*Pharmaceutical Research, Vol. 23, No. 7, July 2006 (© 2006)*
DOI: 10.1007/s11095-006-0279-5

*Research Paper*

# Efavirenz Does Not Interact with the ABCB1 Transporter at the Blood–Brain Barrier

**Grégoire Dirson,[1] Christine Fernandez,[1,2,5,6] Patrick Hindlet,[1] Françoise Roux,[3] Michèle German-Fattal,[4] François Gimenez,[1] and Robert Farinotti[1,2]**

*Received December 22, 2005; accepted February 24, 2006*

***Purpose.*** This work characterizes the interactions between efavirenz (EFV) and P-glycoprotein (P-gp/ABCB1) at the blood–brain barrier (BBB) and predicts the possible consequences on the brain uptake of coadministered P-gp substrates.
***Methods.*** The uptake of EFV was measured in whole brains of rat and mdr1a$^{-/-}$ and mdr1a$^{+/+}$ mice, and in GPNT cells (rat brain endothelial cell line) with and without P-gp inhibitors (PSC833, S9788, Quinidine). The effect of a single dose or multiple doses of EFV on the P-gp functionality was evaluated *in vivo* and *in vitro* by measuring the brain and cell uptake of digoxin, completed by the analysis of the P-gp expression at the rat BBB after repeated administrations of EFV.
***Results.*** Inhibition of P-gp did not alter the uptake of EFV in rat brain and GPNT cells. The EFV brain/plasma ratio in mdr1a$^{-/-}$ mice, lacking the expression of P-gp, was not different from that in mdr1a$^{+/+}$ mice. Moreover, a single dose of EFV did not modify the uptake of digoxin in rat brain and GPNT cells. Finally, the 3-day exposure of GPNT cells to EFV did not have any effect on the uptake of digoxin. Similarly, the 7-day treatment with EFV did not change the uptake of digoxin in rat brain nor the expression of P-gp at the BBB.
***Conclusion.*** EFV is strongly distributed in the brain, but is neither a substrate nor an inhibitor of the P-gp at the blood–brain barrier. On the other hand, EFV did not induce P-gp, allowing to sustain the brain accumulation of associated P-gp substrates such as protease inhibitors. These findings make EFV suitable for combinations circumventing the brain HIV-1 residency.

**KEY WORDS:** blood–brain barrier; efavirenz; efflux; P-glycoprotein; uptake.

## INTRODUCTION

Since the achievement of Highly Active Antiretroviral Therapy in 1996, antiretroviral multitherapies have become increasingly efficient in inhibiting HIV-1 replication and limiting viral resistance. However, the association of treatments has failed to effect an outright cure. This failure has been partly attributed to the poor disposition of antiretroviral drugs (ARVs) in cells and tissues such as the brain, hence are called sanctuary sites (1). The dramatically longer life span of HIV-1 positive individuals has underscored the rising incidence of neurological complications of AIDS with the prevalence of HIV encephalitis and dementia (1,2). The brain capillary endothelium actually constitutes an effective barrier regulating the penetration of endogenous and exogenous molecules in the central nervous system (CNS). The specific properties of the blood–brain barrier (BBB) mainly result from the presence of tight junctions between endothelial cells, restricting paracellular transport, and also from the expression of several transporters such as the ATP-binding cassette (ABC) proteins (3). Some of these ABC transporters are crucial for the pharmacokinetics of drugs, because they have been incriminated in the reduced absorption and distribution and in the increased metabolism and elimination of substrate xenobiotics, thereby protecting organs such as the brain from potentially toxic drugs (4). These transmembrane ATPases were extensively shown to prevent the tissue accumulation of numerous and diverse compounds, by extruding them out of the cell, a phenomenon originally termed as multidrug resistance (4). The efflux of ARVs was notably described for nucleoside reverse transcriptase inhibitors (NRTI) such as zidovudine and lamivudine, both of which are transported by two ABC proteins: the breast

[1] Clinical Pharmacy Unit (EA 2706; Barrières et passage des medicaments), University of Paris-Sud XI, 5 rue Jean-Baptiste Clément, Châtenay-Malabry, 92296, France.
[2] Pharmacy Department, Pitie Salpetriere Hospital, 47 Bd de l'Hopital, Paris, 75013, France.
[3] INSERM U705–CNRS UMR 7157, University of Paris VII & V, Fernand Widal Hospital, 200 rue du faubourg St-Denis, Paris, cedex 10, 75475, France.
[4] CNRS UMR 8078, University of Paris-Sud XI, Surgical Center Marie-Lannelongue, Le Plessis-Robinson, 92350, France.
[5] Service Pharmacie, Groupe Hospitalier PITIE Salpetriere, 47 boulevard de l'Hôpital, Paris, 75013, France.
[6] To whom correspondence should be addressed. (e-mail: christine.fernandez@psl.ap-hop-paris.fr)

**ABBREVIATIONS:** ARV, antiretroviral; BBB, blood–brain barrier; EFV, efavirenz; MDR, multidrug resistance; NNRTI, nonnucleoside reverse transcriptase inhibitor; P-gp, P-glycoprotein.

cancer resistance protein (BCRP/ABCG2) (5) and the multidrug resistance-associated protein (MRP/ABCC) (6). Most protease inhibitors (PIs) are substrates of P-glycoprotein (P-gp/ABCB1), which has been incriminated in the restraint brain uptake of amprenavir, saquinavir, and other PIs (7). P-gp is a major obstacle to the brain accumulation of numerous drugs because of its extensive substrate specificity and its high expression at the BBB (8). Efavirenz (EFV) is a potent and selective nonnucleoside reverse transcriptase inhibitor (NNRTI). EFV is known to cause cerebral adverse effects and displays a cerebrospinal fluid/plasma ratio of 0.69% (9). The extent of EFV brain penetration is poorly documented. Furthermore, a parallel has been drawn between P-gp and the cytochrome P450 3A4 (CYP3A4) because of their synergistic implication in drug metabolism and of their similar substrate spectra (10). As EFV is partly metabolized by CYP3A4 and induces its expression (11), it was expected to influence P-gp functionality and expression. Moreover, EFV displays a high lipophilicity (log $P$ = 4.18) and a cationic structure, like many P-gp substrates. These reasons prompted us to determine whether EFV interacts with this major ABC transporter. Few reports explored the interactions between EFV and P-gp. First of all, a clinical study revealed an impact of the single-nucleotide polymorphisms of P-gp on EFV human plasma levels (12). These observations were later contradicted by a work of Winzer and colleagues (13). This was further explored by ruling out a correlation between intracellular accumulation of EFV and P-gp expression by peripheral blood mononuclear cells (14). In other respects, Störmer *et al.* (15) demonstrated that EFV only interacts with P-gp by inducing its expression and functionality in colon carcinoma cells. This P-gp up-regulation was confirmed in peripheral blood mononuclear cells (16), but was not observed in human (11) and rat (17) intestine. Therefore, we designed a study to characterize the interactions between EFV and P-gp function and expression at the BBB and predict the possible consequences on the brain uptake of coadministered P-gp substrates.

## MATERIAL AND METHODS

### Chemicals

Efavirenz [(*S*)-6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2(*H*)-3,1-bensoxazin-2-one] (Fig. 1) and the SUSTIVA® formulation were kindly provided by Bristol Myers Squibb (Rueil Malmaison, France). Valspodar (PSC833; MW = 1,214.65), S9788, and ritonavir (MW = 720.95) were supplied by Novartis Pharma (Basel, Switzerland), Servier International Research Institute (Courbevoie, France), and Abbott Laboratories (Rungis, France), respectively. Quinidine gluconate salt was purchased from Sigma (Saint Quentin Fallavier, France) and Digoxin Nativelle® from Procter & Gamble Pharmaceuticals (Neuilly sur Seine, France). For radioactivity studies, $^{3}$H-Digoxin (23.4 Ci/mmol–1 mCi/mL in methanol), Soluene®, Hionic fluor® and Ultima Gold® were purchased from Perkin Elmer, Life and Analytical Sciences (Courtaboeuf, France).

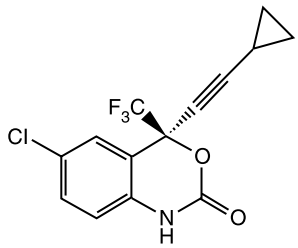


**Fig. 1.** Efavirenz (EFV) chemical structure (MW = 315.68 g/mol).

Solvents were of high-performance liquid chromatography (HPLC) or analytical grade and purchased from local suppliers.

### Dose Preparation

Efavirenz (EFV) was dissolved in DMSO/Tween 80/Glucose 5% (5:17:78, v/v/v) at a maximum concentration of 20 mg/mL for i.p. injections and diluted with 5% glucose solution when needed. Valspodar (PSC833) solution was prepared in ethanol/PEG300 (20:80, v/v). S9788 and quinidine gluconate salt were solubilized in 5% glucose solution (w/v).

Animals were treated with 15 mg/kg PSC833, 50 mg/kg S9788, 100 mg/kg quinidine, or a corresponding volume of the vehicle by intraperitoneal (i.p.) route in a volume of 2 mL/kg for rats and 5 mL/kg for mice.

For induction studies, EFV was given orally as a suspension of Sustiva® capsules in 0.2% carboxy-methylcellulose solution (w/w).

A preliminary oral pharmacokinetic study revealed that the brain/plasma concentration ratio was statistically constant within 12 h following administration (100 mg/kg) with a $T_{max}$ of about 4 h and a $C_{max}$ of about 5 mg/L (data not shown). Furthermore, preliminary i.p. injections allowed us to choose the doses of 15 mg/kg (25 mg/kg in mice for comfortable quantification) and 40 mg/kg in rats to obtain, 1 h after administration, concentrations in the lowest and middle range of those measured in human for substrate and inhibition studies, respectively. The dose of digoxin and the timing of sampling were extrapolated from previous data (18).

### Animals

Animals were housed and handled according to the European Guidelines on Care and Use of Laboratory Animals. They had access to water and food *ad libitum*.

Adult male Wistar rats (250–300 g) were purchased from Charles Rivers Laboratories (L'Arbresle, France). Male CF-1 mice (20–25 g), spontaneously lacking the expression of P-glycoprotein (mdr1a$^{-/-}$) and wild-type CF1 mice (mdr1a$^{+/+}$) were supplied by Charles Rivers Laboratories (Wilmington, MA, USA).

### Cell Line and Growth Media

GPNT cells (from GP8.3 cells), an immortalized rat brain endothelial cell line presenting a high P-gp expression level, were used at passages 25–35 (20,21). GPNT cells were grown on rat-collagen-coated surfaces in a DMEM/ Ham's F12 (1:1) medium with glutamax, 10% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL puromycin, 0.5 ng/mL basic fibroblast growth factor, 4 μg/mL

transferrin, 4 μg/mL insulin, and 3.5 ng/mL selenium in humidified 5% $CO_2$/95% air at 37°C.

### *In Vivo* Studies Design

#### *P-Glycoprotein Influence on EFV Brain Uptake*

*Rats.* Each group of eight rats was initially treated with one of the P-gp inhibitors (PSC833, S9788 or quinidine) or their placebo. Thirty minutes later, rats were dosed with EFV (15 mg/kg) also by i.p. route. One hour later, blood was collected by cardiac puncture under isoflurane anesthesia; after euthanasia, brain was removed. Blood was centrifuged (2,000 g, 10 min, +4°C). Plasma and brains were frozen at −20°C until analysis.

*Mice.* Two groups of 8 mdr1a$^{+/+}$ mice were treated with either PSC833 or its vehicle (i.p.). In the mean time, mdr1a$^{-/-}$ mice were also treated with PSC833 vehicle (i.p.). Thirty minutes after this treatment, all animals received EFV (25 mg/kg) by i.p. route. One hour later, blood was collected by cardiac puncture and brain was removed. Blood was centrifuged (2,000 g, 10 min, +4°C). Plasma and brains were frozen at −20°C until analysis.

#### *EFV's Effect on P-Glycoprotein Functionality*

*Single Dose Study.* Twenty-four male Wistar rats were randomized in three groups. Each group first received either EFV (40 mg/kg), its vehicle (negative control), or PSC833 (positive control) by i.p. route. One hour after the injection of EFV or its vehicle, and 30 min after PSC833 injection, digoxin (0.5 mg/kg) and [$^3$H]digoxin (0.37 MBq/kg) were injected to each animal (i.p.). Six hours later, blood was collected by cardiac puncture under isoflurane anesthesia, immediately followed by animal sacrifice and brain removal. Blood was centrifuged (2,000 g, 10 min, +4°C). Next, plasma and brains were prepared for analysis.

*Repeated Dose Study.* Two groups of male rats ($n$ = 8) underwent a chronic oral treatment for 7 days based on a suspension of Sustiva® (EFV: 100 mg/kg; b.i.d.) or the EFV vehicle (2 mL/kg) as negative control. Eighteen hours after the last gavage, rats were administered a single dose of digoxin (0.5 mg/kg) and [$^3$H]digoxin (0.37 MBq/kg) by i.p. route. Blood and brains were collected 6 h after digoxin injection and prepared for analysis.

#### *EFV's Long-Term Effect on P-Glycoprotein Expression*

*Animal Treatment.* As previously described, two groups of male rats ($n$ = 8) were chronically treated for 7 days with either EFV or its vehicle. Eighteen hours after the last gavage, rats were sacrificed; then brains were removed and stored at −80°C for capillary isolation and semiquantification of P-gp expression.

*Isolation of Brain Capillaries.* Brain microvessels were isolated from male Wistar rat brains by using the capillary depletion method of Triguero *et al.* (19) with slight modifications. After euthanasia, brains were removed from rat skulls and promptly immerged in ice-cold phosphate-buffered saline (PBS; Invitrogen Corporation, Cergy-Pontoise, France). They were then cleared of cerebellum, hind- and midbrain, superficial blood vessels, and meninges. Afterwards, half of the remaining cortex was gently homogenized in ice-cold Dulbecco's modified Eagles medium/fetal calf serum (DMEM/FCS; 90:10; v/v) (Invitrogen) by using a Teflon potter. The homogenates were next centrifuged at 1,000 *g* for 10 min. After elimination of the supernatant, pellets were homogenized in 5 mL of a 25% BSA solution and centrifuged again at 1,500 g for 20 min. Supernatants were thereafter removed and pellets were suspended in 1 mL of the same ice-cold mixture of DMEM/FCS and filtrated at 60 μm. Filtrates containing brain capillaries were centrifuged at 12,000 g for 45 min. Pellets of capillaries were washed and resuspended in ice-cold PBS. Microvessels suspensions were finally centrifuged at 12,000 g for 20 min. Pellets were immediately prepared for protein extraction and semiquantification.

### *In Vitro* Studies Design

#### *P-Glycoprotein Influence on EFV Brain Uptake*

In this experiment, $2 \times 10^6$ cells/well was seeded on 6-well plates and cultured until confluence. Two hours before the experiment, culture media were replaced with the same culture medium without puromycin (a P-gp substrate). The 6-well groups of GPNT cells were first treated with either PSC833 (3 μM) or its vehicle (ethanol: 0.3% final) in the culture medium for 30 min. Thereafter, cells were incubated for 1 h with the same respective media also containing 30 μM EFV in DMSO (0.5% final, v/v). Once the media were discarded, cells were promptly washed four times with ice-cold phosphate buffered saline (PBS without $Ca^{2+}$/$Mg^{2+}$) and scraped in 400 μL of this saline. A volume of 300 μL of this cell suspension was centrifuged at 1,000 g for 10 min. Cell pellets were prepared for HPLC analysis.

#### *EFV's Effect on P-Glycoprotein Functionality*

*Single Dose Study.* Confluent GPNT cells grown from a load of $4 \times 10^5$ cells on 24-well plates were deprived of puromycin 2 h prior to the experiment. Cells were then washed three times with phosphate-buffered saline (PBS with $Ca^{2+}$/$Mg^{2+}$) at 37°C and were preincubated for 30 min in a Hanks' balanced salt solution supplemented with 0.3% BSA (w/w) and 0.8 mg/mL glucose, and containing either EFV (10 μM), its vehicle (DMSO: 0.5% final, v/v) for the negative control, or PSC833 (3 μM) in ethanol (0.3% final, v/v) as referent inhibitor of digoxin P-gp-mediated efflux ($n$ = 6 wells). This medium was replaced for 1 h with 0.3 mL of the same respective media containing also digoxin (100 nM) and [$^3$H]digoxin (18.5 kBq/mL). The incubation medium was then removed, and cells were washed four times with ice-cold PBS. Cells were finally lysed and gently shaken in 0.3 mL 1% triton solution for 30 min. Cell lysates were prepared for analysis.

*Repeated Dose Study.* Cells were plated and cultured as previously described. Confluent cells were incubated for 72 h in the puromycin-free growth medium containing either 30 μm EFV, its vehicle (DMSO: 0.5% final, v/v), or 10 μM ritonavir, as referent P-gp inducer. After three washes with PBS (+$Ca^{2+}$/+$Mg^{2+}$) at 37°C, cells were exposed for 1 h to 0.3

mL Hanks' balanced salt solution supplemented with 0.3% BSA (w/w) and 0.8 mg/mL glucose, and containing digoxin (100 nM) and [$^3$H]digoxin (18.5 kBq/mL). Cell wells were then processed as described for the single dose study.

## Analyses

### *Semiquantification of P-gp Expression by Western Blot*

Proteins were extracted from the crude membranes with a TENTS solution (1 M Tris, 0.5 M EDTA, 3 M NaCl, 10% Triton, 20% SDS) with a protease inhibitor cocktail (1 mM PMSF, 2 mM benzamidine, 7.3 μM pepstatine, 5 μg/mL aprotinin, and 20 μg/mL leupeptine) for 60 min under gentle shaking. Once centrifuged (12,000 g; 20 min.), the supernatants were stored at −20°C before quantification of total proteins by using the Bicinchoninic acid protein assay kit (Sigma). Ten micrograms of proteins was dissolved in electrophoresis sample buffer containing β-mercapto-ethanol and separated on a 8% SDS-polyacrylamide gel. Resolved proteins were transferred to nitrocellulose membranes and subjected to immunoblot analysis. The blots were blocked for 1 h in TBS buffer containing 0.05% Tween 20 (TTBS) and 10% nonfat dry milk. After washing with TTBS, the blots were incubated for 2 h at room temperature with a 1:200 dilution of monoclonal anti-P-gp antibody C219 (Dako, Glostrup, Denmark) or with a 1:5,000 dilution of anti-β-actin antibody clone AC74 (Sigma-Aldrich, St. Louis, MO, USA). After five 10-min washes in TTBS, they were further incubated 1 h at room temperature with antimouse horseradish peroxidase-conjugated antibody diluted 1:10,000 (Dako). The membranes were washed five times for 10 min in TTBS and then probed with the Western lightning chemiluminescence reagent (Perkin Elmer). The intensity of the bands was quantified by using Scion Image (NIH, Scion Corporation, Bethesda, MD, USA).

### *Efavirenz Assay*

EFV was extracted from samples and quantified following an HPLC method adapted from a previously described method (22). Briefly, the HPLC system consisted of a Shimatzu pump (LC-10AS; Shimadzu, Croissy-Beaubourg, France) and an auto sampler (Waters 717 Plus; Waters, St. Quentin en Yvelines, France). The chromatographic separation of EFV and the internal standard (ritonavir) was performed at a constant flow rate of 1 mL/min of mobile phase through a LiChrospher® 100 Reverse phase-18 column (5 μm), (LiChroCART® 125-4; Merck, Darmstadt, Germany) protected by a LiChrospher® 100 RP-18 (5 μm) Guard column (LiChroCART® 4-4; Merck). Absorbance was measured at 245 nm with a Shimatzu UV spectrophotometric detector (SPD-6A; Shimadzu).

The mobile phase was composed of a 25 mM phosphate buffer with 0.5% diethylamine adjusted to pH 3.5 and acetonitrile (52:48, v/v).

*Animal Samples.* A 500-μL volume of a solution of ritonavir (4 μg/mL in methanol) was mixed with 100 μL plasma or 200 mg of brain. After vortexing for 10 s, samples were maintained at +4°C for 1 h. Extraction of antiretroviral drugs was achieved by adding 1 and 2 mL acetonitrile in spiked plasma and brain, respectively. Brain and plasma samples were then homogenized by using an Ultra-Turax (TP 18/10; Janke & Kunkel, Staufen, Germany). Homogenates were then centrifuged at 3,000 g for 10 min. Supernatants were evaporated to dryness at 45°C under air stream. Residues were finally reconstituted with mobile phase (150 μL) and 100 μL was injected into the HPLC system.

*Cell Samples.* Cell pellets were suspended in 150 μL mobile phase, whereas naïve cell pellets were loaded with varying concentrations of EFV in 150 μL mobile phase for calibration. Cell suspensions were left overnight at 4°C for EFV extraction. Supernatants of the centrifuged suspensions (12,000 g for 10 min) were finally injected (100 μL) into the HPLC system for the analysis of EFV. Pellets from the remaining 100 μL of the initial cell suspensions were used to normalize results with the total amount of proteins quantified by the Bicinchoninic acid protein assay kit (Sigma).

### *Digoxin Assay*

[$^3$H]Digoxin concentration of each sample was determined with a β-scintillation counter (LS6000LL, Beckman, Galway, Ireland).

*Animal Samples.* Brains were weighed and lysed in Soluene® for 12 h at 60°C, then transferred to polypropylene

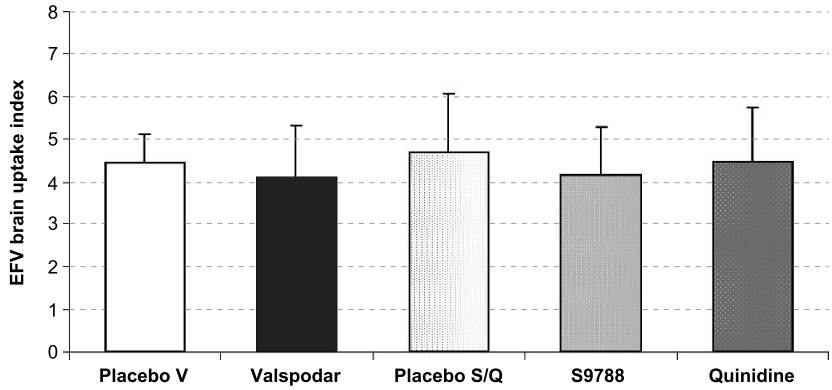


**Fig. 2.** Mean (±SD) EFV brain uptake as the brain/plasma concentrations ratios (mL/g) in rats ($n$=8) after a single i.p. pretreatment either with valspodar (15 mg/kg) *vs.* its vehicle (Placebo V), S9788 (50 mg/kg), or quinidine (100 mg/kg) *vs.* their vehicle (Placebo S/Q), preceding an i.p. administration of EFV (15 mg/kg).

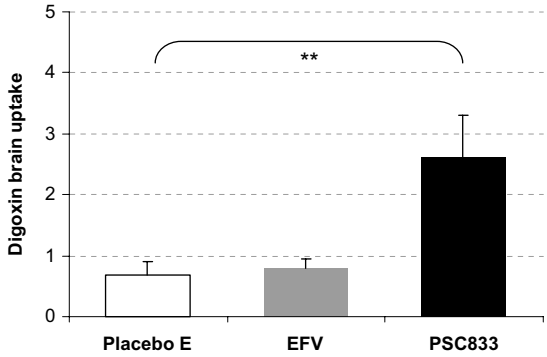


**Fig. 3.** Mean (± SD) digoxin brain uptake as the brain/plasma concentration ratios in rats ($n$ = 8) after a single i.p. pretreatment with EFV (40 mg/kg), its vehicle (Placebo E) or valspodar (15 mg/kg), preceding an i.p. administration of digoxin (0.5 mg/kg) (** $p <$ 0.05).

vials with 10 mL of Hionic Fluor® scintillation liquid. Meanwhile, 5 mL Ultima gold® scintillation liquid was added to 200 μL of each plasma sample in polypropylene vials.

*Cell Samples.* Cell lysates were collected and added to 5 mL Ultima Gold® scintillation liquid in polypropylene vials. Two nonradioactive cell lysates per group were used to normalize results as described above.

### *Statistics*

Results were presented as mean ± SD. Statistical analyses were performed by using GraphPad Software (San Diego, CA, USA). Results of experimental treatments and corresponding controls were compared by using nonparametric Mann-Whitney test. Resulting differences were considered statistically significant (*) or very significant (**) when two-tailed $p$ values were less than 0.05 and 0.01, respectively.

## RESULTS

### *In Vivo*

#### *Influence of P-gp on EFV Brain Uptake*

The influence of P-gp on EFV brain uptake was studied by pretreating rats with several P-gp inhibitors (valspodar, quinidine, S9788), which have different specificities (Fig. 2). The ratio of brain/plasma concentrations was used to evaluate brain uptake. The uptake observed for EFV was high in each group with mean values ranging from 4.17 to 4.70 mL/g, but it was not significantly modified by any pretreatment (coefficient of variation between 15.2% and 29.2%).

A similar experiment was performed on CF-1 mdr1a$^{-/-}$ mice naturally lacking the P-gp expression. The mean brain uptake of EFV were 2.90 ± 0.56 and 2.79 ± 0.26 for control mdr1a$^{-/-}$ and mdr1a$^{+/+}$ mice, respectively, and 2.86 ± 0.43 mL/g for valspodar-pretreated mdr1a$^{+/+}$ mice, with no significant differences.

#### *Effect of EFV on P-gp Functionality*

*Single Dose Treatment.* Pretreating rats with EFV resulted in no significant effect on the brain/plasma concentration ratio of digoxin compared to that of animals pretreated with placebo. On the contrary, valspodar inhibited digoxin P-gp efflux with a 3.89-fold increase in the mean value of digoxin brain uptake compared to placebo-pretreated rats (Fig. 3).

*Multiple Dose Treatment.* Figure 4A and B shows the P-gp expression in capillary-enriched fractions of rat brain after a chronic EFV treatment (100 mg/kg twice a day for 7 days). After normalization with the β-actin signal intensity, the semiquantification of P-gp expression revealed no significant

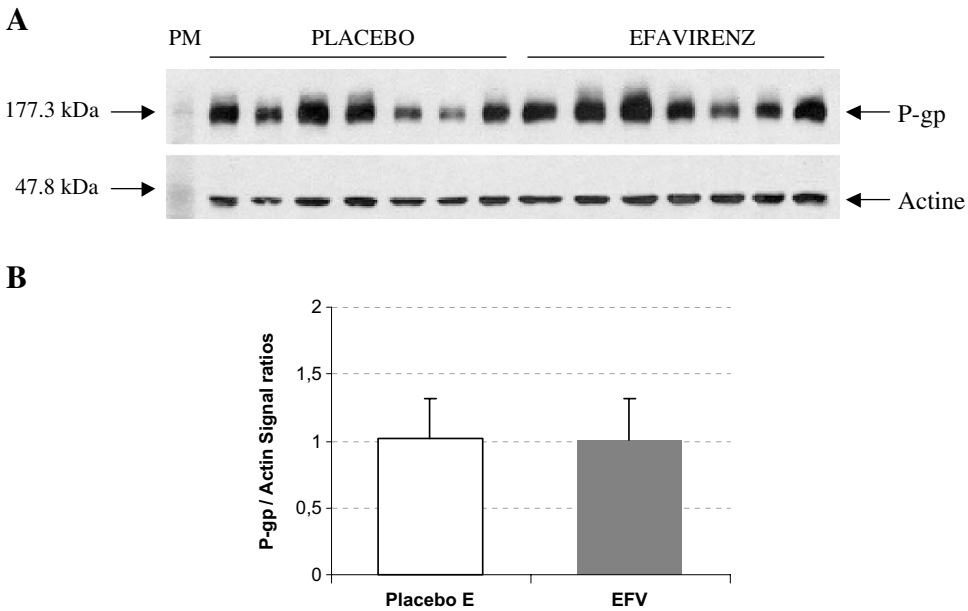


**Fig. 4.** Western blot (A) and signal intensity (B) analyses of P-gp expression in isolated rat brain capillaries after oral administrations (b.i.d.) of EFV (100 mg/kg) or its vehicle (Placebo E) during 7 days.

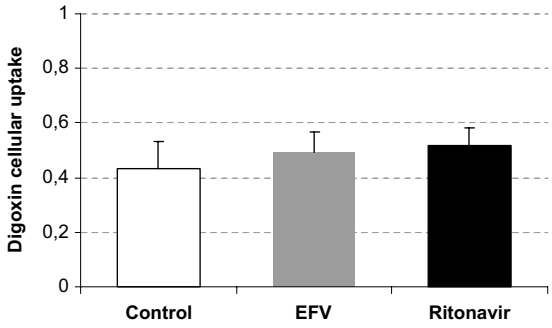


**Fig. 5.** Mean (+ SD) digoxin uptake (pmol/μg total proteins) by GPNT cells ($n$ = 6) pretreated for 3 days with EFV (30 μM), its vehicle (control), or ritonavir (10 μM).

difference in the P-gp/β-actin signal ratios of control (1.01 ± 0.31) and chronic EFV-treated animals (1.02 ± 0.29).

P-gp functionality was assessed after rats were treated with the same EFV regimen. Chronic EFV did not significantly modify digoxin brain uptake (1.22 ± 0.34 mL/g) when compared to the control mean value (0.9 ± 0.28 mL/g). This result on P-gp functionality was consistent with the lack of effect on its expression.

### *In Vitro*

#### *Influence of P-gp on EFV Brain Uptake*

Using GPNT cells, we focused on EFV transport at the brain endothelial cell scale. The exposure of GPNT to a P-gp inhibitor did not affect the uptake of EFV by these cells, with mean values of 3.64 ± 0.92 and 3.52 ± 0.63 pmol of EFV per μg of total proteins (pmol/μg proteins) for control and valspodar-pretreated cells, respectively.

#### *Effect of EFV on P-gp Functionality*

*Single Dose Treatment.* GPNT cells were also used with the same compounds to check whether the *in vivo* observations were specifically attributable to the BBB. The uptake of digoxin significantly ($p \ll 0.01$) improved by roughly 4.2-fold after pretreatment with the P-gp inhibitor PSC833 (1.87 ± 0.17 pmol/μg proteins) compared to controls (0.45 ± 0.07 ng/μg proteins), whereas EFV pretreatment had no significant effect (0.39 ± 0.05 pmol/μg proteins).

*Multiple Dose Treatment.* P-gp-mediated uptake of digoxin was also examined in GPNT cells that were chronically exposed to EFV and ritonavir. The experiment revealed that ritonavir failed to induce digoxin efflux, and EFV did not modify the uptake of digoxin by GPNT cells (Fig. 5). This last observation was consistent with *in vivo* results.

## DISCUSSION

Our results showed that, *in vivo* and *in vitro*, EFV is not a P-gp substrate and that it has no influence on the expression and functionality of P-gp at the blood–brain barrier. As it is now clear that P-gp bears several pharmacophores (23) explaining the large diversity of substrate compounds, three inhibitors with different physicochemical properties were used to increase the probability of neutralizing the hypothetic transport of EFV by P-gp. The results first displayed a high EFV brain uptake when administered to rats. However, the preadministration of S9788, Quinidine, or Valspodar, considered as one of the most potent P-gp inhibitors, did not modify brain concentrations of EFV in rats. The experiment was also performed in wild-type CF-1 mice, to confirm results because species-related differences in the P-gp structure may potentially displace substrate specificity. The CNS uptake of EFV was finally studied in spontaneously muted CF-1 mice lacking the P-gp expression ($mdr1a^{-/-}$) in the brain (24) *vs.* wild-type CF-1 mice ($mdr1a^{+/+}$). Similar results to those observed in rats were obtained in wild-type CF-1 mice with valspodar. EFV brain disposition in $mdr1a^{-/-}$ mice remained unchanged with valspodar and was not different from that in $mdr1a^{+/+}$ mice, whereas, comparatively, the brain uptake of the P-gp substrate bromocriptine was reported to be increased 28-fold in the same model (25). However, the breast cancer resistance protein (BCRP or ABCG2), another ABC transporter associated with the MDR phenomenon, was found to be overexpressed at the BBB in P-gp-deficient CF-1 mice (26). Consequently, it was not ruled out that the loss of EFV brain efflux in the absence of P-gp expression could be offset by a greater EFV efflux due to BCRP overexpression. To dismiss this eventuality, the experiment was reproduced with the P-gp/BCRP inhibitor elacridar, which led to the same results (data not shown)—suggesting that BCRP overexpression did not influence EFV brain uptake in $mdr1a^{-/-}$ mice. Furthermore, the use of the GPNT cell line provided a relevant tool to specifically study P-gp function at the brain endothelial cell level. In this model, it was also noted that P-gp inhibition by valspodar did not alter EFV uptake. Taken together, the present information obtained in rats, mice, and cultured brain endothelial cells concur to establish that EFV is not a P-gp substrate, which is in agreement with previous studies performed on different cellular models (15).

The conclusion that EFV is not transported by P-gp brings an important element when considering the combinations of several ARVs and their interactions related to P-gp function. Inhibition and/or induction of P-gp functionality has been reported to be achieved by ARVs, especially PIs such as ritonavir or amprenavir (7). Their administration could alter the pharmacokinetics of associated P-gp substrates. Because P-gp does not influence the brain disposition of EFV, PIs will not modify the brain concentration and toxicity of this NNRTI, when combined.

The study of EFV as a P-gp modulator was intended to predict the possible effect of this NNRTI on the brain entry of associated P-gp substrates and, in particular, PIs. Higher concentrations of ARVs would further inhibit viral replication and limit the development of viral resistance against EFV in tissues poorly reached by ARVs. Therefore, the direct influence of EFV on P-gp function was examined at the BBB. Results showed that EFV did not alter the rat brain concentration of digoxin, a P-gp substrate (27). This was confirmed on GPNT cells, in which digoxin uptake remained unchanged with EFV. It was thus concluded that EFV has no ability to inhibit P-gp efflux,

confirming previous data on other cellular models (15). Consequently, EFV would not have a synergistic effect on the efficacy of associated PIs in P-gp-expressing cells and tissues susceptible to the virus.

Long-term treatments are often associated with altered expression and functionality of proteins implicated in metabolism and transport as described for CYP3A4 induced by EFV (11). Therefore, a third series of experiments focused on the response of endothelial cells *in vivo* and *in vitro* to chronic exposure to EFV in terms of P-gp functionality and expression. After a 1-week treatment with EFV, the rat brain uptake of digoxin remained unchanged. This corroborated the statistically unchanged level of P-gp expression following the same treatment. This was consistent with previous reports at the rat intestinal level (17). However, the difficulty of finding a referent P-gp inducer at the BBB questioned whether P-gp expression might be modulated at the BBB. Indeed, few molecules have been described to induce P-gp at the BBB level *in vivo* (28) and *in vitro* (29), notably morphine and PXR ligands such as rifampicin and dexamethasone. In preliminary experiments on wild-type CF1 mice, these compounds failed to reduce digoxin brain uptake (data not shown). Using morphine and rifampicin, Zong and Pollack (30) reported a modest induction of the expression and functionality of brain endothelial P-gp in mice with regard to that observed in the intestine with rifampicin (31). This could be linked to integrated regulatory mechanisms possibly implicating other cell types such as astrocytes (32). This regulation would bring about an almost maximum basal expression level of P-gp at the BBB, which could only slightly be further increased (33). In consequence, this weak induction may easily be masked by interindividual or polymorphisms-linked variability.

The use of endothelial cell monoculture limiting external variability factors could enable slight variations in P-gp function to stand out. The study was thus completed with the examination of P-gp functionality following the chronic exposure of GPNT cells to EFV and ritonavir. Similar to *in vivo* results, the measure of digoxin uptake by GPNT cells was statistically unchanged after EFV as well as ritonavir treatment, although the PI is also known to induce P-gp (15,34). Like numerous peripheral P-gp inducers, ritonavir might induce P-gp at a peripheral level without displaying the same effect at a central level (33). Besides, Berruet *et al.* ( 17) and Mouly *et al.* (11) demonstrated no significant modifications in the expression and/or functionality of P-gp at the intestine epithelium of rat and human, respectively, after chronic oral treatment with EFV. The absence of effect of EFV on P-gp at a peripheral level tends to confirm the present results at the central level. Globally, our whole induction data strongly suggest that EFV is not a P-gp inducer at the BBB. Therefore, EFV will not further restrict brain penetration of P-gp substrates such as PIs after repeated administrations, thus maintaining their intrinsic brain efficacy.

## CONCLUSION

In conclusion, the present work examined the interactions between EFV and P-gp, a major component in the pharmacokinetics of drugs. First, our study revealed that even if the passive diffusion of EFV through cell membranes may largely exceed and thereby mask its hypothetical efflux by P-gp, this transporter has no influence on the uptake of EFV at the BBB. Hence, EFV brain penetration should not be modified by P-gp modulators. Second, analysis of the functionality and expression of P-gp following a single or chronic exposure to EFV *in vitro* and *in vivo* discarded the potentiality of EFV to modulate the transporter function at the BBB. The combination of EFV with P-gp substrates subsequently will not have a depletory impact on the CNS efficacy of these associated drugs, notably PIs, as long as the BBB is not disrupted.

## ACKNOWLEDGMENTS

This research project was granted by the doctoral CIFRE convention no. 651/2003 established between the Infectiology & Immunology Department (Thu Huyen Nguyen, MD, and Dan Chiche, MD) of the Bristol-Myers Squibb group (Rueil-Malmaison, France), the National Association for Technical Research (Paris, France) and the Clinical Pharmacy Unit (EA 2706; Châtenay-Malabry, France). We would like to thank Pr. J.C. Gantier and Dr. M. Buyse for expert assistance in animal experimentations, and Dr H. Chacun for technical support in radioactivity studies.

## REFERENCES

1. J. E. Bell. An update on the neuropathology of HIV in the HAART era. *Histopathology* **45**:549–559 (2004).
2. J. C. McArthur. HIV dementia: an evolving disease. *J. Neuroimmunol.* **157**:3–10 (2004).
3. D. J. Begley. Understanding and circumventing the blood–brain barrier. *Acta Paediatr. Suppl.* **92**:83–91 (2003).
4. A. H. Schinkel and J. W. Jonker. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv. Drug Deliv. Rev.* **55**:3–29 (2003).
5. X. Wang, T. Furukawa, T. Nitanda, M. Okamoto, Y. Sugimoto, S. Akiyama, and M. Baba. Breast cancer resistance protein (BCRP/ABCG2) induces cellular resistance to HIV-1 nucleoside reverse transcriptase inhibitors. *Mol. Pharmacol.* **63**:65–72 (2003).
6. J. D. Schuetz, M. C. Connelly, D. Sun, S. G. Paibir, P. M. Flynn, R. V. Srinivas, A. Kumar, and A. Fridland. MRP4: a previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat. Med.* **5**:1048–1051 (1999).
7. F. Gimenez, C. Fernandez, and A. Mabondzo. Transport of HIV protease inhibitors through the blood–brain barrier and interactions with the efflux proteins, P-glycoprotein and multidrug resistance proteins. *J. Acquir. Immune Defic. Syndr.* **36**:649–658 (2004).
8. D. J. Begley. ABC transporters and the blood–brain barrier. *Curr. Pharm. Des.* **10**:1295–1312 (2004).
9. H. J. von Giesen, H. Koller, A. Theisen, and G. Arendt. Therapeutic effects of nonnucleoside reverse transcriptase inhibitors on the central nervous system in HIV-1-infected patients. *J. Acquir. Immune Defic. Syndr.* **29**:363–367 (2002).
10. M. F. Fromm. Importance of P-glycoprotein at blood-tissue barriers. *Trends Pharmacol. Sci.* **25**:423–429 (2004).
11. S. Mouly, K. S. Lown, D. Kornhauser, J. L. Joseph, W. D. Fiske, I. H. Benedek, and P. B. Watkins. Hepatic but not intestinal CYP3A4 displays dose-dependent induction by efavirenz in humans. *Clin. Pharmacol. Ther.* **72**:1–9 (2002).

12. J. Fellay, C. Marzolini, E. R. Meaden, D. J. Back, T. Buclin, J. P. Chave, L. A. Decosterd, H. Furrer, M. Opravil, G. Pantaleo, D. Retelska, L. Ruiz, A. H. Schinkel, P. Vernazza, C. B. Eap, and A. Telenti. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. *Lancet* **359**:30–36 (2002).
13. R. Winzer, P. Langmann, M. Zilly, F. Tollmann, J. Schubert, H. Klinker, and B. Weissbrich. No influence of the P-glycoprotein genotype (MDR1 C3435T) on plasma levels of lopinavir and efavirenz during antiretroviral treatment. *Eur. J. Med. Res.* **8**: 531–534 (2003).
14. L. M. Almond, P. G. Hoggard, D. Edirisinghe, S. H. Khoo, and D. J. Back. Intracellular and plasma pharmacokinetics of efavirenz in HIV-infected individuals. *J. Antimicrob. Chemother.* **56**:738–744 (2005).
15. E. Stormer, L. L. von Moltke, M. D. Perloff, and D. J. Greenblatt. Differential modulation of P-glycoprotein expression and activity by non-nucleoside HIV-1 reverse transcriptase inhibitors in cell culture. *Pharm. Res.* **19**:1038–1045 (2002).
16. B. Chandler, L. Almond, J. Ford, A. Owen, P. Hoggard, S. Khoo, and D. Back. The effects of protease inhibitors and nonnucleoside reverse transcriptase inhibitors on P-glycoprotein expression in peripheral blood mononuclear cells *in vitro*. *J. Acquir. Immune Defic. Syndr.* **33**:551–556 (2003).
17. N. Berruet, S. Sentenac, D. Auchere, F. Gimenez, R. Farinotti, and C. Fernandez. Effect of efavirenz on intestinal P-glycoprotein and hepatic p450 function in rats. *J. Pharm. Sci.* **8**:226–234 (2005).
18. S. Song, H. Suzuki, R. Kawai, and Y. Sugiyama. Effect of PSC 833, a P-glycoprotein modulator, on the disposition of vincristine and digoxin in rats. *Drug Metab. Dispos.* **27**:689–694 (1999).
19. D. Triguero, J. Buciak, and W. M. Pardridge. Capillary depletion method for quantification of blood–brain barrier transport of circulating peptides and plasma proteins. *J. Neurochem.* **54**:1882–1888 (1990).
20. A. Regina, I. A. Romero, J. Greenwood, P. Adamson, J. M. Bourre, P. O. Couraud, and F. Roux. Dexamethasone regulation of P-glycoprotein activity in an immortalized rat brain endothelial cell line, GPNT. *J. Neurochem.* **73**:1954–1963 (1999).
21. Y. T. Pham, A. Regina, R. Farinotti, P. Couraud, I. W. Wainer, F. Roux, and F. Gimenez. Interactions of racemic mefloquine and its enantiomers with P-glycoprotein in an immortalised rat brain capillary endothelial cell line, GPNT. *Biochim. Biophys. Acta* **1524**:212–219 (2000).
22. V. Proust, K. Toth, A. Hulin, A. M. Taburet, F. Gimenez, and E. Singlas. Simultaneous high-performance liquid chromatographic determination of the antiretroviral agents amprenavir, nelfinavir, ritonavir, saquinavir, delavirdine and efavirenz in human plasma. *J. Chromatogr., B, Biomed. Sci. Appl.* **742**:453–458 (2000).
23. A. Garrigues, N. Loiseau, M. Delaforge, J. Ferte, M. Garrigos, F. Andre, and S. Orlowski. Characterization of two pharmacophores on the multidrug transporter P-glycoprotein. *Mol. Pharmacol.* **62**:1288–1298 (2002).
24. D. R. Umbenhauer, G. R. Lankas, T. R. Pippert, L. D. Wise, M. E. Cartwright, S. J. Hall, and C. M. Beare. Identification of a P-glycoprotein-deficient subpopulation in the CF-1 mouse strain using a restriction fragment length polymorphism. *Toxicol. Appl. Pharmacol.* **146**:88–94 (1997).
25. S. Vautier, L. Lacomblez, H. Chacun, V. Picard, F. Gimenez, and R. Farinotti. Interactions between the dopamine agonist, bromocriptine and the efflux protein, P-glycoprotein at the blood–brain barrier in the mouse. *Eur. J. Pharm. Sci.* **27**:167–174 (2006).
26. S. Cisternino, C. Mercier, F. Bourasset, F. Roux, and J. M. Scherrmann. Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood–brain barrier. *Cancer Res.* **64**:3296–3301 (2004).
27. M. Ben Reguiga, L. Bonhomme-Faivre, S. Orbach-Arbouys, and R. Farinotti. Modification of the P-glycoprotein dependent pharmacokinetics of digoxin in rats by human recombinant interferon-alpha. *Pharm. Res.* **22**:1829–1836 (2005).
28. W. Loscher and H. Potschka. Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Prog. Neurobiol.* **76**:22–76 (2005).
29. B. Bauer, A. M. Hartz, G. Fricker, and D. S. Miller. Pregnane X receptor up-regulation of P-glycoprotein expression and transport function at the blood–brain barrier. *Mol. Pharmacol.* **66**:413–419 (2004).
30. J. Zong and G. M. Pollack. Modulation of P-glycoprotein transport activity in the mouse blood–brain barrier by rifampin. *J. Pharmacol. Exp. Ther.* **306**:556–562 (2003).
31. C. J. Matheny, R. Y. Ali, X. Yang, and G. M. Pollack. Effect of prototypical inducing agents on P-glycoprotein and CYP3A expression in mouse tissues. *Drug Metab. Dispos.* **32**:1008–1014 (2004).
32. N. J. Abbott. Astrocyte–endothelial interactions and blood–brain barrier permeability. *J. Anat.* **200**:629–638 (2002).
33. J. H. Lin. Drug–drug interaction mediated by inhibition and induction of P-glycoprotein. *Adv. Drug Deliv. Rev.* **55**:53–81 (2003).
34. M. Kageyama, H. Namiki, H. Fukushima, S. Terasaka, T. Togawa, A. Tanaka, Y. Ito, N. Shibata, and K. Takada. Effect of chronic administration of ritonavir on function of cytochrome P450 3A and P-glycoprotein in rats. *Biol. Pharm. Bull.* **28**: 130–137 (2005).