Mechanism-based pharmacokinetic-pharmocodynamic modelling of opioids : role of biophase distribution and target interaction kinetics

Groenendaal, D.

Chapter 4

MEMBRANE TRANSPORT OF OPIOIDS: RELATIVE CONTRIBUTION OF P-GLYCOPROTEIN MEDIATED TRANSPORT AND PASSIVE PERMEABILITY *IN VITRO*

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ABSTRACT

Restricted transport across the blood-brain barrier (BBB) can be a critical factor in the development of mechanism-based pharmacokinetic/pharmacodynamic (PK-PD) models for the central effects of opioids. In this study, the P-glycoprotein (Pgp) interaction and the apparent transmembrane passive permeability rates (P_{app}) of a wide range of opioids were investigated, This was performed *in vitro*, using monolayers of MDCK:MDR1 and LLC-PK1:MDR1a cells. The opioids alfentanil, fentanyl, sufentanil, loperamide and morphine were able to inhibit Pgp mediated efflux of ^{3}H-digoxin in MDCK:MDR1 and LLC-PK1:MDR1a cells, whereas no inhibition was observed for butorphanol, nalbuphine, and the morphine metabolites M3G and M6G. Moreover, active transport by Pgp was found for loperamide and morphine. The P_{app} values were determined in the presence of the Pgp inhibitor GF120918. High transmembrane passive permeability rates (>500 nm/sec) were found for alfentanil, fentanyl, sufentanil and butorphanol, whereas the permeability rates of loperamide (206 nm/sec), nalbuphine (156 nm/sec) and morphine (16 nm/sec) were relatively low. It is concluded that the contributions of both Pgp mediated transport and transmembrane passive permeability rates are of importance for the influence of BBB transport on the PK-PD relationships of opioids. Alfentanil, fentanyl, sufentanil and butorphanol have high passive permeability rates and the relative contribution of Pgp mediated transport, if any, is therefore considered as not significant. In contrast, nalbuphine, morphine and loperamide have low passive permeabilities and therefore the net transmembrane transport is significantly influenced by Pgp mediated transport.

INTRODUCTION

The objective of pharmacokinetic-pharmacodynamic (PK-PD) modelling is the prediction of the time course of drug effects under physiological and pathological conditions (Breimer & Danhof 1997). At present there is a clear trend towards the development and application of mechanism-based PK-PD models. Mechanism-based PK-PD models differ from empirical descriptive models in that they contain specific expressions to characterise the processes on the causal path between drug administration and effect. These processes include 1) biophase distribution, 2) target site activation, 3) transduction and 4) the influence of *in vivo* homeostatic feedback mechanisms (Danhof *et al.* 2005). An important feature of PK-PD models is further the strict distinction between drug and system specific characteristics (van der Graaf & Danhof 1997). Our interest is to develop a mechanism-based PK-PD model for the central effects of opioids. In these investigations alfentanil, fentanyl, sufentanil, nalbuphine, butorphanol, morphine and loperamide (figure 1) are used as model drugs, because they differ widely in affinity and intrinsic efficacy at the μ-opioid receptor.

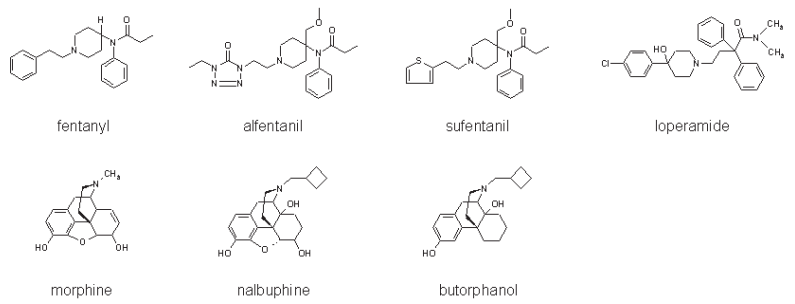

Figure 1: The chemical structures of the opioids investigated in these studies. Two categories of opioids can be identified; the ones derived from fentanyl and the ones derived from morphine.

To determine the complex *in vivo* concentration-effect relationships of these opioids, the transport across the blood-brain barrier (BBB) needs to be taken into consideration.
Recently, the effects of the opioids alfentanil, fentanyl and sufentanil have been studied *in vivo* in a chronically instrumented rat model, using the amplitude in the 0.5-4.5 Hz frequency band of the electroencephalogram (EEG) as a pharmacodynamic endpoint (Cox *et al.* 1998). However, the role of BBB transport as a mechanism for the observed hysteresis of these opioids has so far not been explored in detail. This is important since active transporters at the BBB may influence both the rate and extent of transport into the brain, and thereby the estimation of the parameters in the complex PK-PD relationships of centrally acting drugs (de Lange *et al.* 2005).

Transport across the BBB can be divided into passive and active transport processes (de Lange & Danhof 2002). Passive transport of compounds across the BBB is dependent on physicochemical properties, such as lipophilicity, degree of ionisation and number of hydrogen bonds (van Bree *et al.* 1988). Active transport can be divided into carrier mediated transport, receptor mediated transport and endocytosis (de Boer *et al.* 2003). Several transporters are present at the BBB to transport endogenous compounds such as amino acids, glucose etc. An important efflux transporter expressed at the luminal face of the BBB is P-glycoprotein (Pgp). Pgp is a member of the adenosine triphosphate-binding cassette super family and is encoded by the multidrug resistance gene (MDR1) (Thiebaut *et al.* 1987). This transporter has been shown to have an important influence on the BBB transport of a wide range of drugs (de Lange & Danhof 2002; Schinkel *et al.* 1995; Schinkel *et al.* 1996).

In previous investigations, morphine and loperamide have been identified as Pgp substrates in both *in vitro* models, comprising of either brain capillary endothelial cells or LLC-PK1:MDR1 cells, and *in vivo* models, in rats and mice (Wandel *et al.* 2002, Letrent *et al.* 1999b; Mahar Doan *et al.* 2002; Schinkel *et al.* 1995, 1996). Furthermore, PK-PD studies in rats have revealed that after oral pre-treatment with the specific Pgp inhibitor GF120918, the anti-nociceptive effect of morphine was prolonged due to its prolonged half-life in the brain (Letrent *et al.* 1998, 1999a). Alfentanil and sufentanil were not identified as Pgp substrates within the abovementioned investigations in *in vitro* models, whereas inconsistencies have been reported for fentanyl (Henthorn *et al.* 1999; Wandel *et al.* 2002). In addition, for fentanyl, *in situ* brain perfusion studies indicated Pgp mediated efflux as it was found that the brain uptake of fentanyl is slightly increased (1.2 fold) in MDR1a (-/-) mice when compared to MDR1a (+/+) mice (Dagenais *et al.* 2004; Mahar Doan *et al.* 2002; Schinkel *et al.* 1995, 1996). Nalbuphine, a semi-synthetic opioid analgesic, was also found to be a Pgp substrate in a MDCKII-MDR1 cell-system (Mahar Doan *et al.* 2002), whereas to our knowledge so far no studies have performed on butorphanol.

The aim of the present study was to identify the membrane transport characteristics with respect to the relative contribution of Pgp mediated (efflux) transport and passive transmembrane permeability rate (P_{app}), for a series of opioids including alfentanil, fentanyl, sufentanil, nalbuphine, butorphanol, morphine and loperamide. The chemical structures of these opioids are shown in figure 1. The opioids were tested in an *in vitro* cell system, comprising of monolayers of MDCKII:MDR1 or LLC-PK1:MDR1a cells. Regression analysis was used to investigate the relationships between P_{app} and physicochemical properties of the opioids. These properties included polar surface area, lipophilicity (cLogP) and the predicted BBB permeability based on the number of hydrogen donors and acceptors, according to the Abraham equation (Abraham *et al.* 1994).

MATERIALS AND METHODS

Materials

Fentanyl citrate, morphine sulphate, nalbuphine hydrochloride, butorphanol tartrate, loperamide hydrochloride, morphine-3-glucuronide, morphine-6-glucuronide and lucifer yellow were obtained from Sigma Aldrich (United Kingdom). Alfentanil hydrochloride and sufentanil citrate were kindly provided by Janssen Pharmaceutica (Beerse, Belgium). GF120918 and SB-243213 were supplied by GlaxoSmithKline. ^{3}H-amprenavir (specific activity 0.13 Ci/mmol), ^{3}H-digoxin (specific activity 37 Ci/mmol) and ^{3}H-loperamide (specific activity 10 Ci/mmol) were obtained from Amersham.

The stock solutions of the opioids and GF120918 were prepared in DMSO (Sigma Aldrich, United Kingdom). The GSK internal standard solution was prepared in ammonium acetate/methanol (50/50 v/v). The lucifer yellow solution was prepared in Millipore water (18.2MΩ.cm). All other chemicals were of analytical grade.

All cell-culture reagents were obtained from Invitrogen (United Kingdom). BD Falcon™ HTS 24-Multiwell Inserts (24-well, 0.31 cm^2, 0.1 µm pore size) were obtained from Becton-Dickinson (United Kingdom).

Cell lines

MDCKII:MDR1; Madine Darby Canine Kidney type II cells transfected with the human MDR1 gene (MDCKII:MDR1) were cultured in DMEM – glutamax media, formulated with D-glucose (4.5 g/l), L-alanyl-glutamine and phenol red and supplemented with penicillin (10000 U/ml)-streptomycin (10000 µg/ml) and 10% (v/v) fetal calf serum at 37 °C and 5% CO_2. Cells were trypsinised every 4 days. For the studies, the cells were seeded onto BD Falcon™ HTS 24-Multiwell Inserts at a seeding density of 50000 cells/well and grown for 3 days in DMEM full media.

LLC-PK1:MDR1a; Porcine kidney epithelial cells transfected with the mouse MDR1a gene (LLC-PK1:MDR1a) were cultured in M199 media, formulated with Earle's salts, L-glutamine, phenol red and sodium bicarbonate (2.2 g/L) and supplemented with penicillin (10000 U/ml)-streptomycin (10000 µg/ml) and 10% (v/v) fetal calf serum at 37 °C and 5% CO_2. The cells were trypsinised every 4 days and sub-cultured in M199 media containing 2 mM vincristine as a selection agent. For the studies, the cells were seeded onto BD Falcon™ HTS 24-Multiwell Inserts at a seeding density of 75000 cells/well and grown for 3 days in M199 media without the selection agent vincristine.

General experimental procedures on transmembrane transport studies

Transport experiments were performed using monolayers of both the MDCKII:MDR1 and the LLC-PK1:MDR1a cells. As an integrity check, prior to the experiment, the trans-epithelial electrical resistance (TEER) of each monolayer was measured with an EVOM™ voltohmmeter (World Precision Instruments, Stevenage, United Kingdom). The experiments were performed in transport buffer consisting of DMEM containing

25 mM HEPES without phenol red and sodium pyruvate. The experiments were started with pre-incubation of the monolayers for 15 min at 37°C with transport buffer to which either the Pgp inhibitor GF120818 or the vehicle (0.5% DMSO) was added. After the pre-incubation period, the transport buffer was removed and the test solutions were added. All test solutions were prepared from stock solutions, that were prepared in 100% DMSO (opioids at 100 mM; GF120918 at 2 mM). To prepare the test solutions, the stock solutions were diluted further with transportbuffer. The test solutions ultimately contained 0.5% DMSO. The monolayers were incubated with the test solutions for a test period of 90 min at 37°C under continuous shaking. All experiments were performed automatically using the robotic TECAN™ genesis workstation (TECAN, Reading, United Kingdom).
The reference drugs for membrane integrity towards paracellular transport (lucifer yellow, 10 µM) and for Pgp-efflux functionality (amprenavir, 3 µM) were included in each experiment to test the integrity and quality of the monolayer. Lucifer yellow was added to each well, whereas amprenavir was only added to two wells as a positive control. After the test period of 90 min, 100 µl samples were collected to be analysed on the concentrations of the references lucifer yellow and amprenavir, as well as the test compound.

Inhibition of Pgp mediated efflux of ^{3}H-digoxin, the "^{3}H-digoxin transport inhibition factor"

The experiments on inhibition of Pgp mediated transport by either the Pgp inhibitor GF120918 or by the seven selected opioids were performed in duplicate using monolayers of both the MDCKII:MDR1 and the LLC-PK1:MDR1a cells. Transport measurements were performed in basolateral-to-apical direction (b→a). This would correspond to BBB transport in the direction from brain into blood (efflux).
In addition to alfentanil, fentanyl, sufentanil, nalbuphine, butorphanol, morphine and loperamide, the morphine metabolites, morphine-3-glucoronide (M3G) and morphine-6-glucoronide (M6G) were also included in these studies. Stock solutions of opioids (100 mM) and GF120918 (2 mM) were prepared. The monolayers were pre-incubated with transport buffer containing either GF120918 (2 µM) or opioid (100 µM) at both the basolateral and apical side. At time = 0, the experiment was started by addition of ^{3}H-digoxin (3 µM) for testing the inhibitory action of the opioids and the morphine metabolites. As a positive control ^{3}H-amprenavir was added to two of the wells and lucifer yellow was added to all the wells to check for monolayer integrity. The ^{3}H-digoxin transport inhibition factor was calculated (see data analysis).

Pgp substrate assessment studies; the "Pgp substrate efflux ratio"

Pgp substrate assessment studies were performed in duplicate using monolayers of the MDCKII:MDR1 cells. Transport experiments were performed both into the apical-to-basolateral (a→b) as the basolateral-to-apical (b→a) direction. The stock solutions of the opioids contained final concentrations of 3 mM in DMSO. The pre-incubation solutions

contained vehicle (0.5% DMSO) or GF120918 (2 µM in 0.5% DMSO) in transport buffer. The test solutions contained vehicle (0.5% DMSO) or GF120918 (2 µM), lucifer yellow and one of the opioids (3 µM) or amprenavir (3 µM). The "Pgp substrate efflux ratio" was calculated (see data analysis).

Sample analysis

^{3}H-amprenavir, ^{3}H-loperamide and ^{3}H-digoxin samples were dried down at 37°C and analysed with a Topcount NXT™ microplate scintillation counter (Perkin Elmer, Beaconsfield, United Kingdom), 2 min per sample.

Alfentanil, fentanyl, sufentanil and butorphanol were analysed by dual high-performance liquid chromatography with tandem mass spectrometry (HPLC-LC/MS/MS). The system consisted of an API-365 (Applied Biosystems, Warrington, United Kingdom) LC/MS/MS employing positive ion turbospray ionisation with a CTC HTS PAL autosampler (CTC Analytics, Hitchin, United Kingdom). HPLC was conducted on a 50 mm x 2.1 mm HyPURITY column (ThermoHypersil, Runcorn, United Kingdom) at a flow rate of 0.8 ml/min and a split ratio of 1:2. The mobile phase consisted of two solvents: (A) 10 mM ammonium acetate pH 4 and (B) 100% acetonitrile. The gradient profile was set at 0 min on 80% A and 20% B, at 1 min on 0% A and 100% B and at 1.1 min on 80% A and 20% B. The total run time was 1.5 min. Data acquisition was performed with PE Sciex version 1.1 (Applied Biosystems, Warrington, United Kingdom) and data were reported as the ratio of test compound peak area over internal standard peak area.

Nalbuphine and morphine samples were analysed by high performance liquid chromatography with electrochemical detection (HPLC-ECD) as described previously (Groenendaal *et al*. 2005 – chapter 3). Briefly, the nalbuphine samples were transferred into glass tubes and the internal standard butorphanol (50 µl, 2500 ng/ml) was added. The samples were acidified with phosphoric acid (0.15 mM, pH 2.3, 500 µl) and extracted with 3 ml of ethyl acetate. After extraction, the organic layer was discarded. Subsequently, the samples were alkalinised with carbonate buffer (0.15 mM, pH 11, 500 µl) and extracted with 5 ml of ethyl acetate. The organic layer was transferred into clean glass tubes and evaporated to dryness under reduced pressure at 37 °C. For morphine samples, only the alkaline extraction with carbonate buffer and ethyl acetate was performed. The HPLC system consisted of an LC-10AD HPLC pump (Shimadzu, Kyoto, Japan), a Waters 717 plus autosampler (Waters, Milford, MA, USA), a pulse damper (Antec Leyden, Rijnsburg, The Netherlands) and a digital electrochemical amperometric detector (DECADE, software version 3.02) from Antec Leyden. The electrochemical detector consisted of a VT-03 electrochemical flow cell combined with a 25 µm spacer and an Ag/AgCl reference electrode operating in the DC mode at a temperature of 30 °C, set at a voltage of 0.85V and 0.75V for nalbuphine and morphine, respectively.

Chromatography was performed on C18 ODS Ultrasphere 5 µm column (4.6 mm I.D. x 150 mm) (Alltech, Breda, The Netherlands) equipped with a refill guard column. The mobile phase was a mixture of 0.1 M phosphate buffer (pH 5.5) and methanol (65:35, v/v) for nalbuphine and butorphanol and a mixture of 0.1 M phosphate buffer (pH 4) and methanol (75:25, v/v) for morphine and contained a total concentration of 5 mM KCl and 20 mg/l EDTA. The mobile phase for morphine also contained 2.0 mM octane-sulfonic acid. The flow rate was set at 1 ml/min. Data acquisition and processing was performed using the Empower integration software (Waters, Milford, MA, USA).

The lucifer yellow samples were analysed by a Polarstar® fluorescence microplate reader with λ_{ex}=430 nm and λ_{em}=538 nm (BMG-Labtech, Aylesbury, United Kingdom).

Data analysis

For the Pgp inhibition studies, the amount of ^{3}H-digoxin transported from the basolateral to apical (b→a, "brain to blood") side of both monolayers of the MDCKII:MDR1 and the LLC-PK1:MDR1a was calculated in the presence and absence of either the vehicle, the Pgp inhibitor GF120918 or the opioid. The ^{3}H-digoxin transport inhibition factor was calculated as the amount of ^{3}H-digoxin transported from b→a in the presence of the opioid or GF120918, respectively, divided by the amount of ^{3}H-digoxin transported from b→a in the presence of the vehicle (control).

For the Pgp substrate assessment studies, the Pgp substrate efflux ratio was calculated by the amount of opioid or the Pgp substrate ^{3}H-amprenavir as the positive control being transported from basolateral to apical (b→a) side, divided by the amount of opioid or ^{3}H-amprenavir transported from apical to basolateral (a→b) side of the MDCKII:MDR1 monolayer. Involvement of Pgp mediated efflux was considered significant for values of the Pgp substrate efflux ratio >1.5 (Mahar Doan *et al.* 2002).
To exclude potential contributions of other transporters to this Pgp substrate efflux ratio, experiments were also performed in the presence of the Pgp inhibitor GF120918, for which this substrate efflux ratio should decease to 1, if the asymmetrical membrane transport of the opioid was indeed only caused by Pgp.

The transmembrane passive permeability rate; the "P_{app}" values

The apparent transmembrane passive permeability rate P_{app} (nm/sec) of the compounds was calculated using the equation:

$$P_{app} = -\left(\frac{V_R \cdot V_D}{(V_R + V_D) \cdot A \cdot t}\right) \cdot \ln\left\{1 - \frac{CR(t)}{\langle C(t)\rangle}\right\} \qquad (1)$$

where V_D and V_R are the donor (basolateral) and receiver (apical) chamber volumes (cm^3) respectively, A is the area of the monolayer (cm^2), t is the time after the start of the experiments (s), $C_R(t)$ is the drug concentration in the receiver (apical) chamber and

$\langle C(t) \rangle$ is described by equation 2:

$$\langle C(t) \rangle = \frac{V_D \cdot C_D(t) + V_R \cdot C_R(t)}{V_D + V_R} \quad (2)$$

where $\langle C(t) \rangle$ describes the average concentration of the compound on both sides of the monolayer, V_D and V_R are the donor (basolateral) and receiver (apical) chamber volumes (cm^3) and $C_D(t)$ and $C_R(t)$ are the donor (basolateral) and receiver (apical) concentrations at time t (Tran *et al.* 2004; 2005). This calculation of P_{app} takes into account the loss of drug from the donor compartment, which results in a better estimation of the P_{app}.

In all studies, the ^{3}H-amprenavir substrate efflux ratios, the apparent transmembrane passive permeability rate of lucifer yellow and the mass balance values were used as controls for the quality (integrity and Pgp functionality) of each monolayer.

The mass balance was calculated with the following equation:

$$\langle C(t) \rangle = \frac{V_D \cdot C_D(t) + V_R \cdot C_R(t)}{V_D + V_R} \quad (3)$$

where *%MB* is the mass balance, A_{rt} is the drug amount in receiver chamber at time (t), A_{dt} is the drug amount in donor chamber at time (t) and A_{d0} is the drug amount in the donor chamber at t=0.
The data from experiments were only included when the value for the Pgp substrate efflux ratio of ^{3}H-amprenavir was > 16, the value of the transmembrane passive apparent permeability rate of lucifer yellow < 50 nm/sec and the value for the mass balance > 70 %.

It should be noted that for nalbuphine literature values were used (Mahar Doan *et al.* 2002). The nalbuphine efflux ratio was determined at a donor concentration of 10 µM. The Pgp substrate efflux ratio for morphine was calculated at a donor concentration of 100 µM only, as at other concentrations no receiver concentrations were detectable. The apparent permeability rate for morphine was calculated at a donor concentration 100 µM, but without GF120918 and a→b.

Quantitative structure activity relationships – physico-chemical relationships
The Log octanol/water partition coefficients (cLogP) and molar refractivity values (CMR) were calculated using Daylight Software v4.71/82 (Daylight Chemical Information Systems Inc., Irvine, CA). Polar surface areas (PSA) were calculated according to Ertl and co-workers (Ertl *et al.* 2000). An *in silico* predictor of passive BBB transport was also determined based on the Abraham equation (Abraham *et al.* 1994):

$$LogBB = -0.038 + 0198 \cdot R_2 - 0.687 \cdot \pi_2^H - 0.715 \cdot \alpha_2^H - 0.698 \cdot \beta_2^H + 0.995 \cdot V_x \quad (4)$$

where *LogBB* is the logarithm of the blood-brain concentration ratio and R_2, π^H_2, α^H_2, β^H_2 and V_x are defined as the excess molar refractivity, dipolarity/polarisability, hydrogen bond acidity, hydrogen bond basicity and the solute McGowan volume, respectively, as described by Platts (Platts *et al.* 1999).

Statistical analysis

The data obtained from the studies on the inhibition of Pgp mediated efflux of ^{3}H-digoxin (the ^{3}H-digoxin transport inhibition factor) and on the Pgp substrate efflux ratio were analysed using an unpaired Student's t-test (two-tailed) or one-way analysis of variance (ANOVA) (Graphpad Instat®, version 3.00). A value of $p<0.05$ was considered a significant difference. Linear regression analysis of the P_{app}, cLogP, PSA and LogBB values was performed using S-plus (version 6.0 professional, release 1, Insightful corporation, USA) without a weight factor at a confidence level of 0.95. All data are expressed as mean ± SEM, unless indicated otherwise. Each experiment was performed in duplicate in at least three separate experiments.

RESULTS

Opioid inhibition of Pgp mediated efflux of ^{3}H-digoxin –"the ^{3}H-digoxin transport inhibition factor"

The inhibitory properties of the seven selected opioids and the morphine metabolites M3G and M6G were studied in MDCKII:MDR1 and LLC-PK1:MDR1a cells. Transport of ^{3}H-digoxin from the basolateral to the apical side of the monolayers was measured in the presence of vehicle, 2 mM GF120918 or 100 mM of the opioids. The results are shown in figure 2.

Alfentanil, fentanyl, sufentanil, morphine and loperamide were able to inhibit the Pgp mediated ^{3}H-digoxin transport in both cell-lines. In contrast, nalbuphine and butorphanol, M3G and M6G could not. According to the ^{3}H-digoxin transport inhibition factor, a ranking can be made. For the opioids the ranking (strong to weak) is as follows: fentanyl, sufentanil, loperamide, alfentanil, morphine and nalbuphine / butorphanol. This ranking was similar for both cell-lines. Furthermore, the inhibition of Pgp mediated efflux of ^{3}H-digoxin by the opioids was significantly weaker than by the Pgp inhibitor GF120918.

Pgp substrate assessment – "the Pgp substrate efflux ratio"

The next step was to determine the Pgp substrate efflux ratio of the opioids in the monolayers of the MDCKII:MDR1 cells. The results are shown in figure 3. No statistically significant differences were found in the Pgp substrate efflux ratios for alfentanil, fentanyl, sufentanil and butorphanol in the presence or absence of GF120918. For loperamide, a Pgp substrate efflux ratio of 4 was found. This efflux ratio decreased to 1.0 in the presence of GF120918, indicating that the asymmetrical transport was

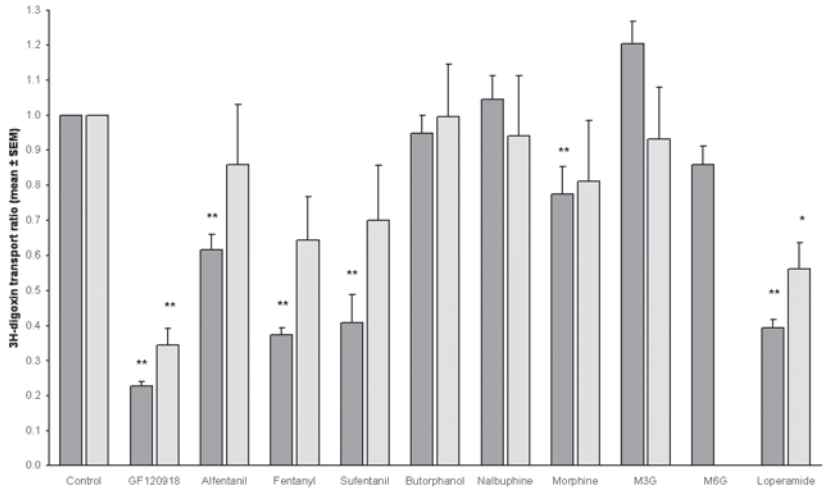

Figure 2: ^{3}H-digoxin transport inhibition factors in the presence of vehicle (control), 2 µM GF120918 or 100 µM opioid in MDCK:MDR1 (solid bars) and LLC-PK1:MDR1a cells (striped bars). The ^{3}H-digoxin transport inhibition factors are represented as a fraction of the control. Statistically significant differences were denoted as ** for P<0.01, * for P<0.05: for control versus opioid/GF120918.

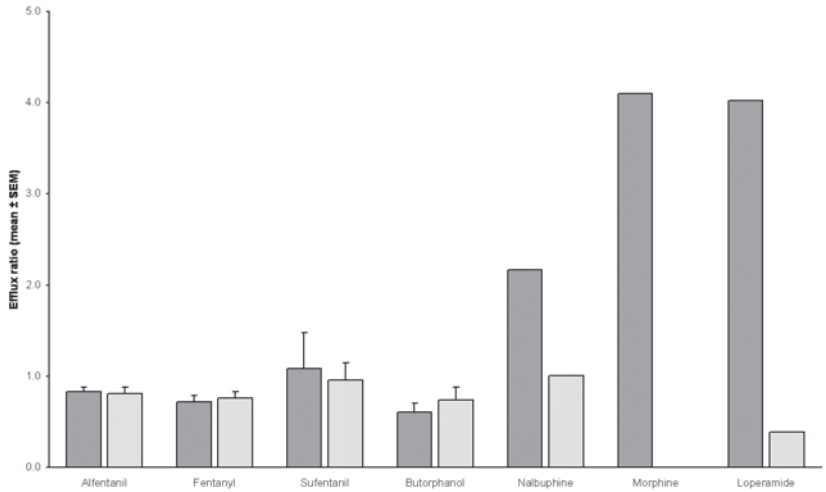

Figure 3: Assessment of opioid transport by Pgp. The Pgp substrate efflux ratios for the tested opioids, using the monolayers of MDCK:MDR1 cells. The efflux ratios were determined in the presence of vehicle (dark bars) as well as in the presence of 2 µM GF120918 (light bars). An efflux ratio larger than 1.5 was considered to reflect a significant involvement of Pgp in membrane transport. For nalbuphine literature values were used (Mahar Doan *et al.* 2002).

caused by Pgp solely. For morphine and nalbuphine the concentrations in the receiving compartment were below the limit of quantification of the assay when using the donor concentration of 3 µM. Therefore, the values obtained at a donor concentration of 10 or 100 µM for nalbuphine and morphine were used, respectively.

Apparent transmembrane passive permeability rates; the P_{app} values

The P_{app} values of the opioids were calculated on the basis of the amount transported across the monolayer over time (nm/s), in both directions, in the presence of GF120918. The results are shown in figure 4.

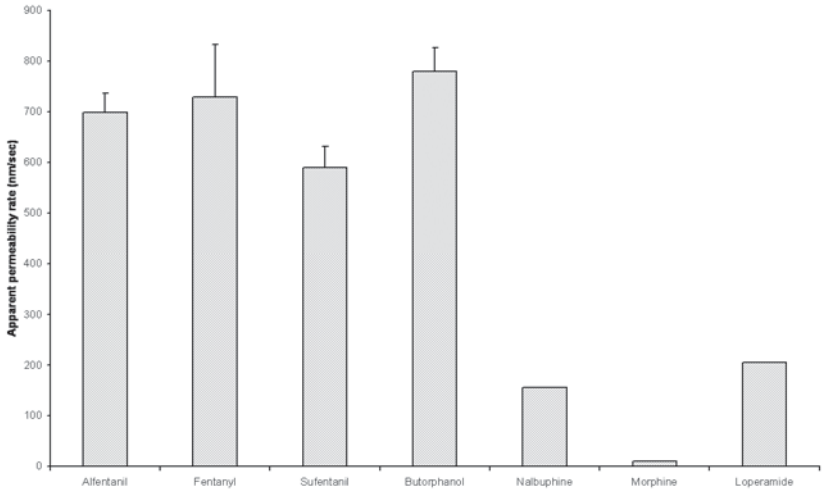

Figure 4: Calculated apparent transmembrane passive permeability rate values of the opioids across the MDCK: MDR1 monolayer, as determined at a donor concentration of 3µM of the opioid in the presence of the Pgp inhibitor GF120918. For nalbuphine literature values were used (Mahar Doan et al. 2002).

P_{app} values > 500 nm/s were found for alfentanil, fentanyl, sufentanil and butorphanol, whereas the P_{app} values of nalbuphine and loperamide were 156 and 206 nm/s, respectively. For morphine, the P_{app} value was 11 nm/s, indicating that almost no morphine is transported across the monolayer within the experimental period of 90 min.

Quantitative structure activity relationships: physico-chemical relationships

The values of P_{app}, cLogP, LogBB, PSA and CMR are listed in table 1. Regression analysis was performed to investigate the possible correlations between the apparent permeability and the physico-chemical properties of the selected opioids. Linear regression was performed with the P_{app} and $LogP_{app}$ values, but no significant correlations were found. However, the $LogP_{app}$ values gave slightly better correlations (figure 5).

Table 1: Physicochemical properties of opioids

			Abraham equation							
Name	**Mw**	**cLogP**	**R_2**	**π^H_2**	**α^H_2**	**β^H_2**	**V_x**	**LogBB**	**PSA**	**CMR**
Alfentanil	416.6	2.1	2.58	3.02	0	2.04	3.26	0.277	85.5	11.5
Fentanyl	336.5	3.6	2.08	2.21	0	1.23	2.84	0.886	23.6	10.4
Sufentanil	386.6	3.6	2.07	2.26	0	1.49	3.11	0.932	32.8	11.3
Nalbuphine	357.5	1.4	2.34	1.91	0.81	2.08	2.62	-0.247	73.2	9.6
Butorphanol	327.5	3.7	2.02	1.7	0.8	1.48	2.61	0.253	43.7	9.5
Morphine	285.4	0.6	2.1	1.68	0.55	1.76	2.06	-0.28	52.9	7.7
Loperamide	477.1	4.7	2.85	2.98	0.35	2.11	3.77	0.566	43.8	14.1

Abbreviations: Mw – molecular weight, cLogP – calculated LogP value, which is indicative for lipophilicity, R_2, π^H_2, α^H_2, β^H_2 and V_x – descriptors for Abraham equation, LogBB = BBB transport on basis of Abraham equation PSA = polar surface area (Å), and CMR = calculated molecular refractivity, which is indicative for size of molecule.

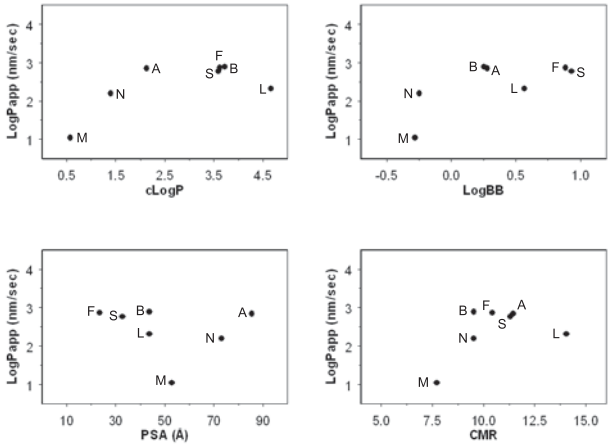

Figure 5: Correlation between the $LogP_{app}$ and the physicochemical properties of the opioids. The compounds are depicted with the first letter of the opioid name.

DISCUSSION

The main objective of these studies was to characterise the membrane transport characteristics of the opioids alfentanil, fentanyl, sufentanil, nalbuphine, butorphanol, morphine and loperamide. The focus was on the relative contributions of the passive membrane permeability rates and Pgp mediated active transport as mechanisms that determine the overall membrane passage. This is important for the development of a mechanism-based PK-PD model for the central effects of opioids, since opioids have to pass the membrane of the the BBB in order to reach their target and to exert their central effect.

Large differences were found between the opioids in the values for passive transmembrane permeability rates (P_{app}) and for the interaction with Pgp. The results show that the contribution of Pgp mediated transport should be taken into account for opioids with a low passive transmembrane permeability rate, whereas for opioids with a high passive permeability rate no significant contribution of Pgp mediated transport on membrane passage is to be expected.
The biophase kinetics of a CNS drug is an important determinant in the time course and intensity of its CNS effects. Apart from plasma pharmacokinetics, the mechanisms that determine CNS biophase kinetics include the rate and extent of BBB transport, and the kinetics of distribution and elimination within the brain. Transport across the BBB can be divided into passive and active transport processes (de Lange & Danhof 2002). Active transport processes are mediated by transporters, such as the Pgp, which has been shown to be an important efflux transporter at the BBB for many drugs. Therefore, the relative contribution of Pgp on overall BBB membrane transport is of interest. Our approach was to characterise the contribution of Pgp mediated transport, relative to passive membrane diffusion, on the overall membrane transport. Investigations were performed for the whole set of opioids, using a robust *in vitro* system composed of monolayers of Pgp-expressing cells. Monolayers of both MDCKII-MDR1 (transfected with the human MDR1 gene that encodes for Pgp) and of LLC-PK1:MDR1a cells (transfected with the mouse MDR1a gene) were used in these studies. These cell systems were chosen because of their very tight junction, which is similar to brain endothelia cells. If significant differences in Pgp mediated transport by the human and rodent gene products would be identified, these findings could be used to improve interspecies extrapolation of PK-PD relationships.

A number of *in vitro* studies has been performed with monolayers of LLC-PK1:MDR1 cells. However, these studies mainly focussed on morphine, loperamide and fentanyl (Schinkel *et al.* 1995, 1996; Wandel *et al.* 2002). Nalbuphine was only investigated once (Mahar Doan *et al.* 2002), whereas butorphanol has never been tested for Pgp interaction. For our ultimate goal, which is the development of a mechanistic PK-PD model for opioids, it was necessary to test all the selected opioids for Pgp interaction in the same experimental design. Moreover, unlike in previous studies, here the relative contribution of Pgp mediated efflux relative to the passive membrane transport component was explicitely addressed.

The inhibition of Pgp mediated efflux of ^{3}H-digoxin by the opioid (the ^{3}H-digoxin inhibition factor) was determined at a concentration of 100 µM of the opioid or 3 µM GF120918. The transport of ^{3}H-digoxin was measured from basolateral to apical direction (what would correspond with brain efflux). Efflux ratios were not calculated because the transport from apical to basolateral was very low and therefore the variability was between the wells was very high when efflux ratios were used. Inhibition of

Pgp mediated transport of ^{3}H-digoxin was found for alfentanil, fentanyl, sufentanil and loperamide, in accordance with the results found by Wandel and co-workers (Wandel *et al.* 2002). Interestingly, in our experiments morphine was identified as a Pgp inhibitor. In contrast, Wandel and co-workers (2002) did not show an inhibition of Pgp mediated efflux of ^{3}H-digoxin when using a 5-fold lower morphine concentration. This indicates that the interaction of morphine and Pgp is relatively weak. No inhibition of Pgp mediated efflux of ^{3}H-digoxin was found for nalbuphine, butorphanol and the morphine metabolites M3G and M6G. GF120918 inhibited the ^{3}H-digoxin efflux to 20%. Interestingly, the ^{3}H-digoxin transport ratio could not be completely inhibited by GF120918 although GF120918 is a very potent Pgp inhibitor. This indicates that other transporters might be involved in the efflux of ^{3}H-digoxin. In general, identical results were obtained using either the MDCKII:MDR1 or the LLC-PK1:MDR1a monolayers, though the variability in the LLC-PK1:MDR1a cells was a little higer. Taken together, these inhibition studies show that Pgp could influence the membrane transport characteristics of alfentanil, fentanyl, sufentanil, morphine and loperamide.

The Pgp substrate efflux ratio was determined for all opioids. For alfentanil, fentanyl and sufentanil no Pgp mediated transport could be found, in accordance with literature (Wandel *et al.* 2002). For loperamide a Pgp efflux ratio value of 4 was found, confirming that loperamide is a strong Pgp substrate (Schinkel *et al.* 1995, 1996; Wandel *et al.* 2002). For morphine and nalbuphine, the Pgp substrate efflux ratio was initially determined at a donor concentration of 3 µM. Under these conditions the concentrations in the acceptor phase were below the limit of quantification of the assay. Therefore, morphine has been investigated at a donor concentration of 100 µM and was clearly detected as Pgp substrate (with an efflux ratio of 4). For nalbuphine, literature values were included (Mahar Doan *et al.* 2002) to calculate a Pgp substrate efflux ratio value of 2. This would indicate that nalbuphine is a substrate for Pgp, while an absence of inhibition of Pgp mediated transport of ^{3}H-digoxin by nalbuphine was found. This apparent contradiction might be explained by the existence of several Pgp binding sites (Martin *et al.* 2000).

The P_{app} values of <50, 50-250, >250 nm/sec were considered as low, moderate and high, respectively. Thus, for morphine a low permeability was found; for loperamide and nalbuphine a moderate permeability, and for alfentanil, fentanyl, sufentanil and butorphanol a high permeability. For alfentanil, fentanyl and sufentanil the high P_{app} values explain why these opioids could not be identified as Pgp substrates. The potential contribution of Pgp mediated transport to the overall membrane transport is too low to be detected, and therefore not of any significance. In contrast, for loperamide and morphine, the P_{app} values were very low, which means that their Pgp mediated transport is of more much relevance in the overall membrane passage. To summarise, these results emphasise that the passive transmembrane permeability rates should be considered when investigating the impact of Pgp interaction on membrane transport.

Our interest was to predict the impact of BBB transport on the *in vivo* PK-PD relationships of opioids. In the current experiment *in vitro* cell systems have been used, made up from kidney epithelium cells, for its robustness. For an extrapolation of the results found in this study to the *in vivo* BBB transport characteristics it should be realised that the BBB is comprised of brain capillary endothelial cells, which may differ from the kidney epithelial cells used in this study in terms of membrane characteristics and paracellular spaces, while also the functionality of Pgp may differ. Such differences may explain why fentanyl, in a study using an *in vitro* cell-system comprising of brain capillary endothelial cells, could be identified as a borderline Pgp substrate (Henthorn *et al.* 1999). However, though small quantitative differences may exist between epithelial and endothelial membranes, the investigation of a whole set of opioids in a similar set-up will provide useful information on transport characteristics, which can be related to the differences observed in the *in vivo* PK-PD relationships. Therefore, based on the P_{app} values, it is to be expected that for morphine, loperamide and nalbuphine, passive transport across the BBB membrane plays a role in the PK-PD relation of centrally mediated effects. For alfentanil, fentanyl, sufentanil and butorphanol, a significant influence of BBB transport can be excluded. However, biophase pharmacokinetic processes like distribution within the brain should still be considered (Liu *et al.* 2005).

Based on the results presented here, a role of Pgp and BBB transport is expected *in vivo* for loperamide, morphine and potentially nalbuphine. Previously, the distribution of ^{3}H-loperamide has been investigated in MDR1a wild-type and knock-out mice. A 13.5 times higher concentration of ^{3}H-loperamide was found in the brain of knock-out mice compared with the wild-type (Schinkel *et al.* 1996). For morphine, Letrent and co-workers showed that the anti-nociceptive effect in rats was prolonged after oral administration of GF120918, due to its prolonged half-life of in the brain (Letrent *et al.* 1998, 1999a). For fentanyl, in MDR1a wild-type and knock-out mice it was shown that Pgp has a very small effect on the distribution and the anti-nociceptive effect (Dagenais *et al.* 2004;Thompson *et al.* 2000). However, it should be noted that variability in the anti-nociceptive data is relatively high and that the influence of Pgp on pharmacokinetics in blood and plasma was not considered in this study. For alfentanil, fentanyl, sufentanil and butorphanol no significant influence of Pgp and BBB transport on their central effects is expected.

In the last years, several *in silico* methods have been developed to predict passive BBB transport on the basis of physico-chemical properties, as reviewed by Clark and Norinder (Clark 2003; Norinder & Haeberlein 2002). These methods include the relationship between polar surface area and BBB penetration (Clark 1999; Kelder *et al.* 1999; Norinder & Haeberlein 2002; van de Waterbeemd *et al.* 1998). Abraham and co-workers have developed an *in silico* method that predicts passive BBB transport on the basis of several descriptors, including excess molar refraction, dipolarity/polarisability,

hydrogen bonding and size of the molecule (Abraham *et al.* 1994). Recently, it has been found that the dynamic polar surface area (non-linear) and logBB as calculated by the Abraham equation (linear) were significantly related to the *in vitro* BBB clearance values of a set of 11 structurally highly related adenosine A_1 receptor agonists (Schaddelee *et al.* 2003). It was therefore of interest to investigate the potential of *in silico* prediction of the *in vitro* values for passive membrane transport for the whole set of opioids in this study. Thus, the relationships between P_{app} values and physicochemical properties were investigated by regression analysis. No significant correlations could be identified. This may be explained by the limited number of opioids included in the present analysis. Moreover, these opioids can be divided in two structurally different groups (figure 1). Taken together, in this stage, no conclusions can be drawn about the predictability of BBB transport of opioids on the basis of physicochemical properties.

It is concluded that the relative contribution of both Pgp mediated transport and passive permeability across the BBB should be considered in the PK-PD relationships of opioids. For alfentanil, fentanyl, sufentanil and butorphanol, all having a high passive permeability value, membrane transport will be insensitive to Pgp. For nalbuphine, morphine and loperamide membrane transport is influenced by active Pgp transport, because of their low passive permeability membrane transport rates.

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