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Inhibition of P-glycoprotein-mediated vinblastine transport across HCT-8 intestinal carcinoma monolayers by verapamil, cyclosporine A and SDZ PSC 833 in dependence on extracellular pH

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Abstract. The ability of the multidrug resistance modifiers R- and R,S-verapamil (VPL), cyclosporine A (CsA) and its non-immunosuppressive derivative SDZ PSC 833 (PSC 833) to inhibit P-glycoprotein (P-gp)-mediated transepithelial flux of tritiated vinblastine was investigated using tight and highly resistant (R $>1{,}400\ \Omega\ cm^2$) monolayer cultures of intestinal adenocarcinoma-derived HCT-8 cells grown on permeable tissue-culture inserts. Apical addition of these chemosensitisers inhibited drug flux (137 pmol h^{-1} cm^{-2}; range, 133–142 pmol h^{-1} cm^{-2}) in the basal to apical secretory direction at clinically relevant concentrations, with PSC 833 showing the highest activity, exhibiting inhibition at concentrations as low as 10 ng/ml (9 n*M*). Acidification of the modulator-containing apical compartment to an extracellular pH (pHo) of 6.8 had no influence on MDR reversal by CsA at 1 μg/ml (0.9 μ*M*; flux inhibition, 52%) or by PSC 833 at 100 ng/ml (0.09 μ*M*; flux inhibition, 60%), in contrast to R,S- and R-VPL, which showed decreased inhibition and caused less accumulation of vinblastine in HCT-8 cells under this condition (flux inhibition of 35% and 23%, respectively, at pHo 6.8 vs 50% and 43%, respectively, at pHo 7.5). P-gp-mediated rhodamine 123 efflux from dye-loaded single-cell suspensions of HCT-8 cells as measured by flow cytometry was not impeded at pHo 6.8 in comparison with pHo 7.5 in standard medium, but at low pHo the inhibitory activity of R-VPL (29% vs 60% rhodamine 123 efflux inhibition) was diminished significantly, again without a reduction in the effect of PSC 833 (rhodamine 123 flux inhibition, 75%). In conclusion, drug extrusion across polarised monolayers, which offer a relevant model for normal epithelia and tumour border areas, is inhibited by the apical presence of R,S- and R-VPL, CsA and PSC 833 at similar concentrations described for single-cell suspensions, resulting in increased (2.2- to 3.7-fold) intracellular drug accumulation. Functional apical P-gp expression, the absence of paracellular leakage and modulator-sensitive rhodamine 123 efflux in single HCT-8 cells indicate a P-gp-mediated transcellular efflux in HCT-8 monolayers. In addition to its high MDR-reversing capacity, the inhibitory activity of PSC 833 is not affected by acidic extracellular conditions, which reduce the VPL-induced drug retention significantly. As far as MDR contributes to the overall cellular drug resistance of solid tumours with hypoxic and acidic microenvironments, PSC 833 holds the greatest promise for clinical reversal of unresponsiveness to the respective group of chemotherapeutics.

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Abbreviations: CsA, cyclosporine A; HEPES, [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)]; MDR, multidrug resistance; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; pHo, extracellular pH; PSC 833, SDZ PSC 833; R, resistance; TEM, transmission electron microscopy; VPL, verapamil

Introduction

Multidrug resistance (MDR) is one of several cellular mechanisms enabling tumour cells to tolerate higher concentrations of cytostatic drugs, being special in simultaneously conferring cross-resistance to important small, hydrophobic chemotherapeutics such as anthracyclines, vinca alkaloids, actinomycin and taxol, among others [19]. In MDR the intracellular drug concentration is lowered by the action of the *mdr*1-gene-encoded P-glycoprotein (P-gp), which functions as a membrane-located adenosine triphosphate(ATP)-dependent drug-efflux pump. A significant role of P-gp-mediated drug resistance has been described for haematological malignancies, and P-gp has been found to be a marker of a dismal prognosis in many solid tumours, either directly causing unresponsiveness to chemotherapy or indicating the co-expression of other mechanisms of resistance [14, 19].

In correspondence to the rather broad substrate specificity of P-gp, a host of compounds have been found to bind to this protein and to inhibit its drug-transport activity in

vitro and in experimental animal models [3]. These chemosensitisers or MDR modifiers with clinical potential include calcium channel blockers [4, 24, 28], with verapamil (VPL) being the most prominent representative, cyclosporine A (CsA) and its non-immunosuppressive derivative SDZ PSC 833 (PSC 833), among many others [3, 4, 25]. Clinical trials of MDR-reversing agents were complicated by the side effects of most compounds, and improvements in the response rates, especially in patients with solid tumours, were far less than expected [6, 14]. This low reversal of drug resistance in MDR-modifier trials in solid tumours may be due to the predominance of other mechanisms of resistance such as alterations in intracellular target proteins or increased drug detoxification in these tumours, or adverse effects of the tumour microenvironment, with limited tumour accessibility and/or efficacy of chemosensitisers [18, 26, 27]. In addition, solid tumours derived from P-gp-positive normal epithelia (jejunum, colon, liver and pancreas) may preserve the polarisation and apical expression of P-gp, in contrast to the uniform distribution in haematological malignancies, and therefore show alterations in drug transport [2, 22].

To study the action of MDR modifiers in a model more relevant to the in vivo situation of solid tumours than single cells, we used a monolayer culture of a P-gp-positive colon carcinoma cell line (HCT-8; human ileocoecal adenocarcinoma) grown on a permeable support, whereby cells act as a functional unit, not as single cells independently from each other. The HCT-8 cell line has been demonstrated to exhibit basic elements of epithelial differentiation such as cellular polarity, formation of tight junctions and directed transepithelial transport, including P-gp-mediated drug fluxes [10, 23]. Separation of the apical and basolateral compartments by tight cell monolayers allows for the independent variation of the experimental conditions, for example to simulate the extracellular acidic conditions of tumour microenvironments or to study drug uptake, incorporation and efflux simultaneously.

In particular we aimed to study the inhibitory activities of R,S-VPL, R-VPL, CsA and PSC 833 on the transepithelial flux and cellular incorporation of [^{3}H]-vinblastine in HCT-8 monolayer cultures under normal tissue-culture conditions and in acidic and alkaline media. These results were compared with measurements of P-gp-mediated efflux of the fluorescent dye rhodamine 123 (Rh 123) in HCT-8 suspension cultures to estimate the relative contribution of MDR to transepithelial transport. The time-dependent decrease in cell-associated Rh 123 can be measured with high sensitivity by flow-cytometric analysis [15]. The results of such studies are expected to be relevant for the normal physiological role of P-gp in the intestine, for the possible side effects associated with clinical chemosensitiser trials and for the interaction of MDR modifiers with P-gp in intestine-derived tumours maintaining features of differentiated epithelial tumours and/or spheroidal structures [10].

Materials and methods

Cell culture. Human ileocecal adenocarcinoma HCT-8 (CCL 244) cells were obtained from ATCC (American Tissue Culture Collection, Rockville, Md.) and were maintained in bicarbonate-buffered 10% fetal bovine serum/RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 4 m*M* L-glutamine and 75 µg gentamycin/ml under tissue-culture conditions (37° C, humidified atmosphere containing 5% CO_2). Confluent monolayers were subcultured every 3–4 days by treatment with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in CA^{2+}- and Mg^{2+}-free PBS.

For measuring P-gp-mediated transepithelial drug transport, HCT-8 cells were seeded into 25-mm-diameter polyester filter cups (Falcon, Becton-Dickinson, Mountain View, Calif.; pore diameter, 0.45 µm, uncoated) at a density of 1×10^6 cells/cup (4 ml) with a basolateral compartmental volume of 3 ml [10]. Under these conditions, filter cups were cultured in six-well plates and media were replaced every 2 or 3 days until cells formed a confluent monolayer (5–7 days). To assess whether the monolayers were tight and capable of acting as a barrier, we measured electrophysiological transepithelial resistance using a WPI Voltohmmeter (World Precision Instruments Inc., Sarasota, USA) equipped with chopstick Ag/AgCl electrodes. For experiments we employed only cell monolayers that retained a transepithelial resistance exceeding 1,400 Ω cm^2 (the resistance value of filters incubated solely with medium, R = 700 Ω cm^2, is subtracted from all data).

Transmission electron microscopy. We rinsed tight HCT-8 monolayers with ice-cold PBS and then fixed them with glutaraldehyde (1% in PBS) for transmission electron microscopy (TEM) thin section. Postfixation occurred with 1% osmium tetroxide in buffer (pH 7.2) for 2 h. Dehydration was done in a graded series of ethanols (up to 96%) and, finally, in propylene oxide. The specimens were embedded in Epon 812 and ultrathin sections were stained with uranyl acetate/lead citrate and examined with an Philips EM 400 microscope.

Western-blot analysis of P-gp in HCT-8 cell extracts. Cells were washed, resuspended in homogenisation buffer (50 m*M* TRIS/HCl, pH = 8.0; 120 m*M* NaCl; 1 m*M* EDTA; 2 m*M* $MgCl_2$; 1% Nonidet P40) and dounced in a glass homogeniser. Aliquots of the resulting cell extracts were stored frozen at –70° C. An extract volume containing 30 µg protein in 20 µl extract volume was diluted with 20 µl solubilising buffer (procedure for detection of P-gp using monoclonal antibody C494 [5] by Western blot according to the manufacturer's instruction; CIS, bio international, Cedex, France) and the sample was incubated at 96° C for 3 min. After cooling, the sample was supplemented with 25 µl urea buffer and run on a 6% polyacrylamide gel (2 h). Blotting was performed overnight in transfer buffer containing 25 m*M* TRIS, 150 m*M* glycine and 2% methanol at constant voltage (30 V) and current (120 mA). The dried nitrocellulose membrane was blocked by incubation in 3% bovine serum albumin/PBS for 2 h and washed with 0.05% Tween 20/PBS.

A 1:3,000 dilution (in 0.5% BSA/PBS) of the C494 monoclonal antibody was added and after 2 h was replaced by a 1:4,000 dilution (in 0.5% BSA/PBS) of anti-mouse peroxidase-labeled rabbit antiserum (Dianova, Hamburg, Germany) followed by detection of the bound enzyme with a chemoluminescence detection kit (ECL Kit, Amersham Life Science, Amersham, UK). For positive controls, canalicular membrane preparations strongly expressing P-gp that had been obtained from regenerating rat liver were isolated and processed as described above [13].

Measurement of [^{3}H]-mannitol permeability. We used tritiated mannitol (26.4 Ci/mmol; Amersham Life Science) flux as an indicator of paracellular epithelial permeability and of the functional state of tight junctions in HCT-8 monolayers [17]. Media of tight monolayers were removed both apically and basolaterally and then replaced at the basolateral site by 3 ml medium containing 0.38 nmol [^{3}H]-mannitol (final concentration, 0.13 µ*M*) and at the apical site by 4 ml R,S-VPL (up to 200 µ*M*), CsA (up to 20 µg/ml) or PSC 833 (up to 20 µg/ml) containing media. Non-confluent monolayers were used in controls. Radioactivity measurements were performed as described below for [^{3}H]-vinblastine.

Directed [^{3}H]-vinblastine sulphate flux with/without chemosensitisers in a physiological extracellular milieu. Media of monolayers were

replaced basolaterally (or apically for reverse flux tests) by 3 ml medium containing 60 nmol [^{3}H]-vinblastine sulphate (11 Ci/mmol; Amersham Life Science; final concentration, 20 μM) and apically by 4 ml medium (or basolaterally without chemosensitizers for reverse flux tests) with 1 or 10 μM R,S-VPL or R-VPL, 100–1,000 ng CsA/ml (0.09–0.9 μM) or 10–500 ng PSC 833/ml (9 nM–0.45 μM) diluted from concentrated stock solutions (1:100–1:10,000 dilution range of 1 mM R,S-VPL and R-VPL, 1 mg CsA/ml, 0.1 mg PSC 833/ml) or equal quantities of the solvent ethanol (final concentration, <1%), respectively. At least 6 experiments were performed in each group. Plates were incubated under tissue-culture conditions at pHo 7.5 and under exclusion of light. The total applied radioactivity of the respective compartments was assessed by liquid scintillation counting of 100-µl media aliquots (5 ml Quicksafe A scintillation fluid; Zinsser, Maidenhead, UK). For measurement of the directed vinblastine flux, 100-µl aliquots were taken from the target compartment at intervals of 30 min during a 5-h period. We calculated the ratios of translocated vinblastine to the initially applied amount (in promille, ‰), put them in time-course graphs and set computer-calculated regression lines whose linear slopes represent the respective flux velocities (Charisma, Micrografx, Richardson, Tex.). Values obtained for flux inhibition in response to different chemomodulators are shown as percentages of inhibition relative to vinblastine fluxes in solvent containing medium.

[^{3}H]-Vinblastine sulphate flux with/without chemosensitisers in different pHo milieus. To investigate whether changes in extracellular [H$^+$] concentration would influence P-gp-mediated [^{3}H]-vinblastine transport and its inhibition by various chemomodulators, we performed experiments as described above under different pHo conditions. Instead of standard RPMI 1640 medium (25 mM bicarbonate), we apically applied bicarbonate-free RPMI 1640 medium (Sera-lab, Crawley Down, Sussex, UK) supplemented with bicarbonate to yield final bicarbonate concentrations of 2.5, 5, 25 and 45 mM, respectively. The media were preincubated for 18 h under tissue-culture conditions (5% CO_2, 37° C, humidified atmosphere) and thereafter exhibited pH values of 6.8, 7.0, 7.5 and 7.8, respectively, as measured closely before experiments. To differentiate the influence of pHo from drug-transport changes due to different bicarbonate concentrations per se, control experiments were performed with 40 mM HEPES-buffered RPMI 1640 media titrated with 1 mM NaOH to pH 7.5 and containing 2.5 and 25 mM bicarbonate, respectively.

Cell-associated [^{3}H]-vinblastine sulphate content in HCT-8 monolayers following incubation with/without chemosensitisers in different pH milieus. At the end of all experiments using [^{3}H]-vinblastine, filter cups were washed with cold PBS and monolayer-coated membranes were removed carefully and placed into vials for scintillation counting. To determine the cell-associated membrane content of vinblastine in particular experimental groups, we evaluated the promille amount of monolayer activity relative to the total initially applied [^{3}H]-vinblastine activity. According to a previously described experimental regimen, we carried out drug accumulation tests in the presence of HEPES-buffered media as well.

Measurement of P-gp-mediated Rh 123 efflux in HCT-8 suspension cultures. HCT-8 cells were harvested, washed with medium and loaded (2×10^7 cells in 5 ml) with 200 ng Rh 123/ml for 30 min in a humidified atmosphere containing 5% CO_2 at 37° C. Cells were washed again and incubated in tissue-culture flasks at a density of 1×10^6 cells/ml as mentioned above. Controls additionally contained the solvent (ethanol) and experimental samples, 10 μM R-VPL and 100 ng PSC 833/ml (0.09 μM), respectively. Aliquots of cell suspensions were collected at the beginning of experiments and at 10-min intervals thereafter for 2 h and were kept at 4° C. Samples were washed with PBS and the cells were resuspended in Cellpack (Sysmex, Tokyo, Japan) for analysis. The fluorescence of 5,000 cells was quantitated using a FACS-Analyzer (Becton-Dickinson, Mountain View, Calif.) equipped with a FACSLite laser source, a filter set for fluorescein isothiocyanate (used for monitoring of Rh 123) and a Consort 30 data-handling utility. Debris and cell aggregates were excluded with a volume side-scatter gate, and the mean peak channel numbers were used for further calculations. Thus, in this study we used flow cytometry to express the pHo dependence of P-gp-mediated Rh 123 efflux and the modulating capacity of the compounds R-VPL and PSC 833 with respect to different milieu conditions [15].

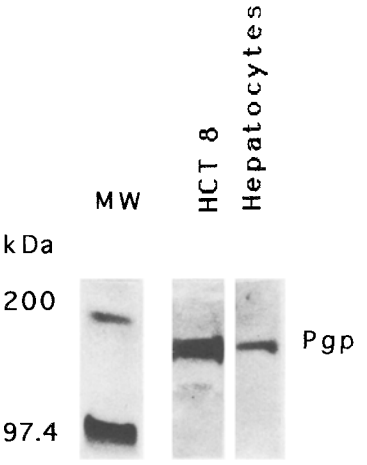

Fig. 1. Western-blot analysis of P-glycoprotein in HCT-7 cell extracts. Using P-gp-directed monoclonal antibody C494, a prominent band was detected within the 170- to 180-kDa region (*middle lane*); molecular-weight-standard proteins were phosphorylase b (97.4 kDa) and myosin (200 kDa; *left lane*), and a positive control was performed with canalicular membrane preparations from regenerating rat liver (*right lane*)

Statistical analysis. Data are expressed as mean values ± SE for n individual monolayers. Significance tests of differences between mean values were done using Student's two-tailed unpaired or paired t-test (Statistica statistical software package, Statsoft Inc., Tulsa, Okla., USA). Results were regarded as significant if $P < 0.05$.

Results

Epithelial characteristics of HCT-8 monolayers

Within 5–7 days of the seeding of HCT-8 cells on filter membranes at high densities, tight monolayers with an average transepithelial resistance of 2,045 ± 22.47 Ω cm^2 (n = 340) were established in tissue culture. TEM observations showed a polarised, differentiated monolayer consisting of absorptive enterocyte-like columnar cells that were interconnected by mature tight junctions and desmosomes and exposed microvilli (data not shown).

Expression of P-gp by HCT-8 cells as a prerequisite of our studies was revealed by Western-blot analysis (Fig. 1). Whole-cell extracts were blotted and probed with the C494 mouse monoclonal anti-P-gp antibody using a highly sensitive chemoluminescence detection system. The band detected in the 170- to 180-kDa region consisted of two closely spaced bands, visible in short exposures, indicating possible heterogeneity of the glycosylation of P-gp in HCT-8 cells. The C494 antibody lacks the cross-reactivities of the C219 and JSB-1 monoclonal antibodies.

In addition, we performed mannitol permeability studies with the aim of assessing whether HCT-8 monolayers could maintain epithelial barrier function, which is the basic condition for transport studies. In monolayers exceeding

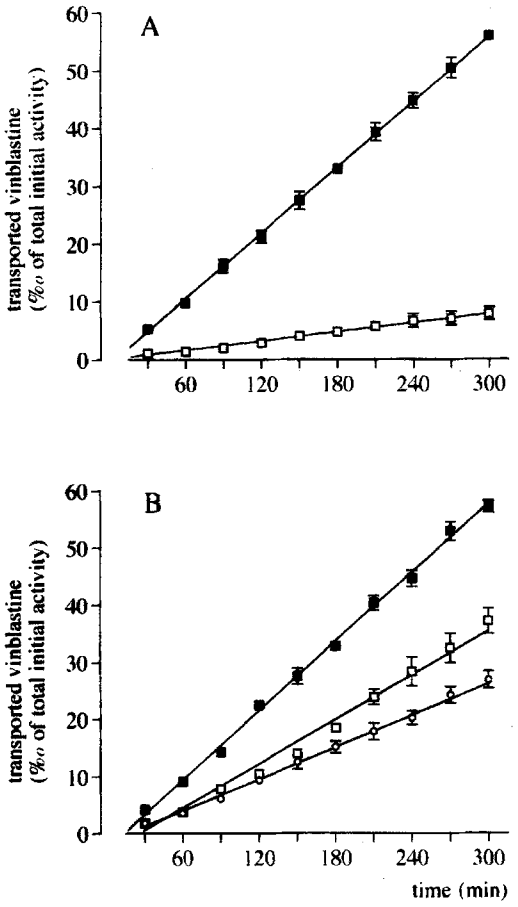

Fig. 2A, B. Basolateral to apical transepithelial vinblastine flux across tight HCT-8 monolayers (n = 6). Lines depict 300-min linear courses of transported vinblastine expressed as proportions (promille) of the total contralaterally applied [^{3}H]-vinblastine activity. **A** Plot revealing a 6- to 7-fold flux preference along the secretory (■) as compared with the absorptive direction (□). Regression line functions were y = –1.15+0.19x and y = –0.12+0.03x, respectively. **B** Basolateral to apical flux (■) as well as its concentration-dependent inhibition by PSC 833 added apically at 10 (□) and 100 ng/ml (○) as plotted against time, performing kinetics as follows: control, y = –2.65+0.2x; 10 ng PSC 833/ml, y = –4.43+0.13x; and 100 ng PSC 833/ml, y = –1.9+0.09x. The initial basolateral [^{3}H]-vinblastine concentration was 20 μ*M*. Data are shown as mean values ± SE, with the square of regression-line correlation indices $r^2 > 0.97$

1400 Ω cm^2, we observed linear mannitol fluxes resulting in a translocation of 1.88%±0.2% (range, 1.1%–3.54%; 100% = 0.38 nmol) of the total contralaterally added mannitol after 5 h. Thus, the absolute transepithelial mannitol flux rates were approximately 0.23 pmol h^{-1} cm^{-2} (range, 0.17–0.53 pmol h^{-1} cm^{-2}), which agrees with previous reported results [17]. Neither addition of chemomodulators (up to 200 μ*M* R,S-VPL, 20 μg CsA/ml or 20 μg PSC 833/ml) nor incubation with different media (pH 6.8–7.8) in vinblastine-transport experiments evoked monolayer leakage, which would be observable as a significant increase in transepithelial mannitol flux (1.98%±0.2%; range, 0.97%–3.34%; 5 h; absolute transepithelial mannitol flux rates were 0.31 pmol h^{-1} cm^{-2}, the range being 0.15–0.52 pmol h^{-1} cm^{-2}). The average transepithelial resistance was not influenced significantly during our experiments (data not shown). On the basis of the functional P-gp tests described below, we assume that cell membrane-associated P-gp is expressed almost exclusively at the apical site of HCT-8 monolayers.

[^{3}H]-Vinblastine sulphate flux with/without chemosensitisers at physiological pHo

Figure 2A illustrates [^{3}H]-vinblastine transport across HCT-8 monolayers in both directions. In either case, drug fluxes progressed linearly for at least 5 h. The rate of transport of vinblastine in the basolateral to apical direction was approximately 137 pmol h^{-1} cm^{-2} (range, 132.5–141.6 pmol h^{-1} cm^{-2}), exceeding the reverse vinblastine flux by a factor of approximately 6–7.

Several chemosensitisers were tested in the present study with respect to their ability to inhibit the secretory transepithelial P-gp-mediated transport of vinblastine, a cytostatic MDR substrate (Figs. 2B, 3). Vinblastine is predicted to pass basolateral membranes passively and to bind to the intracellular drug-binding site of P-gp at the apical cell membrane, followed by adenosine triphosphate(ATP)-dependent extrusion. R,S-VPL, the classic modulator of P-gp function, was tested at concentrations of 1 and 10 μ*M* and inhibited basolateral to apical vinblastine flux to 31.3% ±4.6% and 48.5%±2.2%, respectively (Fig. 3). The R-enantiomere of VPL was tested at the same concentrations and effected vinblastine-transport inhibition of 35.9%±5% and 40.1%±2.9%, respectively. Thus, we observed different potency between R-VPL and the racemate, whereby a significantly (P = 0.025) weaker transport inhibition was evoked by the R-enantiomer at a concentration of 10 μ*M*. At concentrations of 100, 250, 500 and 1,000 ng/ml (range, 0.09–0.9 μ*M*), CsA exhibited vinblastine flux inhibition of 22.5%±5.2%, 41.4%±2.4%, 61.7%±1.7% and 63.4% ±5.3%, respectively. At all concentrations tested, the cyclosporine derivative PSC 833 showed the strongest flux-inhibitory potency, since it exerted flux inhibition of 49.2% ±3.7%, 55%±5.4%, 55.4%±1.7% and 78.3%±2.4% at concentrations of 10, 50, 100 and 500 ng/ml (range, 9 n*M*–0.45 μ*M*), respectively. Figure 2B gives an example of the concentration-dependent vinblastine flux inhibition by PSC 833 in a time-course presentation obtained for the other modifiers in a similar manner.

[^{3}H]-Vinblastine sulphate flux with/without chemosensitisers in different pHo milieus

According to the observation that solid tumours frequently develop acidic pHo values in comparison with adjacent normal tissues, we investigated the influence of different pHo values on transepithelial vinblastine transport and on its attenuation by the above-mentioned chemomodulators. Vinblastine fluxes as measured at the different pHo values without chemomodulators were employed as controls for chemomodulator experiments under the respective conditions. Whereas CsA and PSC 833 showed no statistically significant difference in flux inhibitory capacity in either acidic or alkaline milieus in comparison with controls (pHo

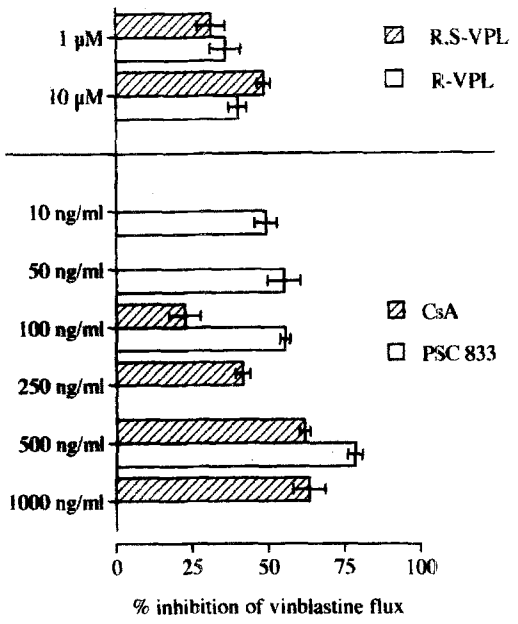

Fig. 3. Effect of chemomodulators on secretory transepithelial vinblastine flux. The continuous apical presence of several tested chemomodulators caused flux inhibition, shown as the percentage of reduction in the control vinblastine-flux velocities. Data represent mean values ± SE ($n = 6$) with regard to R,S- and R-VPL (*upper section*) and to CsA and PSC 833 (*bottom section*)

7.5), we observed a significant pH dependence of both R,S- and R-VPL modulatory effects as illustrated in Fig. 4. At pHo 6.8, the efficacy of both R,S-VPL and R-VPL was diminished by a factor of approximately 1.4 and 2, respectively, in comparison with their inhibition at the physiological pHo of 7.5.

To differentiate pH-exerted influences from those due to variation of the bicarbonate concentration, experiments were repeated for the 2.5- and 25-m*M* bicarbonate media at a pHo value of 7.5 held constant by additional buffering with HEPES. These experiments revealed no significant difference due to various bicarbonate concentrations, since 10 μ*M* R,S-VPL ($n = 5$) and R-VPL ($n = 3$) inhibited vinblastine flux to 59%±0.92% and 50.3%±4.12, respectively, in the presence of 2.5 m*M* bicarbonate in HEPES-buffered media and to 61.6%±4.75% and 49%±4.23%, respectively, in the presence of 25 m*M* bicarbonate in HEPES-buffered media. The higher than normal inhibition observed in response to R-VPL and R,S-VPL in these experiments (approximately 60% at 10 μ*M* for R,S-VPL vs 50% in standard medium) seems to be a result of the HEPES supplementation.

Cell-associated [^{3}H]-vinblastine sulphate content in HCT-8 monolayers following incubation with/without chemosensitisers in different pH milieus

In our model, inhibition of this drug-extruding pump by chemomodulators R,S-VPL and R-VPL (10 μ*M*), CsA (1,000 ng/ml, 0.9 μ*M*) and PSC 833 (100 ng/ml, 0.09 μ*M*) significantly increased drug accumulation as shown in

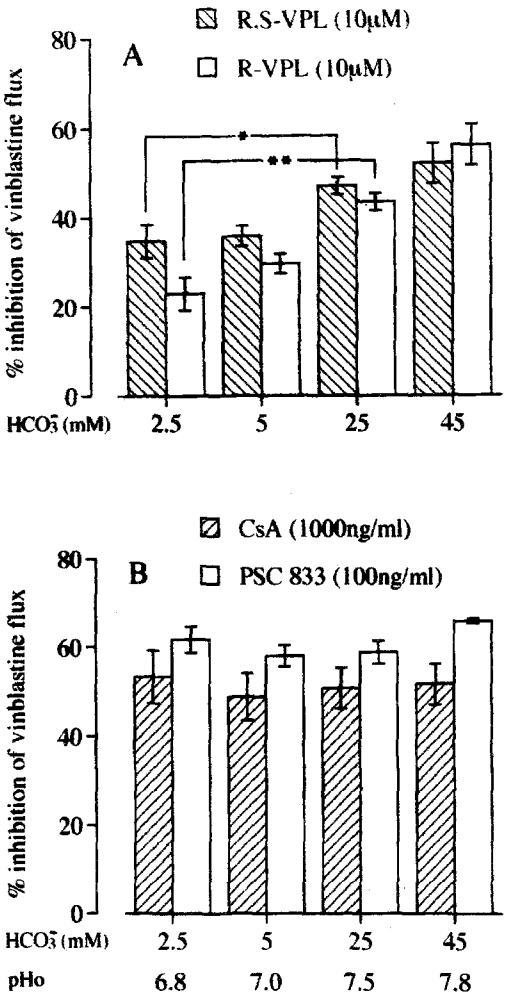

Fig. 4 A, B. Drug-transport-inhibitory potency of chemomodulators at different apical pHo values. The graph illustrates the chemomodulator-induced inhibition of secretory vinblastine flux in different apically applied media, in which buffering with 2.5, 5, 25 and 45 m*M* $NaHCO_3$-/5% CO_2 led to pH values of 6.8, 7.0, 7.5 and 7.8, respectively. **A** As compared with pHo 7.5–7.8, we observed a significant loss of the inhibitory capacity of both R,S-VPL and R-VPL (*$P < 0.05$, **$P < 0.01$; Student's paired *t*-test, $n = 6$) at pHo values of 6.8 and 7.0. Differences in inhibition were significant at between 5 and 25 m*M* bicarbonate for both drugs and between 25 and 45 m*M* bicarbonate for R-VPL. **B** In contrast to **A**, the cyclosporines exhibited pHo-independent flux-inhibition rates (no significant difference was found). Data represent mean values ± SE ($n = 6$)

Table 1. At physiological pHo, drug accumulation was increased by factors of approximately 2.5, 3.7, 2.2 and 2.3, respectively. Again we acertained a significant pHo-dependent effect when we used R,S-VPL or R-VPL, in good agreement with the accompanying flux studies. At pHo 6.8 we just reached 75% and 60% of the cell-associated drug content with 10 μ*M* R,S-VPL and R-VPL, respectively, as compared with the values obtained in experiments conducted at pHo 7.5, although R-VPL induced high relative accumulation at pHo 7.5 and 7.8. CsA-induced drug accumulation correlated weakly with pHo and paralleled the control values, and application of PSC 833 revealed a pHo-independent outcome, with higher relative incorporation occurring at low pH. Experiments performed in the presence

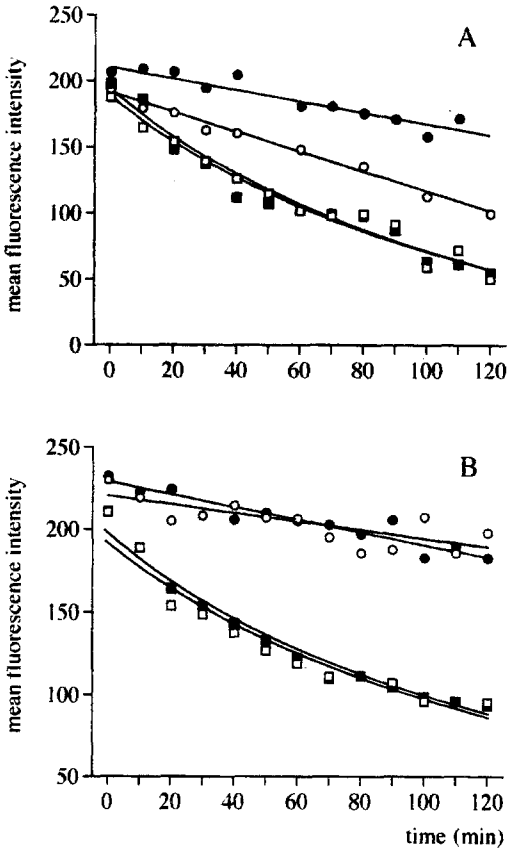

Fig. 5A, B. Rh 123 efflux from HCT-8 single-suspension cells at different pHo values. Means of the intracellular fluorescence intensities of the whole cell population are plotted against the time of incubation. Controls (□, pH 6.8; ■, pH 7.5) effluxed the P-gp substrate Rh 123 in an exponential manner. **A** R-VPL (10 μ*M*) diminished efflux of the fluorescent dye in HCT-8 cells (●, 25 m*M* bicarbonate, pH 7.5; ○, 2.5 m*M* bicarbonate, pH 6.8); however, Rh 123 efflux occurred faster in acidic milieu, indicating a loss of the P-gp-modulating capacity of R-VPL within this range. **B** Inhibition of P-gp-mediated Rh 123 efflux by PSC 833 (100 ng/ml) showed no significant difference between tests performed at pHo 6.8 (○) and pHo 7.5 (●). The square of regression line correlation indices is $r^2 > 0.85$, except for (○) in **B** ($r^2 = 0.59$)

of HEPES-buffered media at constant pHo showed no significant influence of different bicarbonate concentrations on chemomodulatory effects; 10 μ*M* R,S-VPL ($n = 5$) and 10 μ*M* R-VPL ($n = 3$) in HEPES-buffered media containing 2.5 m*M* bicarbonate led to a cellular vinblastine accumulation of 47.3‰ ± 7.62‰ and 48.6‰ ± 3.81‰, respectively, and the same concentration of the two drugs in HEPES-buffered media containing 25 m*M* bicarbonate yielded values of 49.2‰ ± 6.18‰ and 49.7‰ ± 3.98‰, respectively.

Measurement of the inhibitory activities of R-VPL and PSC 833 in assays of Rh 123 efflux in HCT-8 suspensions

Modulation of P-gp-mediated drug efflux was measured in single-cell suspensions by flow-cytometric quantitation of the decrease in the fluorescence of dye-preloaded HCT-8 cells under different experimental conditions during a 2-h incubation period in tissue-culture medium (Fig. 5). In the first 30 min, Rh 123-preloaded HCT-8 cells exhibited an approximately linear decrease in mean cellular fluorescence at normal (7.5) and acidic pHo (6.8). Addition of 10 μ*M* R-VPL inhibited the dye efflux to 60% under normal conditions, whereas at a lower pHo value its MDR-reversing activity was reduced to 28.8% (48% reduction; Fig. 5 A). In contrast to these results, the high P-gp-directed inhibitory activity of PSC 833 (100 ng/ml; pHo 7.6; 60.4% inhibition) was further increased at the lower extracellular pH (Fig. 5 B; pHo 6.8; 75.3% inhibition).

Discussion

Resistance of tumour cells to small and hydrophobic natural cytostatics is mediated by an energy-dependent transmembrane efflux pump, the so-called P-glycoprotein (P-gp), which is expressed at the luminal surface of normal epithelial tissues as well as in specialised endothelia, indicating a functional role in the secretion of substrates that

Table 1. Cell-associated drug content in HCT-8 monolayers[a]

pHo	6.8	7.0	7.5	7.8
Bicarbonate (m*M*)	2.5	5	25	45
Control	11.1 ± 0.68 ($P = 0.014$)*1	13.2 ± 1.05 ($P = 0.012$)*2	13.9 ± 0.75 ($P = 0.032$)*3	17 ± 1.18
R,S-VPL (10 μ*M*)	26.4 ± 1.32 ($P = 0.016$)*4	29.4 ± 3.48 ($P = 0.04$)*5	35.1 ± 2.35	33.9 ± 1.45
R-VPL (10 μ*M*)	31 ± 1.57 ($P = 0.015$)*4	30 ± 2.37 ($P = 0.027$)*5	51.2 ± 5.56	48.1 ± 3.33
CsA (1000 ng/ml)	26.1 ± 2.1 ($P = 0.036$)*1	25.1 ± 2.08 ($P = 0.035$)*2	29.9 ± 2.63	35.5 ± 3.8
PSC 833 (100 ng/ml)	34.5 ± 2.59	33.4 ± 2.12	32 ± 1.27	33.5 ± 1.21

Statistical tests showed significant differences between the following experimental groups: *1pHo 6.8 vs 7.8, *2pHo 7.0 vs 7.8, *3pHo 7.5 vs 7.8, *4pHo 6.8 vs 7.5, *5pHo 7.0 vs 7.5

[a] Cellular [3H]-vinblastine content was obtained by scintillation counting of monolayer-coated supports that were removed immediately after the termination of flux tests (duration of experiments, 5 h). Data represent mean values ± SE for the proportion (promille) of the total amount of basolaterally applied [3H]-vinblastine ($n > 5$) and are shown in dependence on several tested chemomodulators and on different pHo values

are not yet known with certainty [2, 19, 22]. Polarised expression of this protein has been demonstrated in the Caco-2 intestinal cell line, consistent with its presumed role as a secretory detoxifying system in the gastrointestinal tract [11, 12, 16, 19]. This cell line as well as the HCT-8 and T84 human intestinal adenocarcinoma cell lines, capable of forming a polarised epithelial monolayer in vitro when grown on permeable supports, exhibit basal to apical vectorial vinblastine transport [9, 10], which is P-gp-mediated as shown by its sensitivity to the specific MDR modulator verapamil [9–12]. In the present study we obtained tight and highly resistant HCT-8 monolayers by high-density seeding within 1 week and confirmed vectorial vinblastine transport in the secretory direction [10].

The formation of tight junctions, which limit paracellular transport and prevent protein lateral diffusion along the cell membranes, and the apical presence of microvilli were detected in TEM of sections from monolayer cultures. Expression of P-gp by HCT-8 cells was checked in Western blots using the specific C494 monoclonal antibody [5], which does not have the cross-reactivies of the JSB-1 and C219 antibodies. This cell line, which was not selected for increased drug resistance, shows the intermediate P-gp expression typical of clinical intestine-derived tumour specimens with inherent drug resistance [14].

Transepithelial transport of vinblastine in the picomolar range was found to be linear over at least 5 h, and the fluxes obtained were comparable with those reported previously [10]. This HCT-8 monolayer model was used to compare the MDR-reversing activity of drugs of major clinical importance, namely, R,S-VPL, R-VPL, CsA and PSC 833 [3, 4]. Clinically relevant concentrations of the respective modulators were applied apically to investigate their direct interaction with P-gp. Due to the side effects of R,S-VPL and CsA in clinical trials, the less cardiotoxic R-enantiomer of VPL and the non-immunosuppressive cyclosporine derivative PSC 833 were developed to avoid the dose-limiting toxicities of the parent compounds at the high concentrations necessary for chemosensitisation studies [1, 6, 14, 25]. Apical addition of 1–10 μM R,S-VPL or R-VPL, reported to have equal chemosensitising activity [7, 24], resulted in a 30%–50% inhibition of the transepithelial vinblastine flux, indicating partial reversal only at clinically feasible plasma concentrations of 2–3 μM. A dose-dependent inhibition was measured for 100–500 ng CsA/ml (0.09–0.45 μM), a concentration range that can easily be achieved in patients but approaches toxic levels for the upper limit when maintained for extended periods and is associated with maximal CsA-induced immunosuppression [6, 25]. In contrast, a high level of vinblastine-flux inhibition (>45%) is induced by apical addition of PSC 833 at concentrations as low as 10 ng/ml (9 nM), with up to 80% inhibition occurring at 500 ng/ml (0.45 μM), thus proving the extremely high MDR-reversing activity of this non-immunosuppressive derivative, which is currently being tested in phase I trials. These data demonstrate that P-gp-mediated drug efflux can be inhibited to a significant degree by clinically achievable concentrations of MDR modifiers in monolayer cultures.

In contrast to these in vitro results, clinical MDR-reversal studies using VPL, CsA or other modifiers in combination with anthracyclines or epipodophyllotoxins have shown very limited improvement in response rates, especially in solid tumours, as compared with the application of the cytostatic component alone [14, 19]. Since apical polarised expression of P-gp has no adverse effect upon MDR-reversing treatment, other reasons must be held responsible for the low reversal of chemoresistance. The main reason may be the co-expression of several other mechanisms of drug resistance such as modification or lower expression of target enzymes (i.e. topoisomerases) and higher expression of detoxifying systems (i.e. glutathione transferases), among others [14, 18, 26, 27]. In addition, the special tumour microenvironment with hypoxia and acidosis may have adverse effects on the activity of MDR modulators. The pH of the extracellular fluid (pHo) has been measured in tumours by insertion of small-diameter pH electrodes and has been found to be shifted towards more acidic values for larger tumours than that measured in normal tissues (median pH value, approximately 6.9–7.0 vs 7.4–7.5) [21]. The corresponding intracellular tumour pH values determined by ^{31}P-nuclear magnetic resonance (^{31}P-NMR) spectroscopy were near normal. Low pHo values have been demonstrated to reduce significantly the intracellular accumulation and toxicity of cytostatic drugs [20], and we have detected a decrease in the MDR-reversing activity of R-VPL and CsA in drug-sensitivity assays with suspension cultures of colon carcinoma cell lines [8]. To investigate the possible influence of extracellular pH on the activity of chemosensitisers, we exposed the HCT-8 monolayer to media with pH values of 6.8–7.8 that were supplemented with the different MDR modifiers. In agreement with the results obtained in suspension cultures, the activity of both R-VPL and R,S-VPL decreased at more acidic pH values, whereas the inhibition of transepithelial vinblastine transport by CsA and PSC 833 was not significantly affected by the pHo. Although R-VPL induced a higher cellular vinblastine accumulation at normal pHo, a concentration of 10 μM may not be achievable in vivo.

Since the transepithelial flux of vinblastine through HCT-8 monolayers is the net result of P-gp-mediated transport, possible vesicular transport and paracellular fluxes, we investigated the efficacy and pH dependence of R-VPL and PSC 833 in suspension cultures of the same cell line using flow-cytometric measurements of the P-gp-mediated Rh 123 efflux [15]. PSC 833 inhibited Rh 123 efflux to a high degree at normal and acidic pHo, whereas inhibition by VPL decreased significantly at lower pHo, confirming the results obtained in monolayer flux studies. A comparison of PSC 833-induced flux inhibition in single cells versus monolayers indicates that P-gp-mediated transport is the major transepithelial efflux mechanism for the respective substrates.

In conclusion, PSC 833 is a highly potent MDR-reversing drug that inhibits transepithelial MDR-mediated transport at normal and low pH values with equal efficacy, in contrast to the pH-dependent inhibition by VPL. This findings are relevant for epithelia-like structures in tumours and tumour-stroma interphases as well as for the side effects directed towards normal P-gp-positive tissues and for the acidic conditions occurring in microenvironments of solid tumours.

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