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Polarized transport of docetaxel and vinblastine mediated by P-glycoprotein in human intestinal epithelial cell monolayers

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Abstract—The expression of the multidrug transporter P-glycoprotein has been studied in two human intestinal epithelial cell lines. No functional expression of P-glycoprotein was found in the differentiated HT29-18-C_1 cell line. The expression of P-glycoprotein in the Caco-2 cell line was very high, as judged by immunoblotting and by active efflux of vinblastine. The polarized transport of vinblastine in the basolateral to apical direction was temperature and energy dependent, and was reduced by P-glycoprotein inhibitors such as verapamil, chlorpromazine and reserpine. This adds further evidence that the polarized transport of vinblastine across Caco-2 monolayers is mediated by P-glycoprotein. The anticancer drug docetaxel (Taxotere®) was transported in a polarized manner: basolateral to apical permeability was 20-fold higher than in the reverse direction. This polarized transport was inhibited by verapamil, chlorpromazine and reserpine, thus demonstrating that docetaxel is a substrate of P-glycoprotein. The implications of these results for the pharmacokinetics and toxicity of taxoids are discussed.

Key words: Caco-2 cells; P-glycoprotein; vinblastine; docetaxel; intestine; multidrug resistance

ATP-binding cassette transporters form a family of proteins that actively transport various compounds across cellular membranes. This family includes, among others, the P-gp* encoded by the human MDR1 gene. P-gp is a 170–180 kDa membrane protein that pumps various drugs out of cells by an ATP-dependent process. Consequently, the intracellular concentration of these drugs, and hence their toxicity, is reduced [1, 2]. This mechanism explains the MDR phenotype of cancer cells overexpressing P-gp. P-gp is expressed in many human tissues such as liver, intestine and kidney, where it is localized on the apical membranes of cells facing an excretory compartment [3]; P-gp is also expressed on the luminal face of the endothelial cells of the blood–brain barrier [4]. This luminal localization in endothelial and epithelial cells strongly suggests that P-gp may act to secrete toxic compounds or endogenous metabolites and/or limit the absorption of xenobiotics across cellular barriers.

Docetaxel (Taxotere®; RP56976; NSC 628503) is a semisynthetic compound belonging to the taxoid family which has been shown to possess high activity against ovarian, breast and lung carcinomas in early phase II clinical trials [5]. Docetaxel and paclitaxel (Taxol®) both present cross-resistance in multidrug resistant cell lines [6]. Moreover, docetaxel-resistant cells overexpress P-gp mRNA [7]. Docetaxel has also been shown to inhibit the polarized secretion of vinblastine in Caco-2 cell monolayers [8]. However, it remains to be shown that docetaxel is a substrate of P-gp. Using radiolabelled docetaxel, we demonstrate for the first time that docetaxel is actively transported by P-gp across Caco-2 monolayers.

Materials and Methods

Chemicals. Cell culture reagents were supplied by Gibco-BRL (Eragny, France). [^{3}H]VBL sulphate (sp. act. 11 Ci/mmol), [^{3}H]propranolol (sp. act. 27 Ci/mmol) and [^{14}C]-mannitol (sp. act. 57 mCi/mmol) were purchased from Amersham (Les Ulis, France), and [^{3}H]paclitaxel (23 Ci/mmol) from Moravek Biochemicals (Brea, CA, U.S.A.). [^{14}C]Docetaxel (47 mCi/mmol) was synthesized and purified by CEA, Service des Molécules Marquées, Gif-sur-Yvette (France). Monoclonal antibody C219 (Centocor Diagnostics) was purchased from CIS Bio-International (Gif-sur-Yvette, France), and ^{125}I-labelled anti-mouse Ig antibody from Amersham. Liquid scintillation cocktail Ready Solv HP was supplied by Beckman (Gagny, France). All other reagents were supplied by the Sigma Chemical Co. (St Louis, MO, U.S.A.)

Cell culture. The human colon carcinoma cell line Caco-2 was obtained from Dr A. Zweibaum (INSERM U. 178, Villejuif, France) and cultured as described by Wils *et al.* [9]. HT29-18-C_1 cells were obtained from Dr C. Huet (Institut Pasteur, Paris, France) and cultured as described [10]. The multidrug resistant KB/V1 carcinoma cell line was obtained from Dr J. F. Riou (Rhône-Poulenc Rorer, Vitry-sur-Seine, France) and grown as described [2].

Membrane preparations and western blotting. Cells grown in plastic flasks were washed with PBS, then detached by scraping. After centrifugation (1000 *g*, 10 min), the pellet was suspended in lysis buffer (Tris–HCl 10 mM pH 7.5, NaCl 10 mM, $MgCl_2$ 1 mM) and cells were incubated 15 min in ice. Cells were homogenized in a Tenbroeck, then in a Kontes homogenizer. Cell debris and nuclei were removed by centrifugation at 400 *g* for 10 min. Membranes were obtained by centrifugation of the supernatant for 45 min at 40,000 *g*, suspended in PBS and stored in liquid nitrogen. Protein concentrations were measured with the BCA Protein Assay kit (Pierce, Rockford, IL, U.S.A.). Proteins were electrophoresed on 8% SDS–polyacrylamide gels and electroblotted on nitrocellulose filter. The filter was blocked overnight at 4° in PBS containing 0.1% (v/v) Tween 20 and 5% (w/v) non-fat dry milk, incubated with C219 antibody (1 μg/mL in blocking buffer) for 2 hr at room temperature, washed in blocking buffer and incubated with ^{125}I-labelled secondary antibody for 3 hr. After washing and drying, the filter was placed on autoradiography film for 2 days.

Uptake experiments. Cells were seeded in 48-well tissue culture plates, at 12,000 cells/cm^2 and used between days 12 and 15 after seeding. Cells were incubated at 37° for with [^{3}H]VBL (40 nM in transport medium, see below). At the end of incubation, the supernatant was transferred to a scintillation vial, cells were washed rapidly and solubilized with 1 M NaOH–0.1% SDS. Radioactivity associated with the cells plus supernatant radioactivity was

* Abbreviations: AP, apical; BL, basolateral; MDR, multidrug resistance; P-gp, P-glycoprotein; VBL, vinblastine.

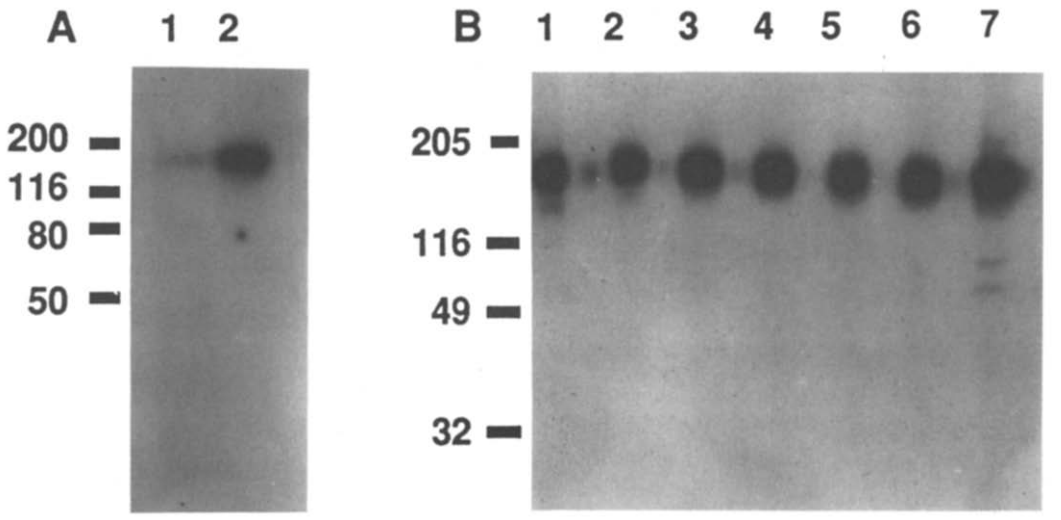

Fig. 1. Immunoblotting of membrane proteins from HT29-18-C_1 (A) and Caco-2 (B) cells with anti-P-gp C219 antibody. Panel A: 100 μg of proteins were loaded in each well. Lane 1: HT29-18-C_1; lane 2: KB/V1. Panel B: 20 μg of proteins were loaded in each well. Lanes 1–6: Caco-2 at day 4, 7, 11, 14, 18 and 22 after seeding; lane 7: KB/V1. Molecular weight markers are indicated (kDa).

taken as total radioactivity and cell associated radioactivity was expressed as percentage of total radioactivity.

Transport experiments. Transport studies with Caco-2 monolayers grown on polycarbonate filters in dual chambers were performed as described [11], except that the incubation medium was BHK21 (Glasgow modified Eagle medium), 25 mM HEPES, pH 7.4. For ATP depletion experiments, the medium used was Hanks balanced salts solution. The cells were preincubated with 5 mM NaN_3 and 50 mM 2-deoxy-D-glucose for 30 min prior to the experiment that was then performed in the presence of NaN_3 and 2-deoxy-D-glucose in both compartments. Data are expressed as means ± SD (N = number of wells).

Results and Discussion

In the present study, the presence of the MDR1 gene product, P-gp, was investigated in two human intestinal epithelial cell lines, HT29-18-C_1 and Caco-2. HT29-18-C_1 is a differentiated subclone derived from the human colon carcinoma cell line HT29. It was found by immunoblotting to express a very low level of P-gp as compared with the multidrug-resistant KB/V1 cell line (Fig. 1A). The Caco-2 cell line has previously been reported to express P-gp, which can be detected by western blot [12] or by immunofluorescence [13]. In the present work, the P-gp was readily detected by western blotting in Caco-2 cell membrane preparations. Furthermore, we examined whether the expression of P-gp was influenced by the growth and differentiation of Caco-2 cells. Membranes prepared from cells at day 4 to day 22 after seeding were compared (under our conditions, cells are confluent 7 days after seeding). As seen in Fig. 1B, no significant changes in the amount of P-gp were detected. The results of VBL uptake experiments confirmed the high expression of P-gp observed by immunoblotting: the uptake of radiolabelled VBL by Caco-2 cells was increased 3.7-fold in the presence of 25 μM verapamil (8.1 ± 0.4% vs 30.3 ± 0.9%, N = 3), while in HT29-18-C_1 cells it was not modified in the presence of verapamil. It is worth noting that the Caco-2 cell line which was P-gp positive was cultured at passages 70 to 100. It seems that the P-gp expression could be lost during long term cultivation, as we did not detect any functional expression of P-gp in a Caco-2 cell line at a higher passage number (data not shown).

The bidirectional fluxes of [^{3}H]VBL (40 nM) were followed across Caco-2 cell monolayers grown on filter in dual chambers. We observed a polarized BL to AP transport of [^{3}H]VBL, as described by Hunter *et al.* [8, 13]. Verapamil (25 μM) reduced the BL to AP flux of [^{3}H]VBL by 50% and induced a 6-fold increase in the AP to BL flux. The BL to AP permeability coefficient of VBL was significantly reduced in the presence of 50 μM chlorpromazine or reserpine, whereas the antiestrogen tamoxifen was ineffective at the same concentration. Consistent with the lack of biochemical expression of P-gp in HT29-18-C_1 cells, no difference was observed between the AP to BL and BL to AP fluxes of [^{3}H]VBL across HT29-18-C_1 cells monolayers (data not shown).

The polarized transport of [^{3}H]VBL across Caco-2 cell monolayers was energy-dependent: in the presence of sodium azide and deoxy-D-glucose, there was a 50% reduction in the BL to AP flux, from $45.2 \times 10^{-6} \pm 4.3 \times 10^{-6}$ cm/sec (N = 3) to $22.6 \times 10^{-6} \pm 2.5 \times 10^{-6}$ cm/sec (N = 4). This treatment did not increase the BL to AP flux of the paracellular marker, mannitol, thus demonstrating that the integrity of the monolayers was maintained (data not shown). The AP to BL flux of [^{3}H]-VBL decreased 2.7-fold at 4° (from $1.8 \times 10^{-6} \pm 0.2 \times 10^{-6}$ cm/sec to $0.7 \times 10^{-6} \pm 0.1 \times 10^{-6}$ cm/sec, N = 4); on the other hand, the BL to AP flux showed a 13-fold decrease (from $45.2 \times 10^{-6} \pm 4.3 \times 10^{-6}$ cm/sec to $3.7 \times 10^{-6} \pm 0.4 \times 10^{-6}$ cm/sec, N = 4). This indicates that the [^{3}H]VBL secretory flux to the AP side of the epithelial monolayer is mediated by a temperature-dependent mechanism, as expected for a P-gp mediated process.

The bidirectional fluxes of [^{14}C]docetaxel (4 μM) were similar to those reported for VBL: the BL to AP transport was 22-fold higher than in the reverse direction (Fig. 2). In the presence of verapamil (100 μM), there was a three-fold increase in AP to BL transport, and the BL to AP flux was reduced by 60%. If the active component of transcellular flux is assumed to be (BL to AP flux) − (AP to BL flux), then this net active [^{14}C]docetaxel flux was reduced by 73% in the presence of verapamil. A polarized, verapamil-sensitive transport of [^{3}H]paclitaxel was also observed across Caco-2 cells (data not shown).

The effect of various P-gp inhibitors on BL to AP flux of [^{14}C]docetaxel was investigated: reserpine (50 μM) reduced the BL to AP transport of docetaxel by 60% (from

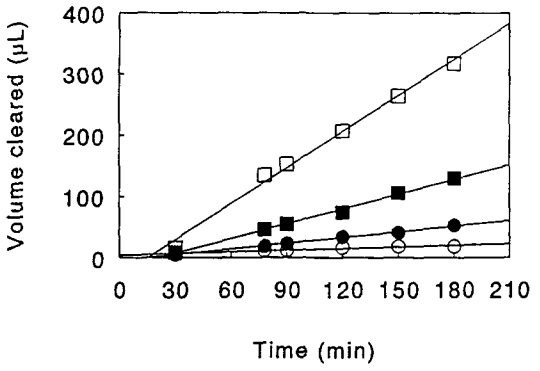

Fig. 2. Transepithelial passage of [^{14}C]docetaxel (4 μM) across Caco-2 monolayers. The volume of docetaxel cleared in the AP to BL (○) or BL to AP (□) direction was calculated as indicated in Materials and Methods and plotted against time of incubation. Passages in the presence of 100 μM verapamil (AP to BL, ●; BL to AP, ■) are also illustrated. Data are means ± SD (N = three wells). Error bars are smaller than the symbols.

$19.1 \times 10^{-6} \pm 1.7 \times 10^{-6}$ cm/sec to $7.4 \times 10^{-6} \pm 0.8 \times 10^{-6}$ cm/sec, N = 3). In the presence of chlorpromazine (50 μM), there was a 20% reduction ($15.3 \times 10^{-6} \pm 0.4 \times 10^{-6}$ cm/sec, N = 3), while tamoxifen (50 μM) did not modify the BL to AP transport ($20.2 \times 10^{-6} \pm 0.7 \times 10^{-6}$ cm/sec, N = 3).

Apart from the possible emergence of resistance during the course of cancer chemotherapy, the interaction of docetaxel with P-gp has other important consequences for the pharmacokinetics and toxicity of this drug. P-gp, which is also present in liver and kidney, could potentially control the excretion of docetaxel not only into bile or urine, but also into the lumen of the gastrointestinal tract. Furthermore, the co-administration of docetaxel with other P-gp substrates or inhibitors could (1) increase the cellular uptake and thus the antitumor action of docetaxel; and (2) dramatically modify the elimination and hence systemic toxicity of this drug. Such an interaction has been described for verapamil, which modulates the pharmacokinetics and toxicity of vincristine [14].

In conclusion, the present study shows that docetaxel is transported via P-gp in the BL to AP direction through Caco-2 cell monolayers. This polarized secretion is reduced by typical P-gp inhibitors.

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