflux has been shown for intact cells.

Insight into the molecular order of a transport process, not following simple Michaelis–Menten kinetics, can be obtained with the help of Eqn 1, from which Eqn 2 can be derived [46, 47].

$$V_o = \frac{V_{max} S^n}{K_m + S^n}, \quad (1)$$

$$\log \{V_o/(V_{max} - V_o)\} = n \log \frac{S}{K_m}. \quad (2)$$

In these equations V_o, V_{max}, n, S and K_m represent the initial velocity, the maximal velocity, the Hill coefficient, substrate concentration, and the Michaelis–Menten constant, respectively. This last equation is formally identical to Hill's empirical relation for the binding of oxygen to haemoglobin [48]. If the transport process is strictly bimolecular, the slope of the line should be 1, which results in the simple Michaelis–Menten equation [49]. We fitted our results to Eqn 2, in which the initial velocity stands for the accumulation rate after pulses

of Vp, and the substrate concentration for the extracellular DN concentration. Using Eqn 2, the Hill coefficients were calculated by linear regression and ranged from 1.6 to 1.8, having correlation coefficients >0.995 as shown in Fig. 7a and b. Hill coefficients of the value 1–2 indicate cooperative interactions.

For the interpretation of this cooperativity in terms of DN transport kinetics through the protein efflux pump, free intracellular DN concentrations are needed. Skovsgaard attempted to measure intracellular concentrations of drugs by measuring the equilibrium concentration of DN in the supernatant of a suspension of homogenized cells [35, 45]. However, drug binding to many cellular components and presumably drug redistribution during fractionation of cellular components may have hampered these estimations. Not knowing cytosolic free drug concentrations, we have used the extracellular concentration for the Hill plot. In fact, the transport of DN can be considered to be a passive influx [5, 35, 50, 51] in series with an active saturable efflux. In this case, when the slope of a Hill plot $n > 1$, this may indicate positive cooperativity of drug binding for the active component [48, 52]. However, for the situation of a low passive leak rate or a low K_m, the slope $n > 1$ could theoretically also be interpreted from simple Michaelis–Menten kinetics. Initial estimations of the passive influx rate from our data or from literature data [53] indicate that this was not the case. It has been reported that P-gp contains two duplicate regions, each having a consensus ATP-binding site [54, 55]. The functional role of these two segments is unclear. Yosimura *et al.* [56] showed a cytoplasmic orientation of the two domains, and Bruggemann *et al.* [57] found two different regions of P-gp which are photoaffinity labeled by azidopine, a P-gp labeling agent. Moreover, after discrete mutations on the predicted nucleotide-binding site of the *mdr*1 gene, results were obtained which suggested that the two halves of *mdr*1 function in a cooperative fashion [58]. The indications for positive cooperativity for drug binding to an active pumping protein are in line with these observations. A more integral model for describing the fate of DN in cells, incorporating more cellular transport data obtained with other methods, will be worked out in the future.

In conclusion, we have presented evidence that at least part of the intrinsic resistance in wild-type colon tumor cells is mediated by the P-gp efflux pump. The presence of cellular saturation kinetics for DN, can be considered as evidence for an active transport of DN over the cell membrane from the cells which may occur via binding at two or more sites on the P-gp efflux pump.

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