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Induction of P-glycoprotein expression and activity by ritonavir in bovine brain microvessel endothelial cells

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Abstract

Extended treatment with human immunodeficiency virus (HIV) protease inhibitors (HPIs) is standard in HIV/AIDS therapy. While these drugs have helped decrease the overall incidence of AIDS defining illnesses, the relative prevalence of HIV/AIDS dementia has increased. HPIs may cause induction of blood–brain barrier (BBB) drug transporters (P-glycoprotein; P-gp) and thereby limit entry of HPIs into brain tissue, increasing the probability that the brain could become an HIV sanctuary site. Using bovine brain microvessel endothelial cells (BMEC) as an in-vitro model of the BBB, the potential for the HIV protease inhibitor ritonavir to cause induction of P-gp activity and expression was examined. BMEC were isolated from fresh cow brain by enzymatic digest and density centrifugation. Primary culture BMEC were co-incubated with ritonavir or vehicle control for 120 h. Quantitative drug accumulation of rhodamine 123 (Rh123) and fluorescence microscopy were used as measures of P-gp activity. P-gp expression was assessed using quantitative Western blotting. Ritonavir decreased Rh123 cell accumulation and increased P-gp immunoreactive protein in a concentration-dependent manner. Fluorescent microscopy mirrored Rh123 quantitative studies. In BMEC pretreated with 30 μM ritonavir, Rh123 accumulation was decreased 40% and immunoreactive P-gp protein increased 2-fold. Collectively, a strong correlation between decreased Rh123 BMEC accumulation and increased P-gp immunoreactive protein was observed (Spearman $r^2 = 0.77$, $P < 0.0001$). Thus extended exposure of BMEC to ritonavir caused a concentration-dependent increase in P-gp activity and expression. Similar findings may occur at the clinical level with prolonged HIV protease inhibitor use, giving insight into the central nervous system as an HIV sanctuary site and eventual development of HIV dementia.

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Introduction

Combination pharmacotherapy, including highly active antiretroviral therapy (HAART), has changed the long-term prognosis and quality of life for human immunodeficiency virus (HIV)/AIDS patients. Use of the HIV protease inhibitors (HPIs) in combination with other anti-HIV medication is effective in lowering HIV RNA in plasma to below detectable levels (Casado et al 2001; Hammer et al 2006). However, traditional HPIs used in HAART have low or variable oral bioavailability (Williams & Sinko 1999) as well as poor central nervous system (CNS) penetration (Schinkel 1999). This is, in part, due to drug transport activity of P-glycoprotein (P-gp; ABCB1) in the intestine and blood–brain barrier (BBB). CNS uptake of HPIs is also limited for other reasons, including CNS microvasculature endothelial tight junctions, and the tendency for HPIs to have high plasma protein binding. Nonetheless, drug transporters play an important role in drug exclusion from the CNS. Brain concentrations of HPIs were 7- to 36-fold higher in mdr1a(−/−) mice after intravenous injection compared with mdr (+/+) animals (Kim et al 1998). Furthermore, pharmacodynamic studies with the P-gp substrate/opioid loperamide (Sadeque et al 2000) and positron emission studies with the P-gp substrate [^{11}C]verapamil (Sasongko et al 2005) have demonstrated that P-gp inhibition at the level of the BBB can have clinical effects. Ritonavir is often added to HIV therapy combinations, in part to exploit its modulating effects on pharmacokinetics (Hsu et al 1998a; Flexner 2000; Gerber 2000; Magnum & Graham 2001). Being a moderate P-gp inhibitor (Profit et al 1999; Washington et al 1998, Perloff et al 2003) and a potent cytochrome P450 3A (CYP3A) inhibitor (Greenblatt et al 2000a, b; Hsu et al 1998a; von

Moltke et al 1998; Culm-Merdek et al 2006), ritonavir acutely increases the bioavailability of other co-administered HPIs, including saquinavir, amprenavir, lopinavir, and indinavir (Hsu et al 1998bc; Merry et al 1997; Sadler et al 2001; Sham et al 1998). CNS uptake of indinavir is enhanced with acute co-administration of ritonavir (van Praag et al 2000). However, previous studies have also suggested drug transporter induction with extended exposure to ritonavir. In rodent studies, repeated administration of ritonavir reduced amprenavir brain concentrations (Polli et al 1999) and increased BBB expression of P-gp (Perloff et al 2004). Direct evidence that extended exposure to ritonavir causes intestinal P-gp induction has been shown in cell culture and rodent studies (Perloff et al 2001, 2004). In human studies, co-administration of ritonavir greatly increased systemic availability of saquinavir (Merry et al 1997; Hsu et al 1998b). With extended exposure to ritonavir, systemic availability of saquinavir decreased, but still remained high (Gisolf et al 2000). This suggested the possibility of enteric P-gp induction, although net inhibition still predominated. In other human studies, enteric and renal P-gp inhibition appeared to be the dominant effect of ritonavir with extended exposure (Ding et al 2004; Penzak et al 2004).

While HAART has dramatically decreased the overall incidence of AIDS-defining illnesses (ADIs), the relative proportion of HIV/AIDS dementia has increased (50% in the case of AIDS dementia) at higher CD4+ cell counts, perhaps reflecting prolonged HIV exposure in the CNS sanctuary site, despite HAART (Dore et al 1999; Sacktor et al 2001). The current study used bovine brain microvessel endothelial cells (BMEC) as an in-vitro model to determine if chronic exposure to ritonavir can induce P-gp expression and activity at the level of the BBB.

Materials and Methods

Chemicals and antibodies

Ritonavir standard was kindly supplied by Abbott Laboratories (N. Chicago, IL). Rhodamine 123 (Rh123), verapamil, heparin, dextran-70, Percoll and trypan blue were obtained from Sigma Chemical Co. (St Louis, MO). Polymyxin B and 4(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from ICN Biomedicals (Costa Mesa, CA). Minimal essential media (MEM), F-12 Ham, calf serum, penicillin, streptomycin, amphotericin B, dispase, and collagenase were obtained from GibcoBRL (Rockville, MD). C219 monoclonal antibody to human P-gp was purchased from Signet Co. (Dedham, MA).

Primary bovine brain microvessel endothelial cells (BMEC) isolation and growth

Acquisition of discarded animal tissue samples for research purposes was not required to undergo review by the Animal Research Committee. Bovine brains were obtained from a local slaughter house, placed in sterile media (MEM with 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B) on ice within 30 min of death and transferred to the laboratory within 1 h. BMEC were purified from bovine cortex gray matter as described previously (Audus & Borchardt 1987; Dallaire et al 1991; Tsuji et al 1992; Jette et al 1993). Briefly, 100 g cortex was minced and digested in 0.8% dispase at 37°C, with shaking, for 2 h. The digest was centrifuged (1000 *g*) and the pellet resuspended in 15% dextran-70. The homogenate was then centrifuged (5800 *g*) for 10 min. The crude microvessel pellet was incubated in 0.1% collagenase–dispase solution at 37°C, with shaking, for 5 h. Crude microvessels were then centrifuged (1000 *g*) for 10 min and the pellet was resuspended in media and layered over a 50% Percoll gradient. After centrifugation (1000 *g*), cell debris (surface layer), red blood cells (pellet), and microvessels (interphase) were separated. Microvessels were collected, washed with media, and transferred to primary cell culture. Cell culture media consisted of 45% MEM, 45% F-12 Ham, 10% calf serum, 25 mM HEPES sodium salt, 100 μg mL^{-1} heparin, 13 mM sodium bicarbonate, 100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2.5 μg mL^{-1} amphotericin B, and 50 μg mL^{-1} polymyxin B (pH to 7.4 with sodium hydroxide). Polymyxin B was removed from the media after three days of culturing. Cells were incubated at 37°C in a humidified chamber with 5% CO_2 and the media was changed every two days until the initiation of experiments.

BMEC P-gp induction studies with ritonavir

BMEC were plated in collagen coated 6-well plates and grown to a density of 50% confluence. At the initiation of induction experiments, 0.5% dimethylsulfoxide (DMSO) was included in the media to ensure drug dissolution. Cells were incubated with increasing concentrations of ritonavir (3, 10, 30 μM) or 0.5% DMSO alone (baseline control) for 120 h (n = 3), at which time confluence was 100%. Pilot studies at 72 h for quantitative activity and expression experiments were very similar to pilot studies at 120 h. The time point of 120 h was chosen to ensure 100% confluence for qualitative microscopy studies; this allowed activity, expression, and microscopy experiments to all be done in the same culture well. Cells were then washed five times with warm (37°C) media (MEM including 0.5% DMSO and 10% calf serum) preceding Rh123 accumulation studies. Each well was used for both Rh123 accumulation studies and subsequent Western blotting.

Drug accumulation studies

BMEC cells were pre-incubated for 30 min with 100 μM verapamil or media (0.5% DMSO) alone. The acute verapamil exposure condition served as an additional control by reversing any P-gp activity. Rh123 solution was then added to each well until a final concentration of 10 μM Rh123 and 1% methanol was reached. After 60-min incubation, the cells were thoroughly washed with warm media five times. Minutes after the final wash, BMEC cell monolayers were analysed by epi-fluorescence microscopy using a Nikon Optishot microscope. Rh123 was visualized using an FITC filter set (ex: 450–490 nm, em: 510–530 nm). Images were captured using a Photometric Quantix Digital Camera and V++ software (Digital Optics, Auckland, New Zealand). Incandescent images of the same

field were also taken to demonstrate similar cell density in the various conditions. Immediately after microscopy photos were taken, cells were solubilized with 0.5% deoxycholate and 1% Triton X. The supernatant from each well was then analysed for Rh123 fluorescence, total cell protein, and P-gp immunoreactive protein.

Western blotting

Western blot procedures were similar to those described by Perloff et al (2001). A 6-μg sample BMEC was loaded into each well. Highly induced/concentrated LS-180 V cell preparations (Perloff et al 2001) were used to generate a relative standard curve at various known total protein concentrations (band signal was linear over a 20-fold concentration range). The calibration curve allowed for comparison of relative P-gp content among samples. Sample protein was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (100 V constant) on a 4–15% gradient polyacrylamide gel at 4°C for 2 h. Samples were then transferred to PVDF membrane for 1.5 h at 100 V in 25 mM Tris buffer (pH = 7.8)/20% methanol. Membranes were immunoblotted using monoclonal mouse anti-human MDR1 (C219, 1:1000) for 24 h. Blots were then probed with the secondary antibody, sheep anti-mouse Ig-horseradish peroxidase. After incubation with secondary antibody, Super Signal Cl-HRP Substrate System (Pierce, Rockford, IL) was used to activate the HRP signal. Blots were then exposed to a cold camera imaging system (Kodak Image Station 440, Kodak Imaging, New Haven, CT). Quantification of protein was completed via computer image analysis with Kodak image software. A standard curve of sum pixel density vs μg LS180 V cell preparation was created and fit to a linear function.

Rh123 fluorometric analysis

Rh123 was quantified with fluorometric analysis at 500 nm (excitation) and 550 nm (emission) using a Perkin Elmer LS50B luminescence spectrophotometer. Picomoles of Rh123 in samples were determined based on calibration curves constructed from a series of standards.

Statistics

In concentration–response experiments (drug accumulation and Western blotting), values are presented as the mean of three individual trials (± s.d.) using three sister culture plates (n = 3); comparison among groups with their respective vehicle controls was done using analysis of variance followed by Dunnett's test.

Results

Extended exposure to ritonavir (120 h) decreased cell accumulation of Rh123 in BMEC compared with the DMSO vehicle controls in a concentration-dependent manner (Figure 1A). The cell accumulation of Rh123 was decreased more than 40% when cells were pretreated with 30 μM ritonavir. Differences in cell accumulation of Rh123 could be eliminated/reversed

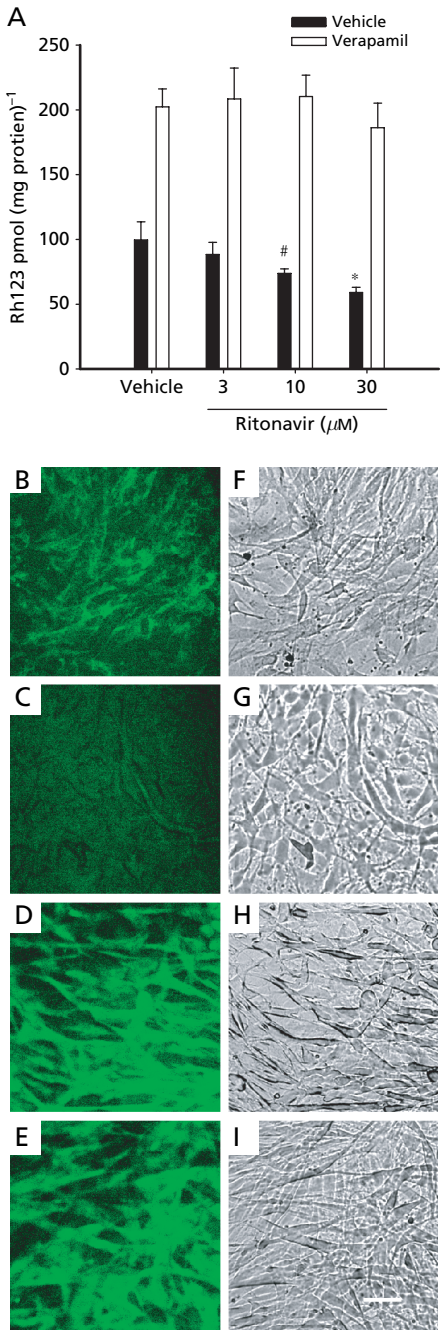

Figure 1 BMEC were treated with 3, 10, and 30 μM ritonavir or 0.5% DMSO vehicle. Pre-incubated (120 h) cells were washed, then incubated with 10 μM Rh123 (1 h), washed, lysed, and analysed for intracellular Rh123. Inhibition controls used 100 μM verapamil acutely. The bars (A) represent the mean (n = 3) ± s.d. Significant differences from the corresponding DMSO control are indicated by * ($P < 0.01$) or # ($P < 0.05$) as determined by analysis of variance followed by Dunnett's post-hoc test. Rh123 cellular accumulation was visualized by epi-fluorescence microscopy using an FITC filter set (B, C). Incandescent light images of the same field demonstrate similar cell density (F, G). After incubation (see Materials and Methods) with 10 μM Rh123 for 1 h, cells were washed, and digital images were taken at 100× magnification within minutes. Control inhibition experiments used 100 μM verapamil acutely (D, E, H, I). Representative fields are shown. Scale bar = 100 μm.

with 100 μM verapamil. Furthermore, acute exposure to 100 μM verapamil caused a 2-fold increase in Rh123 cell accumulation in DMSO vehicle-treated control cells, consistent with substantial baseline P-gp-mediated transport activity of Rh123 even without ritonavir induction.

Fluorescence microscopy of BMEC cells showed an accumulation of Rh123 in the cytoplasm, and modest concentration in the organelles of the 0.5% DMSO pretreated cells (Figure 1B), while ritonavir-pretreated cells showed little diffuse Rh123 accumulation (Figure 1C). Upon acute exposure to 100 μM verapamil, Rh123 intracellular concentration was greatly increased, with intense fluorescence signal throughout the cell (Figure 1D, E). Incandescent images demonstrate similar cell density in each field (Figure 1F–I).

Extended exposure to ritonavir (120 h) resulted in a concentration-dependent induction of P-gp immunoreactive protein in BMEC compared with the DMSO vehicle controls (Figure 2A, B). Western blot quantification (n = 3, mean ± s.d.) showed a 2.0 ± 0.10-fold induction at 30 μM ritonavir (Figure 2B). The effect of ritonavir on P-gp expression was consistent with previous studies (Perloff et al 2001, 2004). Total cell protein and trypan blue cell exclusion was similar in all experimental conditions, with no trypan uptake, verifying cell viability. Western blotting of BMEC P-gp required only a 6-μg sample, and so direct correlation of Rh123 cell exclusion and P-gp immunoreactive protein was possible for individual samples. A strong correlation between increased P-gp immunoreactive protein and decreased cell accumulation of Rh123 was seen (Figure 2C, Spearman rank order correlation, $r^2 = 0.77$, $P < 0.0001$).

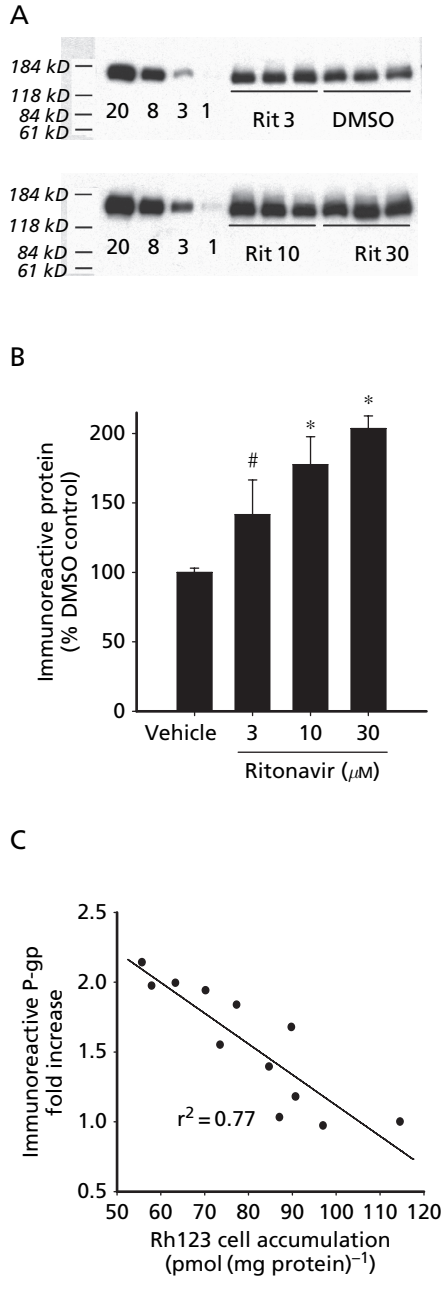

Figure 2 BMEC were incubated with vehicle alone (0.5% DMSO) or ritonavir (Rit 3, Rit 10, Rit 30 μM). Highly induced/concentrated LS-180 V cell preparations were used to generate a relative standard curve (total protein 1, 3, 8, 20 μg). Qualitative Western blotting of immunoreactive P-gp demonstrated a ritonavir concentration-dependent response (A). Mean immunoreactive protein detected (n = 3, ± s.d.) was expressed as percent vehicle control (B). Computer image analysis was used to quantify the immunoblots. Significant differences from the corresponding DMSO control are indicated by * ($P < 0.01$) or # ($P < 0.05$) as determined by analysis of variance followed by Dunnett's post-hoc test. There was a linear correlation between immunoquantified P-gp and Rh123 cell accumulation (C).

Discussion

Ritonavir caused a concentration-dependent induction of P-gp expression and activity in BMEC. P-gp immunoreactive protein was increased 2-fold in BMEC treated with 30 μM ritonavir for 120 h (Figure 2B). Similar results were seen at 72 h (data not shown). In previous in-vivo studies, ritonavir (20 mg kg^{-1} per day) and dexamethasone (80 mg kg^{-1} per day) given for three days by oral gavage increased P-gp immunoreactive protein approximately 30% in rat brain microvessel endothelial cells (RMEC) (Perloff et al 2004). Considering estimated peak ritonavir plasma levels (~2 μM) in rats orally gavaged with 20 mg kg^{-1} per day ritonavir (Denissen et al 1997), induction of P-gp in-vivo RMEC (30% induction at 2 μM ritonavir) correlated well with in-vitro induction of P-gp in BMEC (~40% induction at 3 μM). With typical clinical use (100–600 mg, twice a day), ritonavir plasma concentrations reach 2–15 μM (1.4–11.2 μg mL^{-1}) (Hsu et al 1998a; Gisolf et al 2000; van Heeswijk et al 2000; Culm-Merdek et al 2006). We saw a 50–80% induction of P-gp protein at these concentrations. Furthermore, considering the local transport mechanism of P-gp in the BBB (export and reabsorption, without local drug metabolism), elevated drug concentrations in the microenvironment appeared likely. BMEC cells treated with 30 μM ritonavir for 120 h accumulated only 59% Rh123 compared with controls. This was validated by fluorescence microscopy and was reversed with 100 μM verapamil. Verapamil has been used previously as a P-gp inhibitor in BMEC (Yang & Liu 2004), and is well characterized as a relatively specific P-gp inhibitor (Perloff et al 2001; Profit et al 1999). Acutely, ritonavir affected both P-gp and

multidrug resistance-associated protein (MRP)-related activity, decreasing transport of Rh123 (P-gp substrate) and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (MRP substrate) in BMEC (Bachmeier et al 2005). The brain penetration of fluorescein, an MRP1 index substrate, was not increased in MRP1 knockout mice (Sun et al 2001), however, suggesting that MRP1 activity was minimal in the BBB.

This study focused on P-gp induction and mediated transport, for which we used Rh123, a validated P-gp specific substrate in multiple studies and cell models (Yumoto et al 1999; Litman et al 2001; Perloff et al 2001; van der Kolk et al 2001). Rh123 cell accumulation in BMEC inversely correlated with an increase in P-gp immunoreactive protein. Although induction studies of P-gp in the BBB are sparse in the literature, dexamethasone caused similar P-gp induction in immortalized RMEC (Regina et al 1999), and morphine (10–20 mg kg^{-1}, s.c., for 5 days) caused a 2-fold increase in rat (total) brain P-gp (Aquilante et al 2000). Similarly, rifampin (200 mg kg^{-1}, s.c.) daily for four days increased P-gp content by 50% in mouse brain, using plasma membrane preparations of whole brain homogenates (Zong & Pollack 2003). However, since the previous rodent studies used whole brain preparations, P-gp expression could be attributed to many cell types, including the endovascular cells of the BBB, but also astrocytes, microglia, or other cells in the brain parenchyma that have demonstrated P-gp expression (Lee et al 2001). On a molecular level, the pregnane X receptor (PXR) is considered to be the principal modulator of P-gp induction across species and tissue subtypes (Bauer et al 2004). Ritonavir most likely induced expression of P-gp through the PXR receptor and/or similar orphan nuclear receptors. Recent transgenic studies have verified the role of PXR in P-gp BBB regulation. Mice transfected with human PXR demonstrated an increase in BBB integrity with respect to methadone CNS penetration and a decrease in CNS mediated pharmacodynamics after hPXR activation with classic hPXR ligands (rifampin and hyperforin) (Bauer et al 2004, 2006).

Drug resistance in HIV therapy is assumed to be a direct result of resistant viral mutants. Inadequate plasma concentrations and the existence of viral sanctuaries potentially contribute to the failure of HAART combinations in first line therapy and the eventual development of viral latency (Markowitz 2000; Rousseau et al 2001). Extended use of ritonavir may actually lower the CNS bioavailability of co-administered HIPs due to the induction of drug transporters (and absence of CYP3A in the BBB), despite the increase in systemic plasma concentrations due to inhibition of CYP3A in liver and intestine. This is possible because ritonavir is a relatively strong P-gp inducer (Perloff et al 2000, 2001), a weak to moderate P-gp inhibitor (Washington et al 1998; Profit et al 1999; Perloff et al 2003), and a strong inhibitor of CYP3A (Greenblatt et al 2000a, b; Hsu et al 1998a; von Moltke et al 1998; Culm-Merdek et al 2006). Acute inhibition of intestinal CYP3A and P-gp during oral dosing (when intraluminal intestinal levels of ritonavir may reach millimolar levels) may override the potential activity of induced enteric P-gp and/or CYP3A (Culm-Merdek et al 2006; Mouly et al 2006; Yeh et al 2006). This reversal of induced drug transporter activity may be less likely at the barriers for viral reservoirs, like the brain and testes, where ritonavir concentrations likely fall in the submicromolar range (Kravcik et al 1999; Choo et al 2000).

Forty percent of AIDS patients develop neurological disorders (AIDS dementia complex, characterized by motor, cognitive, and behavioural impairments) that often occur late in HIV infection, perhaps due to the role of the brain as a sanctuary site for HIV virus (Tardieu 1999). A study by Dore et al (1999) demonstrated that, despite the decrease in the total number of AIDS defining illnesses (ADI) since HAART inception in 1996, the percentage of those developing AIDS dementia complex has increased by more than 50% (with median CD4+ counts at 70 mm^{-3} pre-HAART and 170 mm^{-3} post-HAART), while the proportion of other CNS ADI have remained unchanged with minimal changes in median CD4+ cell levels. Higher efficacy against other ADI compared with AIDS dementia complex suggested that decreased CNS penetration of HAART had clinical ramifications (Dore et al 1999). Similar results were seen by Sacktor et al (2001). As patients live longer with HAART, HIV dementia is being reported in patients with higher CD4+ cell counts (~201–350), perhaps reflecting prolonged HIV exposure in the CNS sanctuary site (Sacktor et al 2001).

Conclusions

Extended exposure to ritonavir caused induction of P-gp expression and activity in a BMCE BBB model. Corresponding in-vivo P-gp induction may take place in human HAART recipients and could contribute to the brain as an HIV sanctuary site, with ensuing development of AIDS dementia complex and other HIV/AIDS related neurological disorders.

References

Aquilante, C. L., Letrent, S. P., Pollack, G. M., Brouwer, K. L. (2000) Increased brain P-glycoprotein in morphine tolerant rats. *Life Sci*. **66**: L47–51

Audus, K. L., Borchardt, R. T. (1987) Bovine brain microvessel endothelial cell monolayers as a model system for the blood-brain barrier. *Ann. N. Y. Acad. Sci*. **507**: 9–18

Bachmeier, C. J., Spitzenberger, T. J., Elmquist, W. F., Miller, D. W. (2005) Quantitative assessment of HIV-1 protease inhibitor interactions with drug efflux transporters in the blood-brain barrier. *Pharm. Res*. **22**: 1259–1268

Bauer, B., Hartz, A. M., Fricker, G., Miller, D. S. (2004) Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood-brain barrier. *Mol. Pharmacol*. **66**: 413–419

Bauer, B., Yang, X., Hartz, A. M., Olson, E. R., Zhao, R., Kalvass, J. C., Pollack, G. M., Miller, D. S. (2006) In vivo activation of human pregnane X receptor tightens the blood-brain barrier to methadone through P-glycoprotein up-regulation. *Mol. Pharmacol*. **70**: 1212–1219

Casado, J. L., Dronda, F., Hertogs, K., Sabido, R., Antela, A., Marti-Belda, P., Dehertogh, P., Moreno, S. (2001) Efficacy, tolerance, and pharmacokinetics of the combination of stavudine, nevirapine, nelfinavir, and saquinavir as salvage regimen after ritonavir or indinavir failure. *AIDS Res. Hum. Retroviruses* **17**: 93–98

Choo, E. F., Leake, B., Wandel, C., Imamura, H., Wood, A. J., Wilkinson, G. R., Kim, R. B. (2000) Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. *Drug Metab. Dispos*. **28**: 655–660

Culm-Merdek, K. E., von Moltke, L. L., Gan, L., Horan, K. A., Reynolds, R., Harmatz, J. S., Court, M. H., Greenblatt, D. J.

(2006) Effect of extended exposure to grapefruit juice on cytochrome P450 3A activity in humans: Comparison with ritonavir. *Clin. Pharm. Ther*. **79**: 243–254

Dallaire, L., Tremblay, L., Beliveau, R. (1991) Purification and characterization of metabolically active capillaries of the blood-brain barrier. *Biochem. J*. **276**: 745–752

Denissen, J. F., Grabowski, B. A., Johnson, M. K., Buko, A. M., Kempf, D. J., Thomas, S. B., Surber, B. W. (1997) Metabolism and disposition of the HIV-1 protease inhibitor ritonavir (ABT-538) in rats, dogs, and humans. *Drug. Metab. Dispos*. **25**: 489–501

Ding, R., Tayrouz, Y., Riedel, K. D., Burhenne, J., Weiss, J., Mikus, G., Haefeli, W. E. (2004) Substantial pharmacokinetic interaction between digoxin and ritonavir in healthy volunteers. *Clin. Pharmacol. Ther*. **76**: 73–84

Dore, G. J., Correll, P. K., Li, Y., Kaldor, J. M., Cooper, D. A., Brew, B. J. (1999) Changes to AIDS dementia complex in the era of highly active antiretroviral therapy. *AIDS* **13**: 1249–1253

Flexner, C. (2000) Dual protease inhibitor therapy in HIV-infected patients: pharmacologic rationale and clinical benefits. *Ann. Rev. Pharmacol. Toxicol*. **40**: 649–674

Gisolf, E. H., van Heeswijk, R. P., Hoetelmans, R. W., Danner, S. A. (2000) Decreased exposure to saquinavir in HIV-1-infected patients after long-term antiretroviral therapy including ritonavir and saquinavir. *AIDS* **14**: 801–805

Gerber, J. G. (2000) Using pharmacokinetics to optimize antiretroviral drug-drug interactions in the treatment of human immunodeficiency virus infection. *Clin. Infect. Dis*. **30** (Suppl. 2): S123–S129

Greenblatt, D. J., von Moltke, L. L., Harmatz, J. S., Durol, A. L., Daily, J. P., Graf, J. A., Mertzanis, P., Hoffman, J. L., Shader, R. I. (2000a) Alprazolam-ritonavir interaction: implications for product labeling. *Clin. Pharmacol. Ther*. **67**: 335–341

Greenblatt, D. J., von Moltke, L. L., Harmatz, J. S., Durol, A. L. B., Daily, J. P., Graf, J. A., Mertzanis, P, Hoffman, J. L., Shader, R. I. (2000b) Differential impairment of triazolam and zolpidem clearance by ritonavir. *J. Acquir. Immune Defic. Syndr*. **24**: 129–136

Hammer, S. M., Saag, M. S., Schechter, M., Montaner, J. S., Schooley, R. T., Jacobsen, D. M., Thompson, M. A., Carpenter, C. C., Fischl, M. A., Gazzard, B. G., Gatell, J. M., Hirsch, M. S., Katzenstein, D. A., Richman, D. D., Vella, S., Yeni, P. G., Volberding, P. A. (2006) Treatment for adult HIV infection: 2006 recommendations of the International AIDS Society-USA panel. *JAMA* **296**: 827–843

Hsu, A., Granneman, G. R., Bertz, R. J. (1998a) Ritonavir. Clinical pharmacokinetics and interactions with other anti-HIV agents. *Clin. Pharmacokinet*. **35**: 275–291

Hsu, A., Granneman, G. R., Cao, G., Carothers, L., el-Shourbagy, T., Baroldi, P., Erdman, K., Brown, F., Sun, E., Leonard, J. M. (1998b) Pharmacokinetic interactions between two human immunodeficiency virus protease inhibitors, ritonavir and saquinavir. *Clin. Pharmacol. Ther*. **63**: 453–464

Hsu, A., Granneman, G. R., Cao, G., Carothers, L., Japour, A., El-Shourbagy, T., Dennis, S., Berg, J., Erdman, K., Leonard, J. M., Sun, E. (1998c) Pharmacokinetic interaction between ritonavir and indinavir in healthy volunteers. *Antimicrob. Agents Chemother*. **42**: 2784–2791

Jette, L., Tetu, B., Beliveau, R. (1993) High levels of P-glycoprotein detected in isolated brain capillaries. *Biochim. Biophys. Acta*. **1150**: 147–154

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

Kravcik, S., Gallicano, K., Roth, V., Cassol, S., Hawley-Foss, N., Badley, A., Cameron, D. W. (1999) Cerebrospinal fluid HIV RNA and drug levels with combination ritonavir and saquinavir. *J. Acquir. Immune Defic. Syndr*. **21**: 371–375

Lee, G., Dallas, S., Hong, M., Bendayan, R. (2001) Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations. *Pharmacol. Rev*. **53**: 569–596

Litman, T., Druley, T. E., Stein, W. D., Bates, S. E. (2001) From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol. Life Sci*. **58**: 931–959

Mangum, E. M., Graham, K. K. (2001) Lopinavir-ritonavir: a new protease inhibitor. *Pharmacotherapy* **21**: 1352–1363

Markowitz, M. (2000) Resistance, fitness, adherence, and potency: mapping the paths to virologic failure. *JAMA* **283**: 250–251

Merry, C., Barry, M. G., Mulcahy, F., Ryan, M., Heavey, J., Tjia, J. F., Gibbons, S. E., Breckenridge, A. M., Back, D. J. (1997) Saquinavir pharmacokinetics alone and in combination with ritonavir in HIV-infected patients. *AIDS* **11**: F29–F33

Mouly, S., Rizzo-Padoin, N., Simoneau, G., Verstuyft, C., Aymard, G., Salvat, C., Mahe, I., Bergmann, J. F. (2006) Effect of widely used combinations of antiretroviral therapy on liver CYP3A4 activity in HIV-infected patients. *Br. J. Clin. Pharmacol*. **62**: 200–209

Penzak, S. R., Shen, J. M., Alfaro, R. M., Remaley, A. T., Natarajan, V., Falloon, J. (2004) Ritonavir decreases the nonrenal clearance of digoxin in healthy volunteers with known MDR1 genotypes. *Ther. Drug Monit*. **26**: 322–330

Perloff, M. D., von Moltke, L. L., Fahey, J. M., Daily, J. P., Greenblatt, D. J. (2000) Induction of P-glycoprotein expression by HIV protease inhibitors in cell culture. *AIDS* **14**: 1287–1289

Perloff, M. D., von Moltke, L. L., Marchand, J. E., Greenblatt, D. J. (2001) Ritonavir induces P-glycoprotein (P-gp) expression, MRP1 expression, and drug transporter-mediated activity in a human intestinal cell line. *J. Pharm. Sci*. **90**: 1829–1837

Perloff, M. D., Störmer, E., von Moltke, L. L., Greenblatt, D. J. (2003) Rapid assessment of P-glycoprotein inhibition and induction in vitro. *Pharm. Res*. **20**: 1177–1183

Perloff, M. D., von Moltke, L. L., Greenblatt, D. J. (2004) Ritonavir and dexamethasone induce expression of CYP3A and P-glycoprotein in rats. *Xenobiotica* **34**: 133–150

Polli, J. W., Jarrett, J. L., Studenberg, S. D., Humphreys, J. E., Dennis, S. W., Brouwer, K. R., Woolley, J. L. (1999) Role of P-glycoprotein on the CNS disposition of amprenavir (141W94), an HIV protease inhibitor. *Pharm. Res*. **16**: 1206–1212

Profit, L., Eagling, V. A., Back, D. J. (1999) Modulation of P-glycoprotein function in human lymphocytes and Caco-2 cell monolayers by HIV-1 protease inhibitors. *AIDS* **13**: 1623–1627

Regina, A., Romero, I. A., Greenwood, J., Adamson, P., Bourre, J. M., Couraud, P. O., Roux, F. (1999) Dexamethasone regulation of P-glycoprotein activity in an immortalized rat brain endothelial cell line, GPNT. *J. Neurochem*. **73**: 1954–1963

Rousseau, M. N., Vergne, L., Montes, B., Peeters, M., Reynes, J., Delaporte, E., Segondy, M. (2001) Patterns of resistance mutations to antiretroviral drugs in extensively treated HIV-1-infected patients with failure of highly active antiretroviral therapy. *J. Acquir. Immune Defic. Syndr*. **26**: 36–43

Sacktor, N., Lyles, R. H., Skolasky, R., Kleeberger, C., Selnes, O. A., Miller, E. N., Becker, J. T., Cohen, B., McArthur, J. C. (2001) HIV-associated neurologic disease incidence changes: Multicenter AIDS Cohort Study, 1990–1998. *Neurology* **56**: 257–260

Sadeque, A. J., Wandel, C., He, H., Shah, S., Wood, A. J. (2000) Increased drug delivery to the brain by P-glycoprotein inhibition. *Clin. Pharmacol. Ther*. **68**: 231–237

Sadler, B. M., Piliero, P. J., Preston, S. L., Lloyd, P. P., Lou, Y., Stein, D. S. (2001) Pharmacokinetics and safety of amprenavir and ritonavir following multiple-dose, co-administration to healthy volunteers. *AIDS* **15**: 1009–1018

Sasongko, L., Link, J. M., Muzi, M., Mankoff, D. A., Yang, X., Collier, A. C., Shoner, S. C., Unadkat, J. D. (2005) Imaging P-glycoprotein transport activity at the human blood-brain barrier

with positron emission tomography. *Clin. Pharmacol. Ther.* **77**: 503–514

Schinkel, A. H. (1999) P-glycoprotein, a gatekeeper in the blood-brain barrier. *Adv. Drug Deliv. Rev.* **36**: 179–194

Sham, H. L., Kempf, D. J., Molla, A., Marsh, K. C., Kumar, G. N., Chen, C. M., Kati, W., Stewart, K., Lal, R., Hsu, A., Betebenner, D., Korneyeva, M., Vasavanonda, S., McDonald, E., Saldivar, A., Wideburg, N., Chen, X., Niu, P., Park, C., Jayanti, V., Grabowski, B., Granneman, G. R., Sun, E., Japour, A. J., Leonard, J. M., Plattner, J. J., Norbeck, D. W. (1998) ABT-378, a highly potent inhibitor of the human immunodeficiency virus protease. *Antimicrob. Agents Chemother.* **42**: 3218–3224

Sun, H., Johnson, D. R., Finch, R. A., Sartorelli, A. C., Miller, D. W., Elmquist, W. F. (2001) Transport of fluorescein in MDCKII-MRP1 transfected cells and mrp1-knockout mice. *Biochem. Biophys. Res. Commun.* **284**: 863–869

Tardieu, M. (1999) HIV-1-related central nervous system diseases. *Curr. Opin. Neurol.* **12**: 377–381

Tsuji, A., Terasaki, T., Takabatake, Y., Tenda, Y., Tamai, I., Yamashima, T., Moritani, S., Tsuruo, T., Yamashita, J. (1992) P-glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells. *Life Sci.* **51**: 1427–1437

van der Kolk, D. M., de Vries, E. G., Noordhoek, L., van den Berg, E., van der Pol, M. A., Muller, M., Vellenga, E. (2001) Activity and expression of the multidrug resistance proteins P-glycoprotein, MRP1, MRP2, MRP3 and MRP5 in de novo and relapsed acute myeloid leukemia. *Leukemia* **15**: 1544–1553

van Heeswijk, R. P., Veldkamp, A. I., Mulder, J. W., Meenhorst, P. L., Lange, J. M., Beijnen, J. H., Hoetelmans, R. M. (2000) Once-daily dosing of saquinavir and low-dose ritonavir in HIV-1-infected individuals: a pharmacokinetic pilot study. *AIDS* **14**: F103–110

van Praag, R. M., Weverling, G. J., Portegies, P., Jurriaans, S., Zhou, X. J., Turner-Foisy, M. L., Sommadossi, J. P., Burger, D. M., Lange, J. M., Hoetelmans, R. M., Prins, J. M. (2000) Enhanced penetration of indinavir in cerebrospinal fluid and semen after the addition of low-dose ritonavir. *AIDS* **14**: 1187–1194

von Moltke, L. L., Greenblatt, D. J., Grassi, J. M., Granda, B. W., Duan, S. X., Fogelman, S. M., Daily, J. P., Harmatz, J. S., Shader, R. I. (1998) Protease inhibitors as inhibitors of human cytochromes P450: high risk associated with ritonavir. *J. Clin. Pharmacol.* **38**: 106–111

Washington, C. B., Duran, G. E., Man, M. C., Sikic, B. I., Blaschke, T. F. (1998) Interaction of anti-HIV protease inhibitors with the multidrug transporter P-glycoprotein (P-gp) in human cultured cells. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **19**: 203–209

Williams, G. C., Sinko, P. J. (1999) Oral absorption of the HIV protease inhibitors: a current update. *Adv. Drug. Deliv. Rev.* **39**: 211–238

Yang, Z. Y., Liu, G. Q. (2004) Effect of p-glycoprotein inhibitor combinations on drug efflux from rat brain microvessel endothelial cells. *Pharmazie* **59**: 952–956

Yeh, R. F., Gaver, V. E., Patterson, K. B., Rezk, N. L., Baxter-Meheux, F., Blake, M. J., Eron, J. J., Klein, C. E., Rublein, J. C., Kashuba, A. D. (2006) Lopinavir/ritonavir induces the hepatic activity of cytochrome P450 enzymes CYP2C9, CYP2C19, and CYP1A2 but inhibits the hepatic and intestinal activity of CYP3A as measured by a phenotyping drug cocktail in healthy volunteers. *J. Acquir. Immune Defic. Syndr.* **42**: 52–60

Yumoto, R., Murakami, T., Nakamoto, Y., Hasegawa, R., Nagai, J., Takano, M. (1999) Transport of rhodamine 123, a P-glycoprotein substrate, across rat intestine and Caco-2 cell monolayers in the presence of cytochrome P-450 3A-related compounds. *J. Pharmacol. Exp. Ther.* **289**: 149–155

Zong, J., Pollack, G. M. (2003) Modulation of P-glycoprotein transport activity in the mouse blood-brain barrier by rifampin. *J. Pharmacol. Exp. Ther.* **306**: 556–562