Lipophilicities of Baclofen Ester Prodrugs Correlate with Affinities to the ATP-Dependent Efflux Pump P-Glycoprotein: Relevance for Their Permeation across the Blood–Brain Barrier?*

Christiane Leisen,[1] Peter Langguth,[2] Bernd Herbert,[1] Cornelia Dressler,[1] Annette Koggel,[2] and Hilde Spahn-Langguth[1,3]

Received January 6, 2003; Accepted February 4, 2003

Purpose. Distribution to the effect site is a prerequisite for the therapeutic effect and determined by physicochemical properties and affinities to inside- and outside-directed membrane transporters. Based on the hypothesis that lipophilic esters of the GABA-derivative baclofen have a higher affinity to brain tissue, baclofen esters (methyl, ethyl, 1-propyl, 2-propyl, butyl) were studied regarding their penetration through the blood–brain barrier and their affinities to P-glycoprotein (P-gp).

Methods. Octanol-water distribution coefficients (D) served as lipophilicity parameters. Blood and brain concentrations of baclofen and its methyl ester were determined *in vivo* in rats following intraperitoneal administration. Affinities to P-gp were evaluated using a radioligand binding assay based on P-gp-overexpressing cells and [^{3}H]-talinolol as radioligand.

Results. Log D values for baclofen and ester derivatives were -0.96 (baclofen), 0.48 (methyl), 0.77 (ethyl), 1.31 (1-propyl), 1.27 (2-propyl), and 1.42 (butyl). In-vitro studies yielded negligible affinity of baclofen to P-gp, whereas IC_{50}-values for the esters ranged between 1300 μM (methyl) and 290 μM (2-propyl). Affinity parameters correlated well with the lipophilicity parameters.

Conclusions. Despite the P-gp affinity, brain concentrations of methyl ester were significantly higher than those of baclofen, however, baclofen levels following administration of the ester were smaller than with baclofen administration indicating only partial hydrolysis.

KEY WORDS: radioligand binding assay; octanol-water distribution coefficient; baclofen; P-glycoprotein; prodrug.

INTRODUCTION

The centrally acting antispastic agent baclofen [R/S-4-amino-3-(4-chlorophenyl)butyric acid], a γ-amino butyric acid analog, was experienced to exhibit high therapeutic activity and a high therapeutic value in certain – even severe – spastic disorders (1). The effect correlates well with the doses and the concentrations present at the site of action (2). However, therapeutic levels (necessary for these particular indications) are not reached in the central nervous system (CNS), unless the drug is administered intrathecally. Concentrations in the cerebrospinal fluid (CSF) following p.o. dosage of 100 mg rac-baclofen are below the detection limit (<3 ng/g) at plasma levels of 305–720 ng/g. Intrathecal administration of bolus doses of up to 600 μg or infusions at rates of 50–1200 μg/d result in plasma concentrations of 5–20 ng/g or –5 ng/g, respectively, at high local levels. These studies clearly revealed the lack of a significant mutual exchange between drug in the blood- and in the CNS compartment.

In general, for CNS-active agents the extent of distribution to the effect site is a prerequisite for their therapeutic effect and is determined by their physicochemical properties as well as their affinities to inside- and outside-directed transporters located in the blood–brain barrier (BBB; Refs. 3–6). In the brain, capillary fenestration is lacking, and—unless carriers are involved—this barrier is characterized by low permeability for hydrophilic agents. Transporters known to be present in the BBB are, e.g., amino acid and glucose transporters for inside-directed (7–9) and P-glycoprotein (P-gp) for outside-directed transport (10–12). With respect to prodrugs of a higher lipophilicity derived from active compounds with carboxylic functions, ester formation represents a well-known approach. Known prodrug strategies include the esterification of ganciclovir with the amino acid valine (13), the formation of bisphosphonate prodrugs (14) or the formation of ester prodrugs of β-lactam antibiotics (15). Deguchi *et al.* (16) synthesized 1,3-diacetyl-2-ketoprofen glyceride, as potential prodrug of the anti-inflammatory drug ketoprofen and achieved a higher brain uptake than with the parent drug.

Based on the assumption that the ester prodrugs of the gamma-amino butyric acid derivative baclofen would readily be cleaved in the tissue releasing active baclofen at the site of action, baclofen and baclofen methyl ester were compared regarding their ability to penetrate through the blood-brain barrier. Molecular modeling studies with baclofen and its esters indicated that the esters should not have any affinity to pharmacologically relevant binding sites (17) and should, hence, be negligible regarding their CNS and peripheral activity. Their affinities to the major secretory transporter P-gp were studied using a radioligand binding assay considerably modified from Döppenschmitt *et al.* (18).

MATERIALS AND METHODS

Cell Culture

Caco-2 cells (P 58-61) were cultured in Dulbecco's modified Eagle's medium containing glutamine, 20% fetal bovine serum, 1% nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 IE /mL penicillin, and 10 nM vinblastine sulphate (Velbe™, Lilly, Bad Homburg, Germany). All media were purchased from Biochrom KG (Berlin, Germany). The cells were trypsinized weekly using trypsin/EDTA solution (0.25% / 0.02%) and seeded in 75-cm^2 cell culture flasks (Greiner, Frickenhausen, Germany) at a density of five million cells per flask. Cells were grown at 37°C in an atmosphere containing 95% air and 5% CO_2 with 100% relative humidity. After 7 to 10 days the cells were used in the radioligand binding assay (RBA).

* Dedicated to Professor Dr. Peter Nuhn on the occasion of his 65th birthday.

[1] Department of Pharmaceutical Chemistry, Martin-Luther-University Halle-Wittenberg, Wolfgang-Langenbeck-Strasse 4, D-06120 Hall Saale.

[2] School of Pharmacy, Johannes Gutenberg-University, Staudingerweg 5, D-55099 Mainz.

[3] To whom correspondence should be addressed. (e-mail: HSpahnLangguth@impp.de)

0724-8741/03/0500-0772/0 © 2003 Plenum Publishing Corporation

RBA

For the RBA, the medium was removed from the cell culture flasks and the cell-monolayer was washed twice with 5 mL of phosphate-buffered saline. The cells were trypsinized with 2 mL of trypsin/EDTA (0.25%/0.02%). After 30 min, trypsinization was stopped by addition of 18 mL of complete medium. Cells were individualized, counted with a hemocytometer, and separated from the medium by centrifugation (10 min, 23°C, 250 × g).

The cells were washed once with phosphate-buffered saline (20 mL) and resuspended in a 0.01% solution of L-α-lysophosphatidylcholine in Hank's balanced salt solution supplemented with 10 mM 2-morpholinoethanesulfonic acid monohydrate and adjusted to pH 7.0 to a concentration of one million cells per 125 μL (cell suspension). Cell suspension was immediately used in the radioligand binding assay. Solutions of all compounds (baclofen esters, or the parent drug baclofen, respectively) were prepared in Hank's balanced salt solution /2-morpholinoethanesulfonic acid monohydrate 10 mM (pH 7.0) (incubation buffer). The RBA was conducted on 96-well plates. Each incubation mixture consisted of 100 μL of compound solution (different concentrations), 25 μL of radioligand solution (radioligand: 1 μM talinolol containing 20% [^{3}H]-talinolol, (Kix, Volxheim, Germany; www.isotopes.de), and 125 μL of cell suspension (see above). [^{3}H]-Talinolol was chosen as radioligand because of its low non-specific binding. Following incubation and shaking at 1200 rpm/min for 30 min at room temperature, the solutions were filtered through glass fiber filters (Dunn, Asbach, Germany) in a Brandel-Cell-Harvester (Gaithersburg, MD, USA) and unbound radioligand was removed by purging the filters with 5 mL of ice-cold 0.9% sodium chloride solution in bidistilled water. Thereafter, the filters were dissolved in 4 mL of scintillation fluid (Rotiszint 22, Roth, Karlsruhe, Germany) and bound radioactivity was counted in a liquid scintillation counter (Beckman Coulter, Unterschleissheim, Germany).

In Vivo Rat Study Investigating the Disposition Characteristics of Baclofen vs. Baclofen Methylester

Investigations in animals were approved by the respective local ethics committee for animal studies (Kanton Zürich, Regierungspräsidium Dessau/Sachsen-Anhalt). Studies were performed in male White Wistar rats (n = 3), which were anaesthesized in diethylether atmosphere prior to intramuscular application of the narcotic urethane (50%; g/g in sterile sodium chloride solution (0.9%), dose: 1500 mg/kg body weight). 60 min later the respective drug was administered to the rat. Equimolar doses of baclofen (1.0 mg/kg body weight) and baclofen methyl ester hydrochloride (1.24 mg/kg body weight) were administered intraperitoneally. After administration of the single dose, blood and tissue sampling was performed at 5, 20, and 60 min post-dose. Blood samples were immediately frozen and stored at –20°C until analysis. For baclofen methyl ester the blood samples were stabilized to prevent hydrolysis of the esters by addition of NaF (10 g/L) as esterase inhibitor and oxalic acid (2 g/L) according to Baselt *et al.* (19).

To rule out an effect of urethane as *in vivo* esterase inhibitor, studies including methyl ester dosage were repeated employing a different experimental set-up with permanently cannulated and conscious rats. Release of baclofen from its potential ester precursor was compared between the two experimental set-ups.

Brain samples were used without perfusion. The small contribution (approx. 2–5%) to tissue levels by blood that is left in the brain vasculature was assumed to be negligible. Tissue samples were immediately homogenized with tyrode buffer (1:1; m/m) and stored at –20°C as well.

Compounds and Reagents

Baclofen was a gift from Ciba-Geigy (Basel, Switzerland). DL-Chlorophenylalanine, naphthyl ethyl isocyanate, triethylamine and ethanolamine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *o*-Phthaldialdehyde, *N*-acetyl-L-cysteine and buffer components were obtained from E. Merck (Darmstadt, Germany). All other reagents were of analytical grade and all solvents were of high-performance liquid chromatography grade. Water was distilled prior to use.

Synthesis of Baclofen Esters

As described by Herber (17) baclofen esters were synthesized by reaction of baclofen (1.5 g; 7.0 mM) with methanolic hydrochloric acid (2 mol/L; 70 mL) for 24 h at ambient temperature (Fig. 1). Excess HCl gas was removed by refluxing the reaction mixture for 1h. Structure confirmation of the synthesized ester derivatives was based on NMR and MS analyses. Chromatographic purity was >99%. The esters were used as the respective hydrochloride salts.

Octanol–Water Distribution Coefficients (O/W-DC) as Lipophilicity Parameter

For the determination of the octanol-water distribution coefficient baclofen and baclofen esters were dissolved in 0.1 M KH_2PO_4-buffer (adjusted to pH 7.3 with 0.1 M NaOH). Following partitioning between buffer and octanol (1:1; v/v) during 30 min on a horizontal shaker and phase separation by centrifugation (10 min, 0°C, 4000 rpm) the extinction (E) of the aqueous phase was measured at 220 nm using a UV/VIS-spectrometer. Extinction was measured against an octanol-saturated buffer solution to correct for a potential UV absorption of octanol.

Analytical Conditions for Baclofen and Its Methyl Ester

Extraction and Derivatization Procedures

Baclofen Blood Samples. Protein precipitation was performed by centrifugation at 4000 rpm for 10 min (0°C) fol-

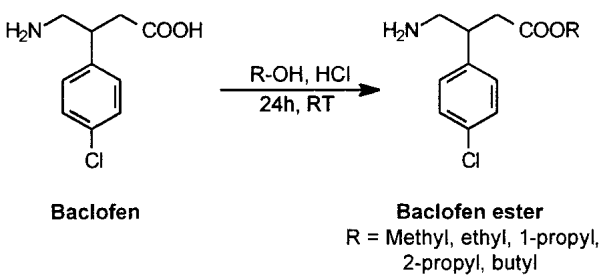

Fig. 1. Baclofen structure and preparation of the respective ester prodrugs.

lowing addition of 25 ng chlorophenylalanine as internal standard (i. st.) and 0.75 mL methanol to 100 mg aliquots of blood samples and following extensive vortexing for 30 s. To the supernatant *o*-phthaldialdehyde (OPA) and *N*-acetyl cysteine (NAC) were added and derivatization was performed at ambient temperature yielding fluorescent isoindole derivatives, which were immediately assayed via HPLC (reaction time 5 min). The stationary phase consisted of a Zorbax C8 column (5 μm, 25 × 0.4 cm; Bischoff Chromatography, Leonberg, Germany) equipped with a LiChrocart C8-guard column cartridge. The mobile phase consisted of a mixture of 1/15 M phosphate buffer (pH 6.6), methanol, and THF (45:50:2.5) (v/v/v) and was delivered at a flow rate of 0.5 mL/min. Fluorescence of the eluate was monitored at 345 nm (excitation) and 443 nm (emission). Average retention times were as follows: baclofen 10.7 min, DL-chlorophenylalanine 17.6 and 20.0 min. Variability of the assay was in the range of 2-10% for the whole linear range (blood: 3400–68 ng/g; brain: 336–68 ng/g), the limit of quantification was 25 ng/g for blood and 15 ng/g for brain samples.

Baclofen Brain Samples. For 1000-mg brain samples (500 mg brain tissue as 1:1 homogenate with tyrode solution) an additional solid-phase extraction was necessary (Baker-bond spe™ Octadecyl, 3 mL, J.T. Baker, Deventer, Netherlands). The method required a three times repeated conditioning of the solid-phase extraction cartridges with 3.0 mL of methanol and was followed by addition of 3 mL of water (twice) prior to transfer of the sample to the cartridge. Subsequently the cartridge was washed with 4.0 mL water, dried, and washed with 0.25 mL of methanol. Elution was carried out with 0.5 mL methanol. The eluate (90 μL) was used for the derivatization and analysis of the sample. The recovery of baclofen in brain samples following solid phase extraction was determined to be 68%. Analytical conditions (chromatography) were identical for blood and brain samples.

Methyl Ester Blood Samples. Derivatization of baclofen esters using the OPA/NAC method turned out to be impossible, probably due to steric hindrance by the ester group (17). Thus an alternative bioanalytical HPLC-method needed to be developed: Due to the lack of the zwitterionic structure of baclofen esters when compared to baclofen, extraction into an organic layer was possible:

After the addition of 500 ng baclofen isopropyl ester as internal standard to a 200-mg sample aliquot, extraction was conducted on a horizontal shaker (15 min) using 3.0 mL of dichloromethane. The mixture was centrifuged for 5 min (4°C, 4500 rpm) and the organic layer was evaporated to dryness (+50°C, N_2). The dried residue was reconstituted in 50 μL of a triethylamine solution (1% in methanol) and 25 μL of naphthyl ethyl isocyanate solution (0.1% in toluene). The derivatization reaction was performed for 30 min at ambient temperature, stopped by addition of 50 μL of ethanolamine solution (1% in methanol), evaporated to dryness, reconstituted in methanol and finally injected into the HPLC system. HPLC conditions were as follows: As stationary phase a Zorbax C8 column (5 μm, 25 × 0.4 cm) equipped with a LiChrocart C8-guard column cartridge was used. The mobile phase consisted of a mixture of methanol, water, and THF (710:276:14; v/v/v) and was delivered at a flow rate of 1.2 mL/min. Fluorescence of the eluate was monitored at 234 nm (excitation) and 333 nm (emission). Variability of the assay was in the range of 2-10% for the whole linear range (blood: 2500–50 ng/g; brain: 2000–50 ng/g), the limit of determination was 20 ng/g for blood and 25 ng/g for brain samples.

Methyl Ester Brain Samples. Baclofen isopropyl ester (500 ng; i. st.) and 2.0 mL of pH 2.5 phosphate buffer were added to a centrifuged 800-mg aliquot of tissue homogenate (1:1, with tyrode solution). After extraction using 2.0 mL of dichloromethane and centrifugation (5 min, 4°C, 4500 rpm), the organic layer was discarded. 1% sodium bicarbonate solution (1 mL) and 2.0 ml of dichloromethane were added to the aqueous layer. This organic layer was separated after centrifugation and evaporated to dryness (+50°C, N_2). The subsequent procedure was identical for brain and blood tissues (see above).

Calculations

DC Estimations

O/W-DCs were calculated according to:

$$DC = \frac{E_{\text{organic layer}}}{E_{\text{aqueous layer}}}$$

RBA Studies

For the determination of P-gp affinity of nonlabeled substances, the displacement of the radioligand [^{3}H]-talinolol by increasing concentrations of the test compound was used. All curve fittings and nonlinear regression analysis were performed using Origin™ version 6.0 (OriginLab Corp., Northampton, MA, USA) applying the following equation:

$$y = y_0 + \frac{a * x^b}{c^b + x^b}$$

where y_0 and a are the displacement for the minimal substrate concentration (0%) and the maximal substrate concentration (100%), respectively, b is the Hill coefficient, and c the IC_{50} value (μM).

Because alprenolol showed a nearly complete displacement of the radioligand at high concentrations (30 mM) it was used to estimate the amount of nonspecific binding and to calculate specific binding:

$$\text{Specific binding} = \text{total binding} - \text{nonspecific binding}$$

Statistical comparisons of mean values were performed using the Student t test and a p value of < 0.05 was considered statistically significant. IC_{50} is defined as the concentration of a compound that inhibits radioligand binding by 50%. Verapamil and talinolol were included as reference compounds in addition to baclofen and its analogs.

In Vivo Studies

The brain-to-blood ratio served as a measure for altered tissue selectivity, since this value is normalized by the respective blood level.

Statistical Calculations

From all single values, arithmetical means and standard deviations (SD_{n-1}) were calculated. Statistical significance of correlations was estimated by performing a linear regression

Table I. Octanol–Water Distribution Coefficients (O/W-DC, Arithmetical Means ± SD) and Respective Log D Values of Baclofen Esters as Well as IC_{50} Values Obtained in the P-Glycoprotein Radioligand Displacement Studies for the Displacement of [^{3}H]-Talinolol

	Number of carbon atoms in side chain	O/W-DC	log D	IC_{50} [μM]
Baclofen	0	0.11	–0.96	4492
Methyl ester	1	3.0 ± 0.3	0.48	1278
Ethyl ester	2	5.9 ± 0.2	0.77	1055
1-Propyl ester	3	20.2 ± 1.6	1.31	315
2-Propyl ester	3(iso)	18.6 ± 0.8	1.27	292
n-Butyl ester	4	26.4 ± 1.1	1.42	430
Talinolol				703
Verapamil				76.7

Note: All compounds were used as racemates.
Values represent means of three repetitive determinations.

analysis and testing the correlation coefficient for statistical significance ($p < 0.05$).

RESULTS

Structural Characteristics, Lipophilicities, and P-gp Binding Parameters

Baclofen as well as baclofen esters were readily soluble in water. Hence, *in vitro* studies were performed without special pretreatment of the compounds and without addition of cosolvents. As expected, O/W-DCs and derived log Ds increased with increasing chain-lengths (Table I). The relationship between the lipophilicity parameter and the length of the ester side-chain was analyzed by linear regression analysis. A clear positive correlation was found with correlation coefficients of $r^2 = 0.9350$ without and $r^2 = 0.8643$ with baclofen. Figure 2 depicts the correlation of log D values and the number of C atoms in the side-chain of baclofen and baclofen ester. Statistical significance was found ($p < 0.05$).

In the radioligand binding studies, almost complete displacement (down to nonspecific binding) of [^{3}H]-talinolol was observed at high displacer levels. The average value for nonspecific binding was 19.4% in the case of baclofen butyl ester under the described experimental conditions. For baclofen and its esters, non-specific binding was generally in the range of 18–25%. Data variability (intraassay) was small for baclofen specimen and one of the two reference compounds, verapamil, while some extent of variability was detected for talinolol. The concentration-dependent displacement of the radioligand allowed the determination of the IC_{50} values for baclofen and its esters, for which typical inhibition curves resulted (Fig. 3A). The *in vitro* studies yielded negligible affinity of baclofen to P-gp. Displacement of [^{3}H]-talinolol was achieved at lower displacer concentrations for the more lipophilic baclofen derivatives indicating higher affinities to the macromolecular binding site and yielding the following ranking with respect to IC_{50} values, which are summarized in Table I:

Verapamil > butyl ester > 2-propyl ester > 1-propyl ester > talinolol > ethyl ester > methyl ester > *baclofen*

This ranking indicates a relationship between IC_{50} value and lipophilicity. The correlation between IC_{50} and log D is depicted in Fig. 3B, including the correlation parameters obtained with linear regression analysis. The correlation coefficients were $r^2 = 0.9321$ without baclofen and $r^2 = 0.9682$ with the low affinity P-gp-substrate baclofen. Statistical significance of the correlation between IC_{50} and log D was found at $p = 0.05$ according to the rank correlation coefficient of Spearman (20).

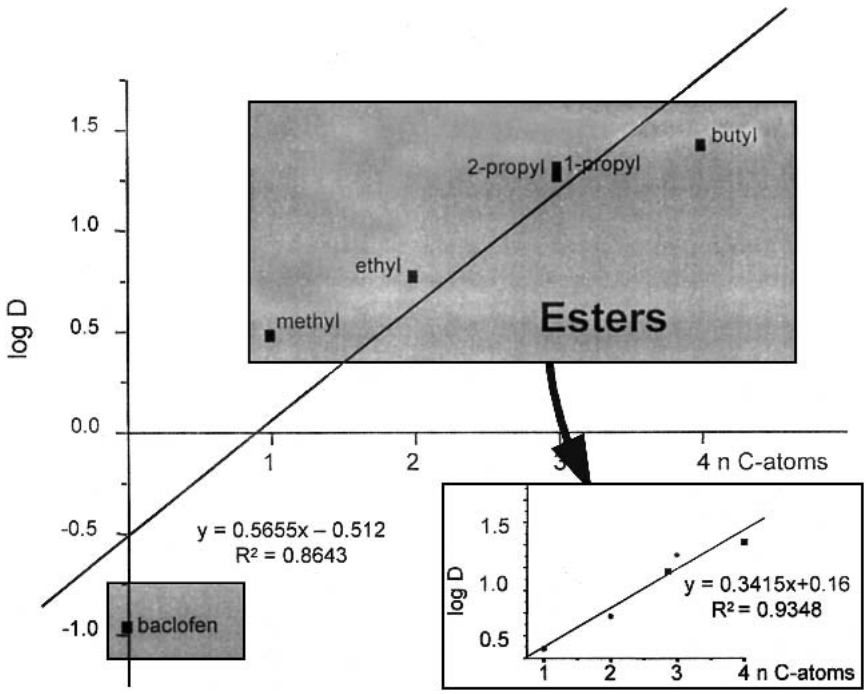

Fig. 2. Correlation of log D and side-chain length.

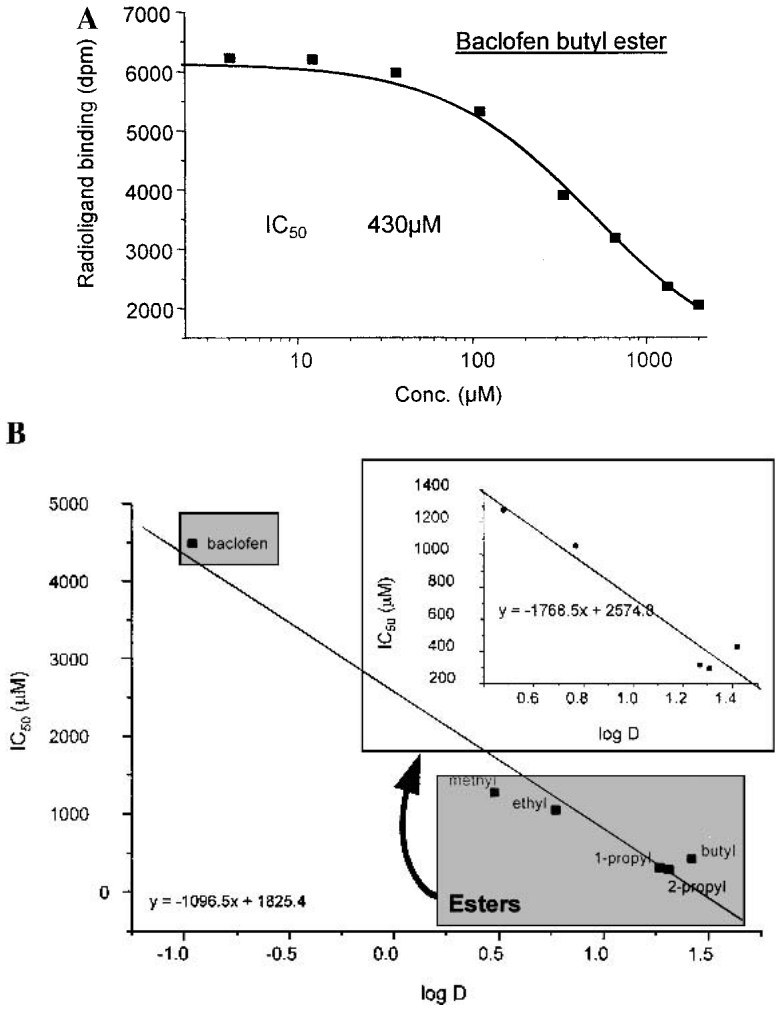

Fig. 3. (A) P-glycoprotein (P-gp) assay. Displacement of the radioligand [^{3}H]-talinolol from binding in P-gp overexpressing Caco-2 cells by increasing levels of butyl ester (example). Non-specific binding amounted to 1254 dpm in this experiment (19.4% of total). (B) Correlation of P-gp affinities and lipophilicities: IC_{50}s vs. log Ds. Esterification, at physiologic pH, of the zwitterionic baclofen removes one of the ionizable groups in the molecule. The "true" comparison in the homologous series would be the protonated (at the carboxyl group) baclofen, which is not possible under given experimental conditions. Therefore, correlation coefficients in the linear regresssion analysis are given with and without baclofen.

Brain Concentration–Time Profiles and Tissue Selectivity: Baclofen vs. Baclofen Methyl Ester

The preliminary concentration-time profiles obtained with baclofen and its methyl ester in blood and brain after intraperitoneal dosage showed a considerably better penetration into the brain for the more lipophilic ester. Ester concentrations in blood were lower and those in the brain were considerably higher for baclofen methyl ester than for baclofen when ester or baclofen, respectively, were dosed (Fig. 4).

In the course of these studies, release of active baclofen from its potential prodrug was also measured. Baclofen blood levels following methyl ester dosage were significantly lower than either the concentrations of parent methyl ester or baclofen levels after application of baclofen, respectively.

The release of baclofen from its potential precursor yielded maximum baclofen levels in blood of 141 ng/g without and 150 ng/g with urethane at 20 min post dose.

In the brain, maximum baclofen concentrations following baclofen methyl ester administration (data not included in Fig. 4) ranged between the limit of quantification, where traces were detectable, and 51 ng/g, while those after baclofen dosage were between the LOQ and 87 ng/g.

Thus, formation of the methyl ester from baclofen had a pronounced effect on tissue selectivity, yielding tissue-to-blood ratios significantly higher for this baclofen derivative than for parent baclofen (Fig. 4), but did not lead to increased baclofen brain concentrations.

DISCUSSION

The fact that baclofen itself does not appear to permeate into the CNS to a sufficient extent was demonstrated in various clinical studies, but is still regarded as surprising, since baclofen was previously found to be a substrate of the neutral amino acid transporter (21), which should also be present in the BBB (3).

In the experimental setup of the present studies, brain tissue collected for the assays was not perfused prior to homogenization, to remove blood left in the vasculature. It must be anticipated that the 2–5% blood remaining is not completely negligible in the case of compounds with low brain

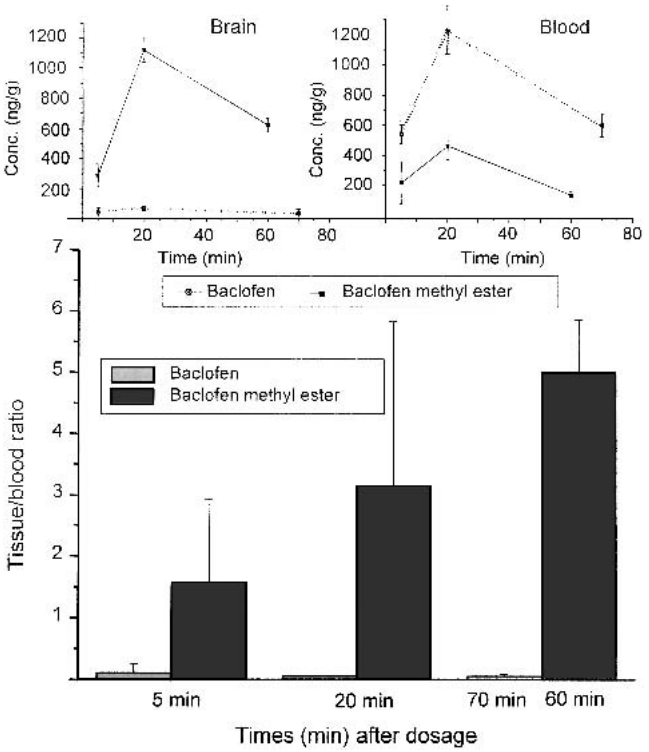

Fig. 4. Top, Preliminary brain and blood profiles for baclofen and its methyl ester as well as baclofen blood levels following methyl ester application Bottom, Brain tissue-to-blood concentration ratios for baclofen and baclofen methyl ester after intraperitoneal application in rats (n = 3, means ± SD) show increased tissue selectivity for the methyl ester of baclofen if compared with baclofen.

penetration (as in the case of parent baclofen). For the ester, which exhibits higher brain levels this does not appear to be relevant.

Stereoselective transport of baclofen through the blood-brain barrier was previously detected, which is in consistence with an active inside-directed transport system (22). Furthermore, it was previously hypothesized that rapid vesicular uptake would reduce the extracellular fluid levels including CSF levels. Baclofen concentrations after intravenous application were 27-fold higher in plasma than in ISF, whole brain tissue and CSF in microdialysis studies (23). It was also hypothesized that the limited distribution of baclofen to the CNS might be due to the affinity to an effective probenecid-sensitive efflux system for organic anions (23). Esterification of baclofen, as it was performed in these studies, eliminates the free carboxyl structure, which appears necessary for affinity to organic anion transporters. In addition to the increased lipophilicity this might serve as explanation for higher brain ester concentrations.

The P-gp binding studies performed would suggest that also the baclofen esters might have problems overcoming the BBB because they may be extruded by the exsorptive transporter P-gp. The enhanced lipophilicity of the baclofen esters generates a higher affinity to the secretory transporter P-glycoprotein, which is also present in the BBB. Nevertheless, the methyl ester readily penetrates into the brain. The P-gp affinity of the methyl ester is lower than for the more lipophilic esters. From these data it is concluded that P-gp in the BBB does not play the major role in brain penetration of this compound. This indicates again that the balance between P-gp affinity and -presumeably passive-membrane permeability determines the fraction of drug entering a particular compartment in spite of the presence of transporters in the respective barrier. Also for other clusters of structurally closely related compounds lipophilicity was revealed as major factor determining the affinity to the verapamil binding site of P-gp, with yet quantitatively undefined impact on the BBB transport. Comparable results were obtained for, e.g., a series of β-adrenoceptor antagonists and a number of CNS-active compounds (24). In the previous publication (18) this interrelationship was already discussed. It was concluded that the P-gp affinity of a compound might not affect its distribution into tissue compartments, when its passive permeability, which mainly depends on lipophilicity and size (molecular weight), is high. This appears to be valid for the investigated group of baclofen congeners as well: *In vivo*, mainly because of the increasing lipophilicity, esterification of baclofen resulted in better brain penetration of baclofen methyl ester as shown by higher brain-to-blood ratios. The high lipophilicity appears to more than compensate for outside-directed transport in the BBB. The high total brain concentrations, which are significantly higher than blood concentrations, indicate an increased affinity of the methyl ester to the lipophilic brain tissue.

The apparent extent of baclofen back-formation from esters *in vivo* in the blood and particularly in the brain was smaller than expected from *ex vivo*/*in vitro* hydrolysis data (17). Additional studies performed showed that the experimental conditions do not affect the extent of hydrolysis, which is identically low for the baclofen methyl ester under different surgical conditions, i.e., with urethane anesthesia or under permanent cannulation without anesthesia in conscious rats.

It might be expected that the small sized baclofen esters with longer side-chains than methyl reach even higher concentrations in the target structure than the parent compound baclofen or its methyl ester. *In vivo* release of baclofen, from its esters and an optimization of the nature of the respective potential prodrug is subject of additional investigations.

Furthermore, it is expected that—as opposed to the poorly protein bound baclofen—the esters exhibit higher protein binding. Further additional studies include an estimation of plasma protein binding, to be able to correlate free CSF levels with unbound blood levels because free concentrations are in equilibrium with CSF and possibly also with brain extracellular fluid levels and, hence, free levels are a more suitable comparator. Disposition studies, including CSF, have been performed for all esters (Herber, 2002) and will be subject of a separate publication.

ACKNOWLEDGMENTS

The present work was supported by a grant of the FCI (Fonds der Chemischen Industrie, Frankfurt, Germany) to H. S.-L. The authors acknowledge the opportunity to perform animal work at the facility for animal studies at the ETH Zürich.

REFERENCES

1. H. Müller, J. Zierski, D. Dralle, O. Hoffmann, and G. Michaelis. Intrathecal baclofen in spasticity. In: H. Müller, J. Zierski, and R. D. Penn (eds.) *Local-Spinal Therapy of Spasticity,* Springer Verlag Berlin, Heidelberg, New York, 1988 pp. 155–214.

2. H. Müller, J. Zierski, D. Dralle, D. Krauß, and E. Mutschler. Pharmacokinetics of intrathecal baclofen. In: H. Müller, J. Zierski, and R. D. Penn (eds.) *Local-Spinal Therapy of Spasticity*, Springer Verlag Berlin, Heidelberg, New York, 1988 pp. 223–226.
3. Y. Kido, I. Tamai, H. Uchino, F. Suzuki, Y. Sai, and A. Tsuji. Molecular and functional identification of large neutral amino acid transporters LAT1 and LAT2 and their pharmacological relevance at the blood-brain barrier. *J. Pharm. Pharmacol.* **53**: 497–503 (2001).
4. H. Matsuo, S. Tsukada, T. Nakata, A. Chairoungdua, D. K. Kim, S. H. Cha, J. Inatomi, H. Yorifuji, J. Fukuda, H. Endou, and Y. Kanai. Expression of a system L neutral amino acid transporter at the blood-brain barrier. *NeuroReport* **11**:3507–3511 (2000).
5. I. Tamai and A. Tsuji. Transporter-mediated permeation of drugs across the blood-brain barrier. *J. Pharm. Sci.* **89**:1371–1388 (2000).
6. I. A. Simpson, N. M. Appel, M. Hokari, J. Oki, G. D. Holman, F. Maher, E. M. Koehler-Stec, S. J. Vannucci, and Q. R. Smith. Blood-brain barrier glucose transporter: effects of hypo- and hyperglycemia revisited. *J. Neurochem.* **72**:238–247 (1999).
7. P. Jolliet-Riant and J. P. Tillement. Drug transfer across the blood-brain barrier and improvement of brain delivery. *Fundament. Clin. Pharmacol.* **13**:16–26 (1999).
8. R. J. Boado, J. Y. Li, M. Nagaya, C. Zhang, and W. M. Pardridge. Selective expression of the large neutral amino acid transporter at the blood-brain barrier. *Proc. Natl. Acad. Sci. USA* **96**:12079–12084 (1999).
9. E. M. Cornford, S. Hyman, and B. E. Swartz. The human brain GLUT1 transporter: ultrastructural localization of the blood-brain barrier endothelia. *J. Cereb. Blood Flow Metab.* **14**:106–112 (1994).
10. M. F. Fromm. P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs. *Int. J. Clin. Pharmacol. Ther.* **38**:69–74 (2000).
11. J. Bart, H. J. Groen, N. H. Hendrikse, W. T. van der Graaf, W. Vaalburg, and E. G. de Vries. The blood-brain barrier and oncology: new insights into function and modulation. *Cancer Treat. Rev.* **26**:449–462 (2000).
12. P. L. Golden and W. M. Pardridge. Brain microvascular P-glycoprotein and a revised model of multidrug resistance in brain. *Cell. Mol. Neurobiol.* **20**:165–181 (2000).
13. M. Sugawara, W. Huang, Y. J. Fei, F. H. Leibach, V. Ganapathy, and M. E. Ganapathy. Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2. *J. Pharm. Sci.* **89**:781–789 (2000).
14. R. Niemi, P. Turhanen, J. Vepsalainen, H. Taipale, and T. Jarvinen. Bisphosphonate prodrugs: synthesis and in vitro evaluation of alkyl and acylmethoxy esters of etidronic acid as bioreversible prodrugs of etidronate. *Eur. J. Pharm. Sci.* **11**:173–180 (2000).
15. L. Mizen and G. Burton. The use of esters as prodrugs for oral delivery of beta-lactam antibiotics. *Pharm. Biotech.* **11**:11345–11365 (1998).
16. Y. Deguchi, H. Hayashi, S. Fujii, T. Naito, Y. Yokoyama, S. Yamada, and R. Kimura. Improved brain delivery of a nonsteroidal anti-inflammatory drug with a synthetic glyceride ester: a preliminary attempt at a CNS drug delivery system for the therapy of Alzheimer's disease. *J. Drug Target.* **8**:371–381 (2000).
17. B. Herber. Brain distribution of drugs – Correlation with lipophilicity and dependence on the administration route. PhD thesis, Martin-Luther-University Halle-Wittenberg, Halle/S., Department of Pharmacy (2002)
18. S. Döppenschmitt, H. Spahn-Langguth, C. G. Regardh, and P. Langguth. The role of P-glycoprotein mediated secretion in absorptive drug fluxes. *J. Pharm. Sci.* **88**:1067–1072 (1999).
19. R. C. Baselt, D. Yoshikawa, and J. Chang. and J. Li. Improved long-term stability of blood cocaine in evacuated collection tubes. *J. Forensic Sci.* **38**:935–937 (1993).
20. L. Sachs. *Applied Statistics*. Springer-Verlag, Berlin, Heidelberg, New York, 1978.
21. M. J. Moll-Navarro, M. Merino, V. G. Casabo, A. Nacher, and A. Polache. Interaction of taurine on baclofen intestinal absorption: a nonlinear mathematical treatment using differential equations to describe kinetic inhibition models. *J. Pharm. Sci.* **85**:1248–1254 (1996).
22. J. B. M. M. van Bree, C. D. Heijligers-Feijen, A. G. de Boer, M. Danhof, and D. D. Breimer. Stereoselective transport of baclofen across the blood-brain barrier in rats as determined by the unit impulse response methodology. *Pharm. Res.* **8**:259–262 (1991).
23. Y. Deguchi, K. Inabe, K. Tomiyasu, K. Nozawa, S. Yamada, and R. Kimura. Study on brain interstitial fluid distribution and blood-brain barrier transport of baclofen in rats by microdialysis. *Pharm. Res.* **12**:1838–1844 (1995).
24. A. Koggel. Influence of secretory transporters on the intestinal permeability of cationic drugs. PhD. thesis, Johannes Gutenberg–University Mainz, Department of Pharmacy (2002).