Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 45–50
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Permeability modulation of human intestinal Caco-2 cell monolayers by interferons

Hiroko Kawaguchi[a], Yukiko Akazawa[a], Yoshihiko Watanabe[b], Yoshinobu Takakura[a,*]

[a]Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan
[b]Department of Molecular Microbiology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

Received 24 September 2003; accepted in revised form 26 June 2004
Available online 7 October 2004

Abstract

We investigated the effects of interferon-β (IFN-β) and IFN-γ on the drug efflux activity of the human intestinal Caco-2 cell line, expressing the P-glycoprotein (P-gp) on the apical membrane. The cells grown on Transwell plates were pretreated with 1000 U/ml IFN-β, IFN-γ or a combination of both for 3 days, and then the transepithelial electrical resistance (TEER) and the vectorial transport of rhodamine-123 (Rho-123) across the cell monolayers were evaluated. Exposure to IFN-γ reduced substantially the TEER, but the effect of IFN-β was minimal? The apparent permeability of Rho-123 in both the basolateral-to-apical and apical-to-basolateral directions was significantly increased by IFN-γ but scarcely by IFN-β. The combination of IFN-γ and IFN-β showed similar effects to IFN-γ alone. Meanwhile, the cellular uptake of Rho-123 from the apical side was not affected by any IFN treatment. The uptake level was increased approximately three times in the presence of verapamil, a P-gp inhibitor, and the increased level was not affected by any IFN treatment, indicating that the efflux activity mediated by P-gp in the monolayers is not altered by these cytokines. Taken together, these results suggest that IFNs modulate the permeability of Caco-2 monolayer through effect on paracellular transport rather than effect on P-gp activity.

Keywords: P-glycoprotein; Transepithelial electrical resistance; Interferon-β; Interferon-γ; Rhodamine-123; Caco-2 cell

1. Introduction

Drug transporters play a vital role in the disposition and elimination of xenobiotics. In particular, P-glycoprotein (P-gp) is an important ATP-dependent membrane transporter which is expressed in many normal tissues, including liver, brain, kidney, adrenal gland and intestinal epithelial cells [1,2]. It is involved in the absorption, distribution and elimination of a variety of clinically important drugs [3,4]. Several studies using mdr1a knockout mice have demonstrated that P-gp expressed in the intestine plays an important role in limiting drug absorption and reducing bioavailability. For example, the oral bioavailability of P-gp substrates, paclitaxel and HIV-1 protease inhibitors is higher in mdr1a(−/−) mice than in mdr1a(+/+) mice [5,6].

P-gp is regulated by a number of substances, including xenobiotics, hormones [7,8] and cytokines [9,10]. Interferons (IFNs), which consist of type I IFN (IFN-α and IFN-β) and type II IFN (IFN-γ), are endogenous cytokines induced in a variety of inflammatory diseases and also used clinically as anticancer or antiviral agents [11,12]. However, the influence of IFNs on P-gp in the intestine is still unclear. The present study was designed to examine the effect of IFN-β and IFN-γ on drug transport or in vitro permeability in human intestinal Caco-2 cells using rhodamine-123 (Rho-123), a typical substrate for P-gp, since Caco-2 cell monolayers have been widely used as a model of small intestinal enterocyte layers in the studies of intestinal transport or permeability [13] and of P-gp expression [14].

Abbreviations: IFN, interferon; Rho-123, rhodamine-123; P-gp, P-glycoprotein; TEER, transepithelial electrical resistance.

* Corresponding author. Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. Tel.: +81 75 753 4615; fax: +81 75 753 4614.
E-mail address: takakura@pharm.kyoto-u.ac.jp (Y. Takakura).

0939-6411/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2004.06.012

2. Materials and methods

2.1. Materials

Nonessential amino acids cocktail (NEAA), L-glutamine, penicillin and streptomycin were obtained from Gibco BRL (Gaithersbug, MD). Rho-123 was purchased from Kanto Kagaku (Tokyo, Japan) and verapamil from Nacalai Tesque (Kyoto, Japan). Highly purified human IFN-β and IFN-γ were kindly donated by Toray Basic Research Laboratories (Kamakura, Japan) and Shionogi Research Laboratories (Osaka, Japan), respectively.

2.2. Cell culture and interferon treatment

Human colon adenocarcinoma Caco-2 cells (ATCC HTB37), obtained from the American Type Culture Collection (Rockville, MD), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l D-glucose, 1% NEAA, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% fetal bovine serum (FBS), in 5%-CO_2 at 37 °C, and were used between 36 and 47 passages. The medium was refreshed every 2 days. For efflux experiments, cells were seeded onto polyester filters (1 cm^2 area, 0.4 μm pore size) (Transwell plate; Corning Coster Co., Cambridge, MA) at a density of 1×10^5 cells/cm^2 and grown for 14–15 days. The volumes of the medium of the apical (insert) and basolateral (well) sides were 0.5 and 1.5 ml, respectively. The formation of functional epithelial monolayers was monitored by measuring the transepithelial electrical resistance (TEER) of cell monolayers with a Millicell-ERS meter (Millipore Corp., Bedford, MA). Cell monolayers were used when the net TEER exceeded 300 Ω(R) cm^2 (usually $\geq$300 R cm^2). To expose cell monolayers to IFN, the medium of the upper and/or lower chambers were replaced with medium containing 1000 U/ml IFN, being refreshed every day.

2.3. Transport and uptake studies

The 15–17 day-maintained Caco-2 monolayers on Transwell plates were used for transport and uptake experiments. The monolayers were, if necessary, treated with IFNs as mentioned above. Rho-123 (5 μM) in Hanks' balanced salt solutions (HBSS) buffered with 10 mM Hepes (pH 7.4) was applied either to the apical (luminal) or basolateral (serosal) side of the cell monolayers. To inhibit the P-gp activity, verapamil (100 μM) was added to both the basal and apical side solutions. At appropriate time intervals, aliquots were taken from the apical (0.1 ml) or the basolateral side (0.1 ml), and then replaced with equal volumes of pre-warmed Hepes-buffered HBSS. The drug contents in the samples were measured fluorometrically as previously described [15]. The apparent permeability coefficients (P_{app}) were estimated from the slope of the early linear portion of the time-course of drug transport across the cell monolayers according to the equation: $P_{app}=(dC/dt)/(AC_0)$, where A is the area of the membrane (cm^2), C_0 is the initial concentration (μM) of the solute in the donor chamber, and dC/dt is the permeability rate (μM/s) of the solute.

To evaluate the uptake level of Rho-123 via P-gp, the intracellular dye at the end of the apical-to-basolateral transport experiments (3 h) was extracted with 1 ml *n*-butanol following three washes with ice-cold HBSS. The extracts were then subjected to fluorometry to determine the steady-state Rho-123 uptake of the Caco-2 monolayers. The remaining *n*-butanol-extracted cells were solubilized with 1 ml of 2% sodium dodecyl sulfate (SDS) 0.1 M Tris–HCl (pH 7.4) per well to determine the protein content by modified Lowry method. The amounts of extracted Rho-123 were normalized by the protein contents.

2.4. Assay for nitrite/nitrate

Nitric oxide production was assessed by measuring the concentration of its stable oxidation products, nitrite and nitrate. Caco-2 cell monolayers were treated with IFNs for 3 days as mentioned above, and after 24 h from the last medium change, media were taken to measure nitric oxide contents using a NO_2^-/NO_3^- Assay Kit (DOJINDO, Japan).

3. Results

3.1. Effect of interferons on TEER of Caco-2 cell monolayers

At first, whether or not IFNs affect TEER of Caco-2 cell monolayers was examined for 3 days after exposure to IFN-β, IFN-γ or their combination at a concentration of 1000 IU/ml on the apical or basolateral surface of the cell monolayers. Basolateral treatment with IFN-γ caused gradual reduction of TEER (up to about 70% of the control value at 3 days), and IFN-β showed no alteration of the TEER level (Fig. 1); Effect of a combination of IFN-γ

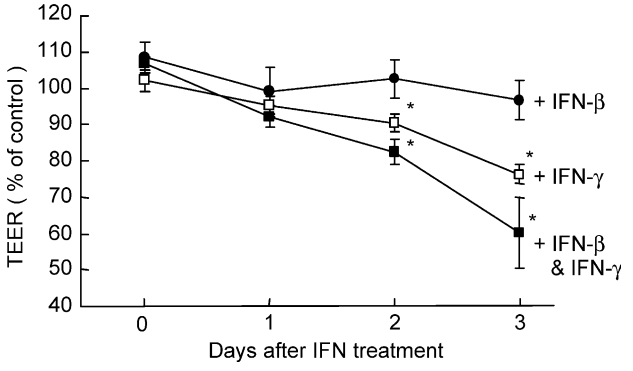

Fig. 1. Effect of IFNs on TEER of Caco-2 cell monolayers. Caco-2 cells were exposed to IFN-β (●), IFN-γ (□), or IFN-β and IFN-γ (■) at 1000 U/ml from basolateral sides. The values represent the means ± SD of three determinations and were analyzed by the Student's *t*-test. *$P<0.01$ vs control. The TEER values for control monolayers ranged from 350 to 440 Ω cm^2.

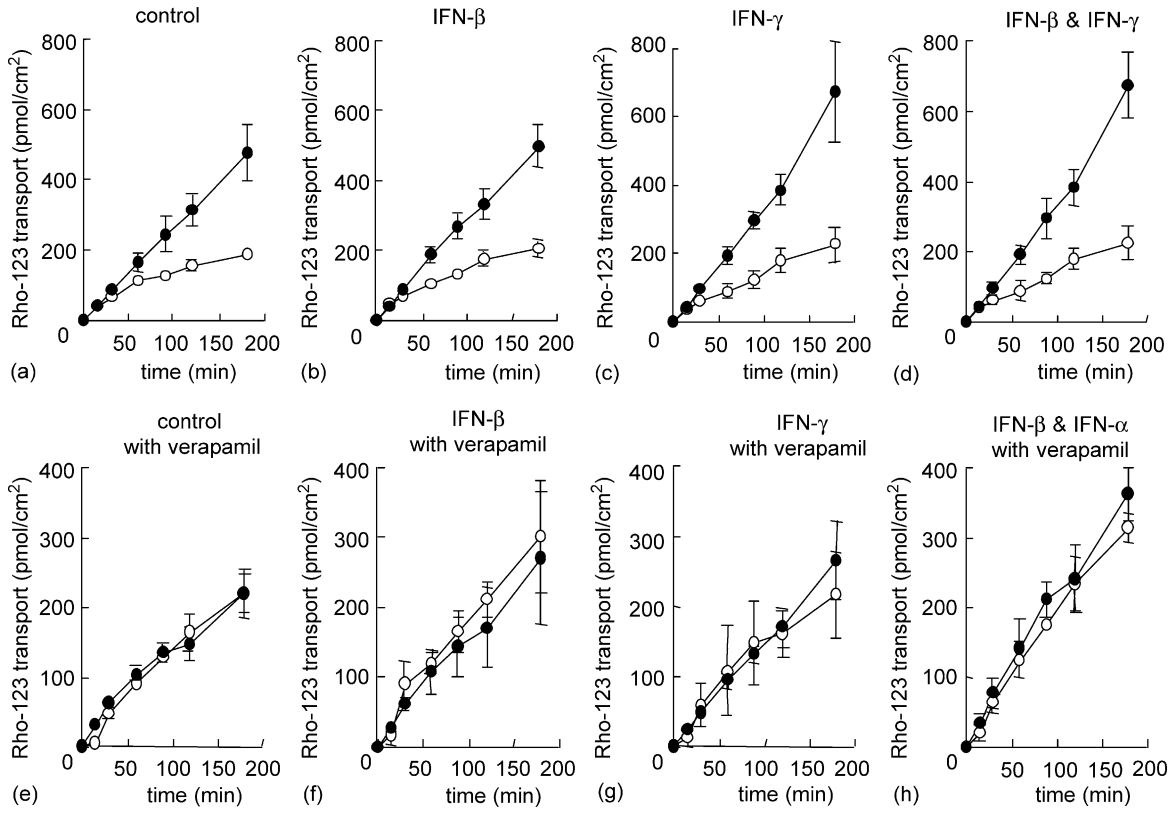

Fig. 2. Effect of IFNs on transepithelial transport of Rho-123 across Caco-2 cell monolayers. Cells were exposed to IFNs at 1000 U/ml for 3 days. Rho-123 (5 μM) was added to the basal or apical side of the monolayers in the absence (upper panel, a–d) or presence (lower panels, e–h) of 100 μM verapamil (both sides). After incubation for 15, 30, 60, 90, 120 and 180 min, aliquots of the medium on both sides were collected and the concentrations of Rho-123 were measured. The values represent the means ± SD of three determinants. ○ Apical-to-basal transport, ● basal-to-apical transport.

and IFN-β was slightly enhanced compared to IFN-γ alone. In any case of apical treatment with IFN, no significant change in TEER was observed (data not shown). These indicate that Caco-2 monolayers are polarized and sensitive to IFNs solely at their basolateral surfaces.

3.2. Effect of interferons on transepithelial transport of Rho-123 across Caco-2 cell monolayers

Fig. 2 shows the effects of IFNs on the transport of Rho-123 across the Caco-2 cell monolayers in the absence or presence of verapamil, an inhibitor of P-gp. In the control cell monolayers (panel a), the transport of Rho-123 in the basolateral-to-apical direction was greater than that in the apical-to-basolateral direction. Whereas treatment with IFN-β (panel b) did not significantly change the Rho-123 transport kinetics, exposure to IFN-γ caused increase of Rho-123 transport in either direction (panel c). Combination of IFN-β and IFN-γ (panel d) showed a similar effect to IFN-γ alone. In the presence of verapamil (panels e–h), preferential Rho-123 transport in the basolateral-to-apical direction disappeared and the transport was refractory for any IFN treatment, suggesting that P-gp is functionally expressed in the apical surfaces of Caco-2 cells. Table 1 summarizes the apparent permeability coefficients (P_{app}) calculated from these transport profiles for Rho-123.

Table 1
Effect on IFNs on permeability of Rho-123 in Caco-2 cell monolayers

Permeability mode	P-gp inhibitor	$P_{app} \times 10^6$ (cm/s)			
		Control	+IFN-β	+IFN-γ	+IFN-β and IFN-γ
A to B	None	3.29 ± 0.23	3.68 ± 0.34	4.10 ± 0.90*	4.04 ± 0.34*
B to A	None	8.81 ± 1.44	9.24 ± 0.47	12.18 ± 2.49*	13.12 ± 1.71*
A to B	+Verapamil	4.23 ± 0.69	5.47 ± 1.76	4.09 ± 1.07	5.99 ± 0.37
B to A	+Verapamil	3.84 ± 0.64	4.80 ± 1.81	4.83 ± 0.97	6.65 ± 0.70

Data represent means ± SD of three experimental determinations. A and B represent 'apical' and 'basolateral' sides, respectively. *$P < 0.05$ vs P_{app} for the same direction under the control in the same row.

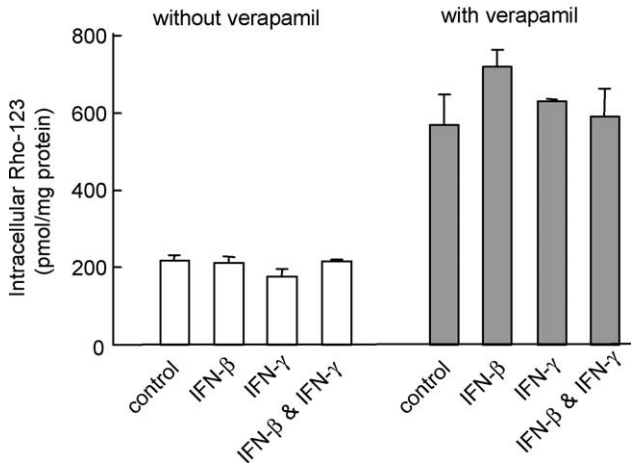

Fig. 3. Intracellular uptake of Rho-123 in Caco-2 cell monolayers. The monolayers were treated with or without IFNs at 1000 U/ml for 3 days. Rho-123 (5 μM) was added to the apical side of monolayers in the absence or presence of 100 μM verapamil (both sides). After incubation for 3 h, the intracellular Rho-123 of each well was extracted with butanol and its concentration was measured. The values, normalized by protein content, are the means ± SD of three experiments.

3.3. Effect of interferons on Rho-123 uptake in Caco-2 cells

To examine whether IFNs affect P-gp transport activity in the Caco-2 monolayers, steady-state levels of Rho-123 uptake from the apical side were measured. Any IFN treatment did not affect intracellular levels of Rho-123 of Caco-2 cells, as shown in Fig. 3 (left half). Addition of verapamil led the uptakes to a three-fold augmented level (Fig. 3, right half), and the augmented levels were almost the same regardless of IFN treatment. These results suggest that the Caco-2 cells express functional P-gp on the apical surfaces in harmony with the kinetics data (Fig. 2 and Table 1) and indicate that both types of IFN do not affect the Rho-123 efflux mediated by P-gp on the apical cell membrane.

3.4. Effect of interferons on accumulation of nitrite/nitrate in Caco-2 cells

To examine the involvement of nitric oxide as a mediator of IFN-γ-induced hyperpermeability, the amount of nitrite/nitrate in the media after IFN treatment was measured (Fig. 4). Whereas no significant differences were seen in the concentrations of NO in the media of IFN-β treated cells, the NO production was significantly increased by IFN-γ treatment. This increase was similar to that in the media of cells treated with both types of IFN.

4. Discussion

In the present study, we have examined the effect of IFN-β and IFN-γ on drug transport across human intestinal Caco-2 cell monolayers. Fifteen- to 17-day-maintained cell monolayers were used, since such a long-term culture allows the cells to express P-gp at a maximal level [16]. The cell monolayers were exposed to IFNs at a concentration of 1000 U/ml of either type of IFN for 3 consecutive days, as this IFN concentration and treatment duration appear to be enough to induce maximal biological responses in cultured cells [17]. In fact, IFN-γ treatment resulted in a significant reduction in the TEER value, which depended on the application side, apical or basolateral: basolateral application of IFN-γ caused a significant reduction of the TEER value, while its apical application did not. On the other hand, IFN-β alone had no significant effect on the TEER but enhanced slightly the effect of IFN-γ. These suggest polarized expression of the functional receptors for IFN-γ and IFN-β on the basolateral surface of the Caco-2 cells. Our observations of IFN effects on Caco-2 cells agree with the previous reports in another human intestinal cell line, T84: only the basolateral surface of T84 monolayers was responsive to IFN-γ, while IFN-αγβ, another type I IFN, displayed no effect [17]. T84 cells also express IL-4 receptors on the basolateral surfaces and IL-4 treatment reduces the epithelial barrier function in a time- and dose-dependent manner [18]. In addition, basolateral surface expression of IFN-γ receptors was seen also in a murine squamous epithelial cell line [19].

Our data demonstrate that while IFNs do not affect the Rho-123 transport activity of P-gp in Caco-2 cells, they can affect permeability of the cell monolayers by modulating paracellular transport potential of the monolayers. Effects of cytokines on the expression and activity of P-gp have been examined in a variety of cultured cells. In rat primary hepatocytes, TNF-α [9] has been shown to induce the expression of P-gp at both the transcription and translation levels and to enhance its transport activity, while IL-6

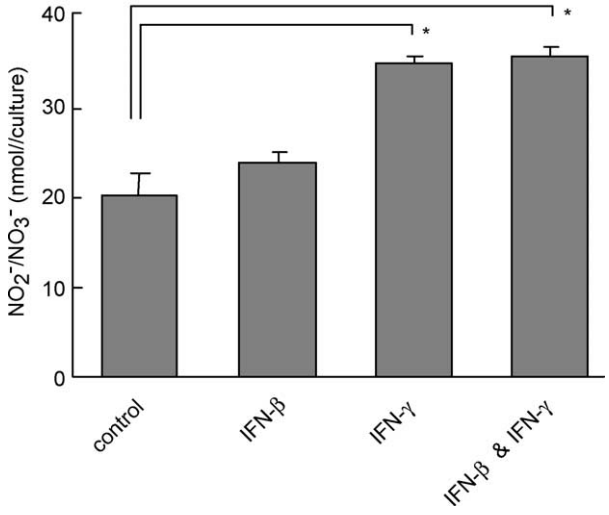

Fig. 4. Accumulation of nitrite/nitrate in supernatants of Caco-2 cell monolayers. The cell monolayers were treated with or without IFNs at 1000 U/ml for 3 days. The values represent the means ± SD of three determinations and were analyzed by the Student's *t*-test. $*P < 0.05$ vs control.

and IL-1β have displayed suppressive effects on the expression and activity of P-gp [8]. In human colon carcinoma cell lines (LoVo, HT115, SW480, and LS174T), IFN-γ, IL-2 and TNF-α caused a reduction in *mdr*1 gene expression after 48 or 72 h treatment [10]. By contrast, P-gp expression in human myeloma cells was unaffected by cytokines, such as IFN-α, IFN-γ, and TNF-α, but was reduced by IL-2 [20]. IFN-α induced the expression of P-gp in multidrug-resistant ChR C5 cells, but enhanced the cellular uptake and cytotoxicity of doxorubicin, a P-gp substrate, in the presence of verapamil [21]. On the other hand, IFN-α increased adriamycin-induced cytotoxicity without any significant change of P-gp expression in a multidrug resistant cell line [22]. In human macrophages, IFN-γ up-regulated P-gp expression and its activity in a dose- and time-dependent manner [23]. Up-regulation by IFN-γ of P-gp expression was also observed in rat primary hepatocytes [24]. This evidence, taken together, indicates that P-gp expression and its activity are modulated by some inflammatory cytokines and the responses differ from one cell type to another.

In this study, IFN-γ caused reduction in the TEER of Caco-2 cell monolayers, and modulated apparent permeability of Rho-123 across the monolayers without direct effect on the P-gp activity in the cells, implying that IFN-γ augments paracellular drug transport of the cells in agreement with the previous report [25]. Since the inducible nitric oxide synthase (iNOs) is IFN-γ-inducible [26], IFN-γ-induced hyperpermeability of epithelial cells may correlate with IFN-γ-induced nitric oxide (NO). Eventually, IFN-γ up-regulated the biosynthesis of NO in Caco-2 cells (Fig. 4) also as reported [27]. Thus, NOs may be an important modulator of epithelial cell permeability, although the underlying mechanisms require further investigation.

In conclusion, our results indicate that P-gp-mediated drug transport activity in Caco-2 cells is itself not influenced by IFN-β or IFN-γ treatment. However, the expression and function of intestinal P-gp in vivo may be modulated indirectly by IFNs because of a large variety of biological activities of the cytokines [26]. Thus, the findings in the present study provide useful information about the effects of clinically important cytokines on human intestinal P-gp.

References

[1] F. Thiebaut, T. Tsuruo, H. Hamada, M.M. Gottesman, I. Pastan, M.C. Willingham, Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues, Proc. Natl. Acad. Sci. USA 84 (1987) 7735–7738.

[2] M.M. Gottesman, I. Pastan, Biochemistry of multidrug resistance mediated by the multidrug transporter, Annu. Rev. Biochem. 62 (1993) 385–427.

[3] M. Yamazaki, H. Suzuki, Y. Sugiyama, Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics, Pharm. Res. 13 (1996) 497–513.

[4] M.F. Fromm, P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs, Int. J. Clin. Pharmacol. Ther. 38 (2000) 69–74.

[5] R.B. Kim, M.F. Fromm, C. Wandel, B. Leake, A.J. Wood, D.M. Roden, G.R. Wilkinson, The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors, J. Clin. Invest. 101 (1998) 289–294.

[6] A. Sparreboom, J. van Asperen, U. Mayer, A.H. Schinkel, J.W. Smit, D.K. Meijer, P. Borst, W.J. Nooijen, J.H. Beijnen, O. van Tellingen, Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine, Proc. Natl. Acad. Sci. USA 94 (1997) 2031–2035.

[7] M. Sukhai, M. Piquette-Miller, Regulation of the multidrug resistance genes by stress signals, J. Pharm. Pharm. Sci. 3 (2000) 268–280.

[8] M. Sukhai, A. Yong, J. Kalitsky, M. Piquette-Miller, Inflammation and interleukin-6 mediate reductions in the hepatic expression and transcription of the mdr1a and mdr1b genes, Mol. Cell. Biol. Res. Commun. 4 (2000) 248–256.

[9] K.I. Hirsch-Ernst, C. Ziemann, H. Foth, D. Kozian, C. Schmitz-Salue, G.F. Kahl, Induction of mdr1b mRNA and P-glycoprotein expression by tumor necrosis factor alpha in primary rat hepatocyte cultures, J. Cell. Physiol. 176 (1998) 506–515.

[10] W. Walther, U. Stein, Influence of cytokines on mdr1 expression in human colon carcinoma cell lines: increased cytotoxicity of MDR relevant drugs, J. Cancer Res. Clin. Oncol. 120 (1994) 471–478.

[11] S. Baron, S.K. Tyring, W.R. Fleischmann, D.H. Coppenhaver, D.W. Niesel, G.R. Klimpel, G.J. Stanton, T.K. Hughes, The interferons: mechanisms of action and clinical applications, J. Am. Med. Assoc. 266 (1991) 1375–1383.

[12] J. Heathcote, Antiviral therapy for patients with chronic hepatitis C, Semin. Liver Dis. 20 (2000) 185–199.

[13] P. Artursson, R.T. Borchardt, Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond, Pharm. Res. 14 (1997) 1655–1658.

[14] H. Gutmann, G. Fricker, M. Torok, S. Michael, C. Beglinger, J. Drewe, Evidence for different ABC-transporters in Caco-2 cells modulating drug uptake, Pharm. Res. 16 (1999) 402–407.

[15] K.K. Nadakavukaren, J.J. Nadakavukaren, L.B. Chen, Increased rhodamine 123 uptake by carcinoma cells, Cancer Res. 45 (1985) 6093–6099.

[16] J. Hunter, M.A. Jepson, T. Tsuruo, N.L. Simmons, B.H. Hirst, Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators, J. Biol. Chem. 268 (1993) 14991–14997.

[17] R.B. Adams, S.M. Planchon, J.K. Roche, IFN-gamma modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding, J. Immunol. 150 (1993) 2356–2363.

[18] S.P. Colgan, M.B. Resnick, C.A. Parkos, C. Delp-Archer, D. McGuirk, A.E. Bacarra, P.F. Weller, J.L. Madara, IL-4 directly modulates function of a model human intestinal epithelium, J. Immunol. 153 (1994) 2122–2129.

[19] K. Nakanishi, Y. Watanabe, M. Maruyama, F. Yamashita, Y. Takakura, M. Hashida, Secretion polarity of interferon (IFN)-β in epithelial cell lines, Arch. Biochem. Biophys. 402 (2002) 201–207.

[20] E.G. Schuetz, W.T. Beck, J.D. Schuetz, Modulators and substrates of P-glycoprotein and cytochrome P450 3A coordinately up-regulate these proteins in human colon carcinoma cells, Mol. Pharmacol. 49 (1996) 311–318.

[21] Y. Kang, R.R. Perry, Effect of alpha-interferon on P-glycoprotein expression and function and on verapamil modulation of doxorubicin resistance, Cancer Res. 54 (1994) 2952–2958.

[22] S. Scala, R. Pacelli, R.V. Iaffaioli, N. Normanno, S. Pepe, G. Frasci, G. Genua, T. Tsuruo, P. Tagliaferri, A.R. Bianco, Reversal of adriamycin resistance by recombinant alpha-interferon in multidrug-resistant human colon carcinoma LoVo-doxorubicin cells, Cancer Res. 51 (1991) 4898–4902.

[23] P. Puddu, S. Fais, F. Luciani, G. Gherardi, M.L. Dupuis, G. Romagnoli, C. Ramoni, M. Cianfriglia, S. Gessani, Interferon-gamma up-regulates expression and activity of P-glycoprotein in human peripheral blood monocyte-derived macrophages, Lab. Invest. 79 (1999) 1299–1309.

[24] Y. Akazawa, H. Kawaguchi, M. Funahashi, Y. Watanabe, K. Yamaoka, M. Hashida, Y. Takakura, Effect of interferons on P-glycoprotein-mediated rhodamine-123 efflux in cultured rat hepatocytes, J. Pharm. Sci. 91 (2002) 2110–2115.

[25] S.V. Walsh, A.M. Hopkins, A. Nusrat, Modulation of tight junction structure and function by cytokines, Adv. Drug Deliv. Rev. 41 (2000) 303–313.

[26] G. Stark, I.M. Kerr, B.R.G. Williams, R.H. Silverman, D.R. Schreiber, How cells respond interferons, Annu. Rev. Biochem. 67 (1998) 227–264.

[27] A.M. Chavez, M.J. Morin, N. Unno, M.P. Fink, R.A. Hodin, Acquired interferon gamma responsiveness during Caco-2 cell differentiation: effects on iNOS gene expression, Gut 44 (1999) 659–665.