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Effect of hr-IL2 treatment on intestinal P-glycoprotein expression and activity in Caco-2 cells

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Abstract

Caco-2 cells were used to investigate the effect of human recombinant interleukin-2 (IL2) on intestinal P-glycoprotein (P-gp) transporter activity in-vitro. More specifically the efflux function of P-gp was studied by measuring the transepithelial transport of rhodamine-123, a fluorescent substrate of P-gp. Its transport was completely inhibited by two specific P-gp inhibitors, ciclosporin A and GG918, in our experiments. Conversely, these two specific P-gp inhibitors inhibited only 50% of transepithelial transport when [^{3}H]vincristine was used as substrate. After Caco-2 cells were treated with 100 IU mL^{-1} (6.1 ng mL^{-1}) IL2 for 24 h, a significant diminution (21%) of P-gp transporter function was observed with rhodamine-123 substrate. This effect was also confirmed after 48 and 72 h of exposure to IL2. However, for higher concentrations of IL2 (1000 and 5000 IU mL^{-1}), diminution of P-gp function only occurred after a longer treatment period (48 h and more). The inhibitory effect of IL2 on P-gp activity was found to be independent of tight junction function as demonstrated by constant transepithelial electrical resistance (TEER) measurements for all experimental conditions encountered in this study (time and concentration of IL2 exposure). Furthermore, the MDR1 mRNA level was found to be strongly repressed in Caco-2 cells exposed with 1000 IU mL^{-1} IL2 for 72 h while the amount of MRP1 mRNA remained unchanged. In conclusion, acute incubation of Caco-2 cells with IL2 induced a decrease of P-gp transporter expression and activity.

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Introduction

Currently, cytokines are widely used in immunotherapy. IL2 was the first cytokine prescribed to treat patients with advanced cancer in combination with anticancer drugs (Elkahwaji et al 1999) or HIV-infected patients under a tritherapy protocol (David et al 2001). IL2 is an immunoregulatory cytokine, which plays a central role and has a pleiotropic effect on patients' immune response. Many cytokines are produced by cells within the lamina propria or by epithelial intestinal cells, which are known to share different cytokine receptors. This suggests an important integration of the epithelial surface of the mucosa with the mucosal immune system (Podolsky 1993; Fiocchi 1997).

Intestinal epithelium is the site of infection and inflammation. Acute intestinal mucosal inflammation has been reported to be associated with activation of peripheral blood mononuclear cells, PBMN (Mahida et al 1997; McAlindon et al 1998), leading to the release of various cytokines, including IL2, transforming growth factor-beta (TGFβ), tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) (Autenrieth et al 1997). In the intestinal mucosa of patients with inflammatory bowel disease (Crohn's disease), a different pattern of cytokines has been reported (Niessner & Volk 1995), in particular an increase of IL2.

The presence of functional IL2 receptors in intestinal epithelial cells (Ciacci et al 1993; Reinecker & Podolsky 1995), which are non-immunological cells, and the in-vitro modulation of intestinal epithelial cell function by IL2 (Dignass & Podolsky 1996) strongly suggest that IL2 plays a role in the physiological functions of intestinal epithelium.

The bioavailability of a drug is often altered in inflammatory disease (Piquette-Miller et al 1998). One cause of such an effect might be the diminution of cytochromes P450

(CYPs) expression and activity, implicated in drug metabolism (Walker et al 1986; Belpaire et al 1989; Morgan 1989). Moreover, it has been reported that IL2 could modulate the expression of CYPs in-vivo, in rodents and in cultured rat hepatocytes (Thal et al 1994; Cantoni et al 1995; Tinel et al 1995). Another cause could be a diminution in the activity of P-glycoprotein (P-gp), an efflux transporter which belongs to the superfamily of ATP-binding cassette (ABC) membrane transport proteins. It has been reported that there is a diminution of the hepatic P-gp expression and activity in rodents after acute inflammation (Piquette-Miller et al 1998; Hartmann et al 2001). In man, intestinal P-gp, encoded by the MDR1 gene, is expressed on the apical membrane of enterocytes where it is assumed to pump xenobiotics from cells back into the intestinal lumen. Recently, an increase in the bioavailability of digoxin (a P-gp substrate) was obtained in mice pre-treated with IL2 (Bonhomme-Faivre et al 2002). Thus, it is of interest to study the effect of IL2 on P-gp in intestinal cells.

The Caco-2 cell model was selected to study the effect of IL2 treatment on functional activity of P-gp transporter because Caco-2 cells express different ABC transporters and several functional cytokine receptors (Reinecker & Podolsky 1995; Gutmann et al 1999). The inhibitory action of IL2 was studied at different concentrations and for various exposure times. The effect of IL2 on the transporter function of P-gp was investigated by measuring the transepithelial transport of rhodamine-123 with and without P-gp specific inhibitors (ciclosporin A and GG918). In parallel, the transepithelial electrical resistance (TEER) of Caco-2 cells was measured. The level of MDR1 mRNA was also quantified following semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and compared with the level of MRP1 mRNA, another efflux transporter which belongs to the superfamily of ABC membrane transport proteins.

Materials and Methods

Chemicals

Human recombinant interleukin-2 (hr-IL2, Macrolin) was purchased from Chiron Laboratories (France). Ciclosporin, Trizol, rhodamine-123 and sodium phosphate were from Sigma-Aldrich Chemical Co. (France).

GG918 (*N*-{4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)-ethyl]-phenyl}-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide, an acridone carboxamide derivative, also known as GF120918) was a gift from GlaxoWelcome Laboratories (France). Ethanol and acetonitrile were purchased from Fisher (France).

[G-^{3}H]vincristine sulfate (2–10 Ci mmol^{-1}; 0.25 mCi mL^{-1}) was purchased from Amersham Pharmacia Biotech (England).

Caco-2 cells culture

Caco-2 cells were obtained from Dr Servin (INSERM U 510, Châtenay-Malabry, France). Cells of passage 65–75 were used in this study. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM-Glutamax I) supplemented with 15% decomplemented fetal calf serum, 1% non-essential amino acids, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. All these components were purchased from Gibco-BRL, Life Technologies (France). Cells were kept at 37°C in a 5% CO_2 atmosphere with 95% relative humidity. They were grown in tissue culture flasks or dishes and the medium was changed every two days. At 80–90% confluence, they were treated with 0.5% trypsin and 0.2% EDTA and seeded at a density of 50 000 cells/mL into plastic flasks or dishes. For mRNA expression experiments, fully differentiated Caco-2 cells were used, corresponding to 18–19 days of culture.

For transport studies, Caco-2 cells were plated at a density of 120 000 cells/mL on tissue-culture-treated Transwell inserts (12 mm diameter, 0.4 μm mean pore size polycarbonate membranes, 1 cm^2 area: Corning-Costar, France) and the medium was changed every day. Three weeks after reaching confluence, transepithelial electrical resistance (TEER) was measured using a Millicell ERS system (Millipore, France) before experiments to test the integrity of cell monolayer. P-gp transport data were recorded once TEER exceeded 200 Ω cm^2, corresponding to 21–22 days of culture.

Cytokine treatment

hr-IL2 was added to the medium at concentrations of 100, 1000 or 5000 IU mL^{-1} for 24, 48, 72 or 96 h. The medium was replaced each day until the day of data collection. All samples were treated on the same day (day 24). The TEER of Caco-2 cell monolayers incubated with or without IL2 was measured at the same time. To identify a possible toxic effect of different IL2 concentrations used, a test of cell viability using Trypan blue staining was performed. For all conditions, the proportion of dead cells did not exceed 5%.

Transport studies

Activity of P-gp was estimated on Caco-2 cell monolayers grown on Transwell filters for 24 days by determining unidirectional transport of rhodamine-123, a fluorescent substrate of P-gp. After removing medium, two washes with DMEM at 37°C were performed. Rhodamine-123 at a concentration of 5 μM in DMEM was added to the basolateral compartment and DMEM alone to the apical compartment. Transport studies were carried out at 37°C in a shaking microplate for 120 min. When ciclosporin A and GG918 (specific P-gp inhibitors) were used, they were added simultaneously with rhodamine-123 to both compartments at a concentration of 10 μM and 2.9 μM, respectively. These two compounds were dissolved in absolute ethanol. Control samples were prepared with the same percentage of solvent (0.6% ethanol). A sample (400 μL) in the apical compartment was used to determine rhodamine-123 concentrations by HPLC with spectrofluorimetric detection, using a Nova-Pak C_8 column (3.9 × 150 mm, particle size 4 μm; Waters, France). The mobile phase consisted of acetonitrile–0.05 M sodium phosphate

buffer, pH 2.83 (25:75 v/v). A sample of 10 μL (1:20 diluted in mobile phase) was injected into the system. When P-gp inhibitors were added under experimental conditions, 20 μL was injected directly. The flow rate was 1 mL min^{-1}. The HPLC system was equipped with a Shimadzu spectrofluorimeter (RF-551), set at wavelengths of 500 nm for excitation and 525 nm for emission. Data acquisition and analysis were performed with a Shimadzu integrator (C-R5A). The quantitative analysis was linear over the range 0.005–0.1 μmol L^{-1} and the limit of quantification was 1 nM. Reproducibility (n = 6, CVs) and repeatability (n = 20, CVs) were 13% and 6.5% at 0.025 μmol L^{-1}, 3.5% and 2% at 0.075 μmol L^{-1} of rhodamine-123, respectively. For experiments with [^{3}H]vincristine, the radiolabelled substrate was added to the basolateral compartment at a concentration of 100 nM in a total volume of 1.5 mL of DMEM (1.125 μCi/well). After 120 min of transport at 37°C, a 400-μL sample from the apical compartment was mixed in a vial with scintillation liquid for counting. The amount of [^{3}H]vincristine transported from the basolateral to the apical compartment was derived from d min^{-1} (disintegration per minute) data given by a liquid scintillation spectrophotometer (Tricarb 2900 TR, Hewlett Packard, France).

RNA isolation and analysis by reverse transcription polymerase chain reaction (semi-quantitative RT-PCR)

Total RNA was isolated from post-confluent, differentiated Caco-2 cell monolayers (18–19 days of culture) by using a total RNA isolation reagent, Trizol (Gibco-BRL Life Technologies, France) according to the protocol provided by the manufacturer.

Total RNA (5 μg) was reverse transcribed by Superscript II RNAse H (Gibco-BRL Life Technologies, France) using oligod$T_{(12-18)}$ as a primer. A semi-quantitative PCR protocol was applied to quantify and compare the expression of specific mRNAs in each sample (Murphy et al 1990).

Several serial dilutions of RNA reverse transcription products (1/40 to 1/20 480) were amplified by PCR to determine the exponential range of reaction. The primers were custom made at Oligo Express (Paris, France) and PCR was performed with a thermocycler (Perkin Elmer).

For the MDR1 mRNA detection, the following primers were used: 5′GTGCTGGTTGCTGCTTACAT3′ (sense) and 5′CCCAGTGAAAAATGTTGCCA3′ (antisense). For amplification of MRP1 cDNA, the following primer pairs were used: 5′GTGCTGGTTGCTGCTTACAT3′ (sense) and 5′CCCAGTGAAAAATGTTGCCA3′ (antisense).

For amplification of human glyceraldehyde phosphate dehydrogenase (GAPDH), known as a housekeeping gene, the following primers were used: 5′ACCACAGTCCATGCCATCAC3′ (sense) and 5′TCCCACCACCCTGTTGCTGTA3′ (antisense).

Each sample was amplified for 35 cycles of denaturation (94°C for 30 s, 60°C for 50 s, 72°C for 50 s). The reaction mixture contained 10 μL of the diluted cDNA template, 1.25 U Taq DNA polymerase (Roche Diagnostics, France), 5 μL 10 × PCR buffer (1.5 mM $MgCl_2$), 1 μL of dNTP reaction mixture (10 mM each), 15 pmol of each primer and water to a final volume of 50 μL. Each PCR included a 50-μL portion of the reaction mixture without cDNA as a negative control.

The PCR products were separated by electrophoresis in 1.5% agarose and gels were stained with ethidium bromide (2 μg mL^{-1}). Densitometric analysis of specific bands obtained with MDR1 or MRP1 primer sets were performed and optical densities were normalised to GAPDH mRNA band intensities.

Table 1 Influence of IL2 exposure on TEER of Caco-2 cells.

		24 h	48 h	72 h	96 h
Control		216±11	212±11	215±9	213±9
IL2 (IU mL^{-1})	100	212±7	214±6	203±7	ND
	1000	205±4	204±12	206±16	199±4
	5000	210±7	209±8	226±16	ND

Units of TEER values are Ω cm^2. Results are mean±s.d. For control, values are the means of 82, 60, 36 and 18 different wells for 24, 48, 72 or 96 h, respectively. For other TEER measurements, values are the mean of 24, 18, 12 or 6 different wells for 24, 48, 72 or 96 h of IL2 exposure, respectively. No statistical difference was observed with analysis of variance ($P > 0.05$). ND, not determined.

Data analysis

The results were expressed as mean±s.d. Statistical comparisons between control samples and those with specific inhibitors and transport experiments in Caco-2 cells treated with IL2 were conducted with Dunnett's test post-hoc following analysis of variance. For data presented in Table 1, control values were firstly subtracted from the measured values and secondly a two-way analysis of variance test was employed. Statistical significance was accepted as $P < 0.05$.

Results

Choice of a functional test for P-gp activity

Preliminary studies were performed to determine the time course of rhodamine-123 transport across Caco-2 monolayers when P-gp inhibitors were present or absent. For the first 30 min, transport was non-linear and data could not be recorded. Linear transport of rhodamine-123 was observed over the next 90 min from the basolateral compartment to the apical compartment (B → A). Transport in the opposite direction (A → B) was found to be 100-fold lower at 120 min (Figure 1). All data were collected at 120 min.

For the purpose of the study, a choice of substrate between [^{3}H]vincristine and rhodamine-123 was needed. To this end, two discriminatory factors were retained: the better transepithelial transport after 120 min and the

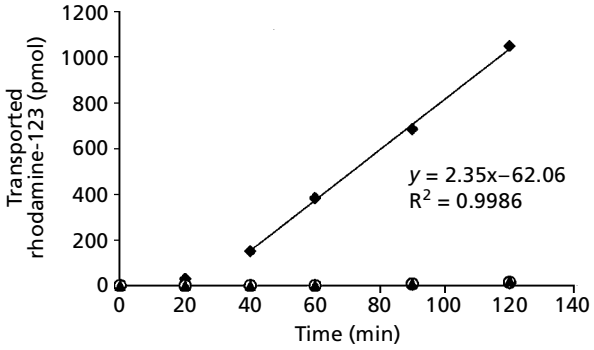

Figure 1 Transepithelial flux of rhodamine-123 across the Caco-2 cell monolayer. Typical curves of transepithelial flux of rhodamine-123. Caco-2 cells were grown on Transwell filters (4.71 cm^2) for 22 days. For each time, 40-μL samples were taken from the well and analysed by HPLC as described in Materials and Methods. Unidirectional rhodamine-123 transport was measured from A to B (○) and from B to A with (▲) or without (◆) ciclosporin A. Each point was the mean of three wells.

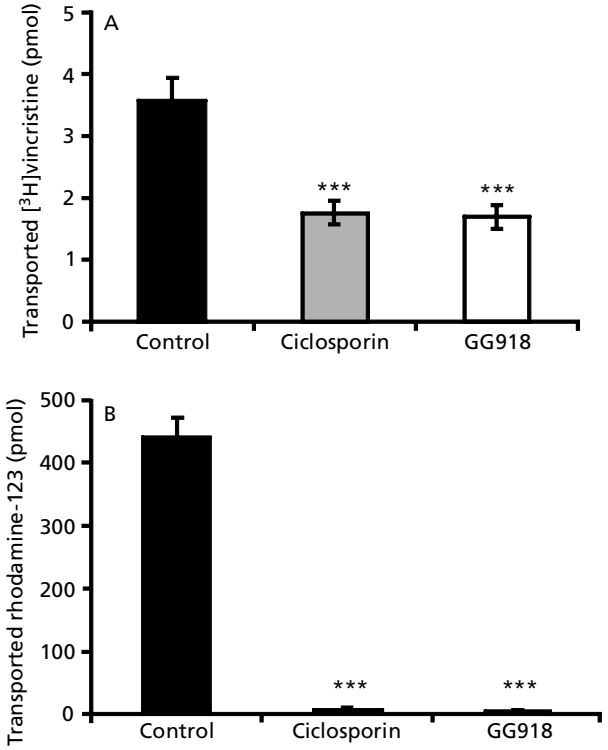

Figure 2 The effect of two specific P-gp inhibitors on the transepithelial transport of two different P-gp substrates. Caco-2 cells were grown on Transwell filters for 22 days and transepithelial transport from the basolateral to the apical compartment of either 100 nM of [^{3}H]vincristine (A) or 5 μM of rhodamine-123 (B) was determined after 120 min at 37°C. Ciclosporin or GG918 were added to both compartments at 10 μM and 2.9 μM, respectively. Data are means±s.d. (n = 6). ***$P < 0.001$, vs control cell samples.

greater inhibition of ciclosporin A and GG918 on transport activity for both substrates.

Figure 2A shows the transport of [^{3}H]vincristine. After 120 min, inhibition by ciclosporin A and GG918 was 50.6% and 52%, respectively. The amount of [^{3}H]vincristine transported across the epithelial layer in 120 min was derived from d min^{-1} to be ~ 10%. Results for the same experiment carried out with rhodamine-123 are reported in Figure 2B. As shown, 31.2% of rhodamine-123 was transported from B→A after 120 min. However, the two specific P-gp blockers, ciclosporin A and GG918, completely inhibited transport (98 and 99%, respectively; Figure 2B).

Comparison of these results revealed that rhodamine-123 was a more appropriate substrate for studying functional P-gp activity.

Time and dose effects of IL2 treatment on activity of intestinal P-gp transporter

Figure 3 shows the effect of different concentrations of IL2 on transepithelial rhodamine-123 transport after different periods of exposure.

When Caco-2 cells were exposed for 24 h to IL2 at its lowest concentration (100 IU mL^{-1}, corresponding to

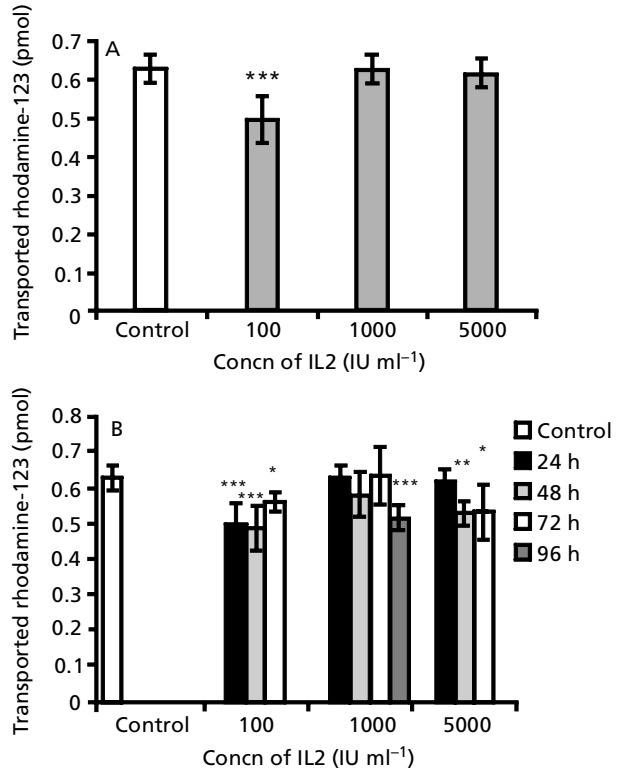

Figure 3 Time and dose effects of IL2 exposure on the intestinal P-gp transporter activity. Caco-2 cell monolayers grown on filters were exposed or unexposed (control) to hr-IL2 100, 1000 or 5000 IU mL^{-1} for 24, 48, 72 and 96 h. A. Total unidirectional transport (B → A) of rhodamine-123 was determined after 24 h of exposure to IL2 at different concentrations. B. Comparisons of IL2 time effects on P-gp function with different IL2 concentrations were made. Data are mean±s.d. (n = 6). Results were confirmed by at least two independent cytokine exposure experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, vs control cell samples.

6.1 ng mL^{-1}), a significant decrease (21%) in rhodamine-123 concentration was observed at 120 min (1.26 ± 0.03 μM vs 0.99 ± 0.05 μM, $P < 0.005$). After 24 h, no decrease in rhodamine-123 transport was observed at higher IL2 concentrations (1000 and 5000 IU mL^{-1}) (Figure 3A). The decrease in rhodamine-123 transport with 100 IU mL^{-1} IL2 was confirmed for 48 and 72 h exposure time (Figure 3B). Decrease in rhodamine-123 transport was delayed and observed only after 48 and 72 h of treatment at 5000 IU mL^{-1} or more at 1000 IU mL^{-1} IL2. The inhibitory power of ciclosporin A and GG918 was similar (98% and 99%, respectively) for all concentrations and exposure times of cytokine treatment in this study (data not shown).

Influence of IL2 on TEER of Caco-2 cells

No significant difference of TEER between control cell samples and Caco-2 cells treated with IL2 was measured (Table 1). This was obtained at different concentrations of IL2 (100, 1000 and 5000 IU mL^{-1}) and after different times of exposure to the cytokine (24, 48, 72 and 96 h).

Effect of IL2 on MDR1 and MRP1 mRNA expression

Semi-quantitative RT-PCR experiments using specific MDR1 primers were investigated to discover whether alterations of the P-gp transport function were correlated to a modification of MDR1 mRNA level. These experiments were carried out on control cells and IL2-treated cells at an intermediate concentration (1000 IU mL^{-1}) and for different durations of cell exposure (24, 48 and 72 h). Amplified specific bands stained with ethidium bromide (Figure 4) were analysed by densitometry and normalised to GAPDH. MDR1 mRNA expression was totally repressed after 72 h of IL2 treatment while the MRP1 mRNA level remained unchanged. This result with MRP1 mRNA confirms the specific effect of IL2 on P-gp.

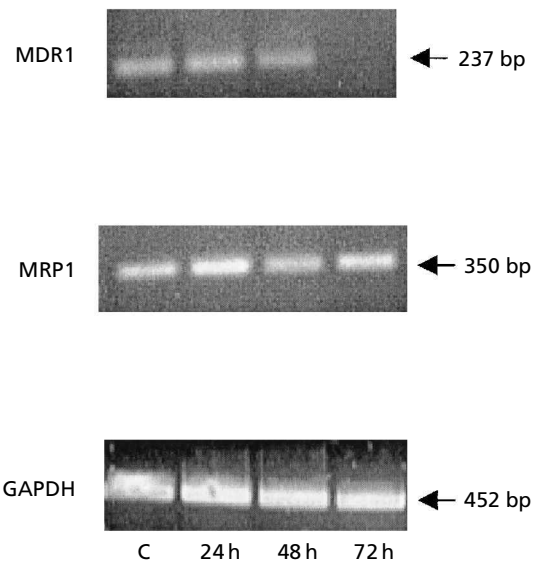

Figure 4 Effect of IL-2 on MDR1 and MRP1 mRNA expression in Caco-2 cells. Total RNA extracted from Caco-2 cells treated or untreated (control; C) with IL2 (1000 IU mL^{-1}) for 24, 48 and 72 h were reverse transcribed and then amplified with specific primers by semi-quantitative RT-PCR protocol. PCR products were separated by electrophoresis and gels were stained with ethidium bromide. Results of this experiment were confirmed twice independently.

Discussion

Our results demonstrate the ability of IL2 to decrease the efflux function of P-gp in Caco-2 cells, with rhodamine-123 as substrate and ciclosporin A or GG918 as specific P-gp inhibitors. The in-vitro Caco-2 cell experimental model was firstly modified to specifically study the efflux function of the P-gp transporter. The inhibitory effects of specific P-gp inhibitors (ciclosporin A and GG918) on transepithelial transport were compared for two different P-gp substrates ([^{3}H]vincristine and rhodamine-123) in differentiated Caco-2 cell monolayers. GG918 has been described as an efficient inhibitor of MDR1 P-gp without an effect on MRP1 and MRP2 transporters (Germann et al 1997; Evers et al 2000). Furthermore, GG918 was found to fully reverse multidrug resistance both in-vivo and in-vitro at 0.05–0.1 μM (Hyafil et al 1993). The maximum GG918 concentration without cytotoxic effect for a continuous exposure was found to be 10 μM (Evers et al 2000). In our experiments, a maximum inhibitory effect was observed at a GG918 concentration of 2.9 μM. No cytotoxic effect on Caco-2 cells was recorded.

With GG918 as a P-gp inhibitor and rhodamine-123 as a P-gp substrate, complete inhibition of the transport was observed, while this inhibition was partial in the [^{3}H] vincristine experiment (Figure 2A, B). Similar results were obtained with ciclosporin A (another reversal of MDR phenotype) as P-gp inhibitor.

We concluded that rhodamine-123 was exclusively transported by P-gp transporter and thus constitutes a good tool to investigate the efflux function of the P-gp transporter.

CYPs and P-gp transporter act in synergy to decrease the bioavailability of xenobiotic substrates. Several studies related to inflammation have already shown a decrease in their hepatic expression (Cantoni et al 1995; Piquette-Miller et al 1998). Furthermore, during mucosal immune response after intestinal inflammation, cytokine secretion, and particularly IL2, affects lamina propria and may also influence epithelial cell function (Reinecker & Podolsky 1995). Moreover, it is well documented that intestinal epithelial cells, including Caco-2 cells, express the IL2 receptor beta and common gamma chain (γc). Their association is necessary and sufficient for effective signal transduction (Nakamura et al 1994). Both act to connect the receptor complex to cytoplasmic signalling intermediates (Gaffen 2001). Signal transduction of IL2 receptors involves a rapid phosphorylation within 2–5 min of stimulation (Reinecker & Podolsky 1995).

Using Caco-2 cells, which have the advantage of expressing both functional P-gp transporter and IL2 receptor, the direct effect of IL2 cytokine on rhodamine-123 transport by P-gp was investigated. After 24 h of exposure to IL2 at the lowest concentration (100 IU mL^{-1}), a significant decrease in the transport of rhodamine-123 was measured whereas no significant effect at this time was obtained with IL2 at 1000 or 5000 IU mL^{-1} (Figure 3A). A minimum of 48 or 96 h of exposure was necessary to decrease P-gp activity with higher concentrations of IL2, 5000 IU mL^{-1} and 1000 IU mL^{-1} respectively. This decrease in functionality is in agreement with previous studies (Stein et al 1996). However, a maximal inhibitory effect was observed after 48 and 72 h of 100 IU mL^{-1} cytokine treatment, by these authors. Due to the specific inhibitory effect of GG918 on P-gp transport function, our results demonstrate that IL2 is able to reduce intestinal efflux function of P-gp. Moreover, a diminution of MDR1 mRNA level in Caco-2 cells exposed to IL2 for 72 h without modification of MRP1 mRNA level confirmed that the alterations of the P-gp transport activity observed were associated with a P-gp down-regulation. These results are in agreement with data obtained with HCT15 and HCT116 cells showing a diminution of MDR1 mRNA expression after 48 h of IL2 treatment at 100 IU mL^{-1} (Stein et al 1996).

Recently, Bonhomme-Faivre et al (2002), from our laboratory, have demonstrated a reduction in P-gp protein expression in the intestine of mice pre-treated with IL2. This decrease was associated with an enhancement of digoxin bioavailability (a P-gp substrate which was not metabolised) in mice.

Several cytokines, such as pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, have been found to decrease TEER values in Caco-2 cells, suggesting a possible effect of these cytokines on tight junction function and then paracellular permeability (Bertilsson et al 2001). In this study, incubation of Caco-2 cells with IL2 had no effect on measured TEER. This rules out a possible effect of the cytokine on the rhodamine-123 paracellular pathway (Table 1). IL2 has been found to increase vascular permeability leading to vascular leak syndrome (VLS) (Heslan et al 1991; Rafi et al 1998; Matsumoto et al 1999). Such findings underline a possible indirect effect of the cytokine on membrane fluidity. However, no data on epithelial cells have been published.

In conclusion, exposure of Caco-2 cells to IL2 induces diminution of P-gp expression and P-gp efflux function. This effect of IL2, observed both in-vivo and in-vitro, may constitute a new therapeutic strategy for enhancing the bioavailability or the efficiency of cytotoxic agents used in cancer treatment or protease inhibitors prescribed in AIDS treatment.

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