

Pharmacokinetics and Pharmacodynamics of Seven Opioids in P-Glycoprotein-Competent Mice: Assessment of Unbound Brain $EC_{50,u}$ and Correlation of in Vitro, Preclinical, and Clinical Data

J. Cory Kalvass, Emily R. Olson, Michael P. Cassidy, Dana E. Selley, and Gary M. Pollack

School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (J.C.K., E.R.O., G.M.P.); and Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond Virginia (M.P.C., D.E.S.)

Received February 11, 2007; accepted June 22, 2007

ABSTRACT

This study was conducted to assess the utility of unbound brain EC_{50} ($EC_{50,u}$) as a measure of in vivo potency for centrally active drugs. Seven μ -opioid agonists (alfentanil, fentanyl, loperamide, methadone, meperidine, morphine, and sufentanil) were selected as model central nervous system drugs because they elicit a readily measurable central effect (antinociception) and their clinical pharmacokinetics/pharmacodynamics are well understood. Mice received an equipotent subcutaneous dose of one of the model opioids. The time course of antinociception and the serum and brain concentrations were determined. A pharmacokinetic/pharmacodynamic model was used to estimate relevant parameters. In vitro measures of opioid binding affinity (K_i) and functional activity [EC_{50} for agonist stimulated guanosine 5'-O-(3-[35 S]thio)triphosphate binding] and relevant clinical parameters were obtained to construct in vitro-to-preclinical and preclinical-to-clinical correlations. The

strongest in vitro-to-in vivo correlation was observed between K_i and unbound brain $EC_{50,u}$ ($r^2 \sim 0.8$). A strong correlation between mouse serum and human plasma EC_{50} was observed ($r^2 = 0.949$); the correlation was improved when corrected for protein binding ($r^2 = 0.995$). Clinical equipotent i.v. dose was only moderately related to K_i . However, estimates of ED_{50} and EC_{50} (total serum, unbound serum, total brain, and unbound brain) were significant predictors of clinical equipotent i.v. dose; the best correlation was observed for brain $EC_{50,u}$ ($r^2 = 0.982$). For each opioid, brain equilibration half-life in mice was almost identical to the plasma effect-site equilibration half-life measured clinically. These results indicate that the mouse is a good model for opioid human brain disposition and clinical pharmacology and that superior in vitro-to-preclinical and preclinical-to-clinical correlations can be achieved with relevant unbound concentrations.

In drug discovery, in vitro assays and preclinical animal studies are widely used to assess compound potency and to identify compound(s) that may have a desirable clinical response. Several options for assessing compound potency are available, including in vitro (receptor binding or functional assays) and in vivo (animal studies to determine dose-response or concentration-response relationships) protocols. In

vitro binding and functional assays, by nature, are designed to estimate the intrinsic affinity or potency at the receptor of interest, whereas in vivo experiments take into account the full spectrum of pharmacokinetic and pharmacodynamic processes that ultimately determine biological response.

Ideally, in vitro potency would translate to or predict in vivo potency. Often, this is the case, and significant correlations between in vivo ED_{50} or EC_{50} and in vitro potency have been established for a variety of therapeutic targets (Leysen et al., 1983; Visser et al., 2003). However, when there is no correlation between in vivo and in vitro potency measures, the validity of the in vitro assay, the animal model, and the target may be questioned. Therefore, establishing strong in vitro-to-in vivo relationships is a necessity for drug development, because it aids in target validation and it boosts con-

This work was supported by National Institutes of Health Grant R01 GM61191 (to G.M.P.), National Institute on Drug Abuse Grant DA10770 (to D.E.S.), and Pfizer Inc. J.C.K. was supported by an Eli Lilly & Co. Foundation Predoctoral Fellowship in Pharmacokinetics and Drug Disposition. Part of this study was presented at 2006 American Association of Pharmaceutical Scientists Annual Meeting and Exposition; 2006 Oct 29–Nov 2; San Antonio, TX.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.107.119560.

ABBREVIATIONS: PK/PD, pharmacokinetic/pharmacodynamic; CNS, central nervous system; BBB, blood-brain barrier; P-gp, p-glycoprotein; GTP γ S, guanosine 5'-O-(3-thio)triphosphate; CFS, cerebrospinal fluid; %MPR, percent maximal possible response; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry.

confidence in the in vitro and in vivo pharmacology models. Historically, in vitro-to-in vivo correlations have been established by comparing an in vitro measure of affinity or potency, such as K_i or EC_{50} from a receptor binding or a cell-based functional assay, with ED_{50} . Although the ED_{50} is not necessarily the best measure of intrinsic drug potency, it has been widely used because it is a straightforward, robust, and readily attainable metric. In vitro-to-in vivo correlations with ED_{50} are most likely to be successful for a compound set, within a discrete pharmacological class, that consists of members with large differences in intrinsic potency and relatively similar pharmacokinetics.

For compounds that evidence large differences in pharmacokinetics, improved in vitro-to-in vivo correlations may be obtained by using EC_{50} as opposed to ED_{50} . Commonly, EC_{50} is estimated from the effect versus plasma concentration relationship. Pharmacokinetic/pharmacodynamic (PK/PD) modeling often is used to obtain estimates of EC_{50} from in vivo data, and it is a powerful tool for enhancing mechanistic understanding of drug disposition and action.

In vitro-to-in vivo potency relationships have maximum predictability when the relationship between biological response and biophase concentration is known. Total plasma and unbound plasma concentrations are most widely used surrogates for biophase concentrations. However, systemic concentrations do not always reflect biophase concentrations, regardless of whether or not protein binding is taken into account. This is especially true for compounds that act on targets within the central nervous system (CNS). The blood-brain barrier (BBB) restricts the CNS distribution of many compounds, and in many cases, it results in temporal dissociation between biophase and systemic concentrations. Therefore, it is important to accurately determine CNS biophase concentration, or a closely related surrogate, to make better in vitro-to-in vivo correlations for centrally active compounds. When compounds have "good" BBB permeability and they are not substrates for transporters, in vitro-to-in vivo correlations can be constructed using unbound plasma or cerebrospinal fluid (CSF) concentrations as a surrogate for CNS biophase concentrations. When unbound plasma or CSF concentrations are not reflective of CNS biophase concentrations, such as when a compound has "poor" BBB permeability or it is subject to active BBB transport, other means for estimating CNS biophase concentrations may be needed. One method for estimating CNS biophase concentrations is simply to multiply total brain concentrations by brain unbound fraction ($f_{u,brain}$).

The present study was conducted to test the hypothesis that the brain $EC_{50,u}$ is the best in vivo measure of CNS intrinsic potency. Seven μ -opioid agonists (alfentanil, fentanyl, loperamide, methadone, meperidine, morphine, and sufentanil) were selected as probe CNS drugs. These agents were selected on the bases of having pronounced differences in potency toward the μ -opioid receptor (Terenius, 1975; Leysen et al., 1983), differing physicochemical properties (i.e., lipophilicity, unbound fractions, and permeability), and differing extent of CNS distribution (i.e., P-gp or non-P-gp substrate) (Dagenais et al., 2004). PK/PD studies were conducted in mice to determine five measures of in vivo potency (ED_{50} : total and unbound EC_{50} for both serum and brain) for each opioid. Estimates of in vitro affinity and potency (K_i and EC_{50} for agonist-stimulated [35 S]GTP γ S binding, respectively) as

well as relevant clinical parameters were obtained and used to construct in vitro-to-preclinical and preclinical-to-clinical comparisons. The most useful measure of in vivo of potency was determined by correlation analysis with the in vitro and clinical potency data.

Materials and Methods

Materials. Alfentanil was purchased from Taylor Pharmaceuticals (Decatur, IL). Fentanyl, loperamide, methadone, morphine, and oxycodone were purchased from Sigma-Aldrich (St. Louis, MO). Meperidine was obtained from Spectrum Chemicals and Laboratory Products (Gardena, CA). Sufentanil was purchased from Abbott Laboratories (North Chicago, IL). [35 S]GTP γ S (1250 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). All opioid drugs used in the [35 S]GTP γ S binding assay were obtained from the Drug Supply Program of the National Institute on Drug Abuse (Bethesda, MD). Adenosine deaminase, GTP γ S, and GDP were purchased from Sigma-Aldrich. All other reagents were obtained from common sources, and they were of reagent grade or better.

Animals. Male CF-1 *mdr1a*(+/+) mice (30–40 g; Charles River Laboratories, Inc., Wilmington, MA) were maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled room with access to water and food ad libitum. All procedures involving mice were approved by either The Institutional Animal Care and Use Committee of the University of North Carolina or The Institutional Animal Care and Use Committee at Virginia Commonwealth University, and they were conducted in accordance with Principles of Laboratory Animal Care (National Institutes of Health Publication 85-23, revised in 1985).

Agonist Stimulated [35 S]GTP γ S Binding in Membrane Homogenates. Mice were sacrificed by decapitation, and the thalamus was dissected on ice. Tissue was homogenized in membrane buffer (50 mM Tris-HCl, 3 mM MgCl₂, and 1 mM EGTA, pH 7.4) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY), centrifuged at 50,000g for 10 min at 4°C, resuspended in membrane buffer, and stored in aliquots of ~2 mg/ml at -80°C until use. Membranes were thawed and diluted 10-fold in assay buffer, centrifuged as described above, resuspended in assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 50 mM KCl, 50 mM NaCl, and 0.2 mM EGTA, pH 7.4), and preincubated for 10 min at 30°C with 4 mU/ml adenosine deaminase to remove endogenous adenosine. Protein was then determined by the Bradford method (Bradford, 1976). Each assay tube contained membranes (10 μ g protein/tube), 0.1 nM [35 S]GTP γ S, 10 μ M GDP, and appropriate agonist in a 1-ml total volume. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured with 20 μ M unlabeled GTP γ S. Assay tubes were incubated for 2 h at 30°C. The reaction was terminated by filtration under vacuum through Whatman GF/B glass fiber filters (Whatman, Maidstone, UK), followed by three washes with ice-cold Tris buffer. Bound radioactivity was determined by liquid scintillation spectrophotometry, at 95% efficiency for 35 S, after overnight extraction of the filters. Agonist-stimulated [35 S]GTP γ S binding was determined from four to six separate experiments performed in duplicate for each opioid. Net agonist-stimulated [35 S]GTP γ S binding was defined as agonist-stimulated minus basal binding. Percentage of stimulation was defined as (net agonist-stimulated [35 S]GTP γ S binding/basal binding) \times 100%. Percentage of maximal stimulation was defined as (net stimulation by agonist/net stimulation by 10 μ M [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin) \times 100%. EC_{50} and E_{max} values were determined by nonlinear regression analyses of concentration-effect curves by iterative curve-fitting using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA).

Pharmacokinetic/Pharmacodynamic Study. Based on the results of pilot experiments, 36 *mdr1a*(+/+) CF-1 mice in total received a single equipotent subcutaneous dose of alfentanil (0.2 mg/kg),

fentanyl (0.09 mg/kg), loperamide (50 mg/kg), methadone (0.6 mg/kg), meperidine (25 mg/kg), morphine (3.6 mg/kg), or sufentanil (0.001 mg/kg). Fentanyl and loperamide were prepared in 50:50 propylene glycol/water, whereas the remaining opioids were prepared in 0.9% saline. Antinociception was assessed at selected time points, and four mice per opioid were sacrificed by decapitation for collection of brain tissue and blood samples. Trunk blood was collected in 1.5-ml microcentrifuge tubes, and it was allowed to clot for ≥ 30 min at room temperature. Serum was harvested after centrifugation. Brain and serum samples were stored at -20°C until analysis by HPLC-MS/MS.

Assessment of Antinociception. Antinociception was assessed with the hot-plate latency test as described previously (Chen and Pollack, 1997). Before opioid administration, baseline hot-plate latency was determined for each animal in triplicate. Hot-plate latency was defined as the time interval between placement on the hot-plate (55°C ; Columbus Instruments, Columbus, OH) and the first observation of a jump or lick of a hind limb. Only animals with an average baseline latency < 25 s were used in this study. A cut-off latency of 60 s was established to avoid tissue damage. The degree of antinociception, expressed as percent maximal possible response (%MPR), was calculated as follows:

$$\% \text{MPR} = \frac{\text{Test latency} - \text{Control latency}}{60 - \text{Control latency}} \times 100\% \quad (1)$$

Evaluation of Protein Binding. Plasma and brain unbound fractions were determined in a 96-well equilibrium dialysis apparatus (HTDialysis, Gales Ferry, CT) using a previously reported method (Kalvass and Maurer, 2002). In brief, fresh mouse plasma and brain tissue were obtained the day of the study. Spectra-Por 2 membranes obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA) were conditioned in HPLC water for 15 min, followed by 30% ethanol for 15 min, and 100 mM sodium phosphate, pH 7.4, buffer for 15 min. Brain tissue was diluted 3-fold with 100 mM sodium phosphate, pH 7.4, buffer, and it was homogenized with a sonic probe. The drug of interest was added to samples of plasma and brain tissue homogenate (3 and 1 μM , respectively), and 150- μl aliquots ($n = 6$) were loaded into the 96-well equilibrium dialysis apparatus and dialyzed against an equal volume of 100 mM sodium phosphate, pH 7.4, buffer for 4.5 h in a 155-rpm shaking water bath maintained at 37°C . Prior experience with the equilibrium dialysis apparatus indicated that equilibrium would be achieved by the end of the specified incubation period (data not shown). After incubation, 10 μl of matrix (plasma or brain homogenate) and 50 μl of buffer were removed from the apparatus and added directly to HPLC vials containing 100 μl of an appropriate internal standard in methanol. A 50- μl aliquot of control buffer was added to the brain homogenate and plasma samples, and a 10- μl aliquot of either control brain homogenate or control plasma was added to the buffer samples, to yield identical matrix composition for all samples before analysis. The samples were vortex-mixed and centrifuged, and the supernatant was analyzed by the HPLC-MS/MS as described below. Plasma unbound fraction was calculated from the ratio of concentrations determined in buffer versus plasma samples. Equation 2, which accounts for the effect of tissue dilution on unbound fraction (Kalvass and Maurer, 2002), was used to calculate the brain unbound fraction:

$$\text{Undiluted } f_u = \frac{1/D}{((1/f_{u, \text{measured}}) - 1) + 1/D} \quad (2)$$

where D represents the -fold dilution of brain tissue, and $f_{u, \text{measured}}$ is the ratio of concentrations determined in buffer versus brain homogenate samples.

Quantitation of Serum and Brain Concentrations. Opioid brain and serum concentrations were measured using previously described methods with slight variation (Kalvass et al., 2007a). In brief, brain samples were homogenized in water [1:2 (v/v)] via sonic probe. An aliquot (2–25 μl) of homogenate or serum was transferred

to an HPLC vial, and protein was precipitated with 4 to 125 volumes of methanol containing internal standard (5 ng/ml loperamide for alfentanil, fentanyl, meperidine, methadone, and sufentanil; 20 ng/ml methadone for loperamide; and 100 ng/ml oxycodone for morphine). The sample was vortex-mixed and centrifuged, and the supernatant was analyzed by HPLC-MS/MS. Samples were injected (2–10 μl ; autosampler, CTC Analytics, Zwingen, Switzerland) onto a Gemini 110A column (2.0×30 mm; $5 \mu\text{m}$) (Phenomenex, Torrance, CA) maintained at room temperature. The total run time was 3 min. Analytes were eluted with a linear gradient consisting of 10 mM ammonium acetate, pH 6.8 (A), and methanol (B) produced by two LC-10ADVP binary pumps (Shimadzu, Kyoto, Japan). An initial condition of 5% B was ramped to 95% B over 2 min, held for 0.5 min, and then returned initial condition of 5% B in a single step to re-equilibrate the column. During the run, the flow rate was increased from 750 to 1500 $\mu\text{l}/\text{min}$ over the first 2 min, held at 1500 $\mu\text{l}/\text{min}$ for 1 min, and then returned the initial flow rate of 750 $\mu\text{l}/\text{min}$ in a single step. For the morphine samples, the initial conditions were held for 0.5 min before ramping the gradient and flow rate. The entire column effluent was diverted from the source of the PE Sciex API-4000 quadrupole mass spectrometer (Turbo V Ionspray source, 700°C ; PerkinElmerSciex Instruments, Boston, MA) for the first 0.8 min and last 0.5 min of the run. Alfentanil, fentanyl, loperamide, methadone, meperidine, morphine, oxycodone, and sufentanil were measured in positive ionization mode using multiple reaction monitoring (417.3 \rightarrow 268.3, 337.1 \rightarrow 188.3, 477.4 \rightarrow 266.0, 248.3 \rightarrow 220.3, 310.3 \rightarrow 265.2, 286.1 \rightarrow 201.1, 316.0 \rightarrow 298.0, and 387.2 \rightarrow 238.4, respectively). Standard curves were prepared in brain homogenate, serum, plasma, or buffer, and they were identical in composition to corresponding samples. Accuracy of standards and interassay variability was within $\pm 20\%$.

Pharmacokinetic/Pharmacodynamic Analysis. A compartmental modeling approach with distribution between serum and brain tissue was used to describe opioid pharmacokinetics. The pharmacokinetic model in Fig. 1 was fitted simultaneously to the serum concentration- and brain concentration-time data using nonlinear least-squares regression (WinNonlin 4.1; Pharsight Corporation, Mountain View, CA). The brain volume (V_{Br}) was fixed at 13.4 ml/kg, assuming a specific gravity of 1.0 g/ml (Kalvass et al., 2007b). All other pharmacokinetic parameters were obtained from fitting the kinetic model to the data. The pharmacodynamic parameters EC_{50} and γ were determined from fitting a sigmoidal E_{max} model to the antinociception versus brain concentration (C) data.

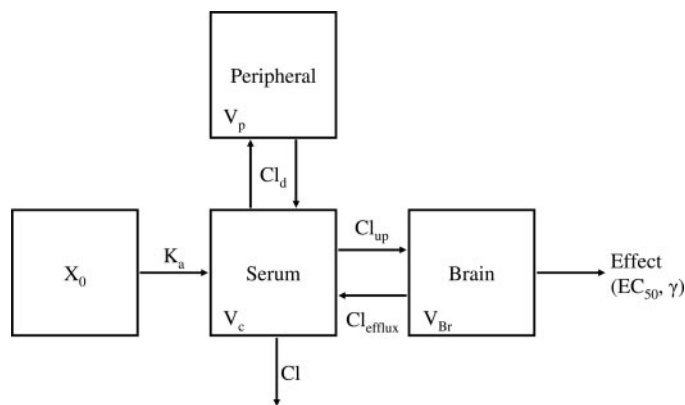


Fig. 1. Pharmacokinetic/pharmacodynamic model for opioid disposition and antinociception in mice. Pharmacokinetic parameters were obtained by fitting the model to the time course of serum and brain concentrations in *mdr1a*(+/+) mice after subcutaneous administration. The absorption rate constant (K_a), central volume (V_c), systemic clearance (Cl), V_p , Cl_d , brain uptake clearance (Cl_{up}), and brain efflux clearance (Cl_{efflux}) were estimated for each opioid. The V_{Br} was fixed. The effect parameters EC_{50} and γ were obtained by fitting a sigmoidal E_{max} model to the brain concentration versus antinociception data. E_{max} was defined as 100%.

$$\%MPR = \frac{E_{\max} \times C^\gamma}{EC_{50} + C^\gamma} \quad (3)$$

E_{\max} was defined as 100%. Serum EC₅₀ was calculated from the following equation:

$$\text{Serum EC}_{50} = \frac{\text{Brain EC}_{50}}{K_{p, \text{brain}, \text{ss}}} \quad (4)$$

The ED₅₀ was calculated, assuming linear pharmacokinetics, from the maximum brain concentration predicted by the PK model (brain C_{\max}), the opioid dose (X_0), and the brain EC₅₀.

$$ED_{50} = \frac{\text{Brain EC}_{50}}{\text{Brain } C_{\max}} \times X_0 \quad (5)$$

The time course of the brain-to-serum ratio ($K_{p, \text{brain}}$) for each opioid was used to estimate the brain equilibration rate constant (k_{eq}) and steady-state brain-to-serum ratio ($K_{p, \text{brain}, \text{ss}}$) for that compound according to the following equation:

$$K_{p, \text{brain}} = K_{p, \text{brain}, \text{ss}}(1 - e^{-k_{\text{eq}} \times t}) \quad (6)$$

The brain equilibration half-life ($t_{1/2, \text{eq}, \text{brain}}$) was obtained from k_{eq} as follows:

$$t_{1/2, \text{eq}, \text{brain}} = \frac{0.693}{k_{\text{eq}}} \quad (7)$$

Results

The dose administered to produce equivalent antinociception in mice varied by more than 4 orders of magnitude between the most and least potent opioid (0.001 versus 50 mg/kg for sufentanil and loperamide, respectively). Likewise, the calculated mouse ED₅₀ varied by nearly 5 orders of magnitude (Table 1). The large range of effective doses was advantageous for subsequent construction of relationships between various in vitro and in vivo metrics of response.

The pharmacokinetic/pharmacodynamic model adequately described the time course of antinociception, as well as the brain tissue concentration versus time and serum concentration versus time relationships, for each of the opioids studied (Fig. 2, A–G). With the exception of fentanyl, the systemic pharmacokinetics of all of the opioids were most effectively modeled with a single compartment system and first-order absorption from the site of administration (Table 2; Fig. 2). The disposition of fentanyl required addition of a peripheral

distributional compartment with associated parameters [apparent peripheral distributional space (V_p); distributional clearance (Cl_d)]. Estimates of relevant pharmacokinetic parameters for each of the opioids are reported in Table 2.

Pharmacokinetic/pharmacodynamic modeling revealed counterclockwise hysteresis in the antinociception versus serum concentration relationship for each opioid (Fig. 3), consistent with delayed distribution between serum concentrations and biophase concentrations. In contrast, all of the opioids exhibited a sigmoidal relationship between antinociception and brain tissue concentration, with no evidence of significant hysteresis behavior associated with temporal dissociation between CNS pharmacokinetics and pharmacodynamics (Fig. 4). Brain EC₅₀ and γ values are reported in Table 1. Brain EC₅₀ estimates differed by more than 3000-fold between the most (sufentanil) and the least (meperidine) potent opioid. Similarly the serum, unbound serum, and unbound brain EC₅₀ estimates evidenced a wide range among the seven opioids. Depending on the particular EC₅₀ value used as a metric (total brain, unbound brain, total serum, or unbound serum), the rank order of opioid potency differed considerably as a consequence of large differences in $K_{p, \text{brain}, \text{ss}}$, $f_{u, \text{plasma}}$, and $f_{u, \text{brain}}$ among the opioids.

$K_{p, \text{brain}, \text{ss}}$ values differed by more than 50-fold among the opioids (Table 1; Fig. 5). The lowest $K_{p, \text{brain}, \text{ss}}$ was observed for loperamide (0.115), and the highest was for meperidine (6.8). The $t_{1/2, \text{eq}, \text{brain}}$ ranged from 1 to 74 min, with alfentanil and morphine having the shortest and longest $t_{1/2, \text{eq}, \text{brain}}$, respectively (Table 2).

The in vitro K_i (obtained from the literature) and GTP γ S EC₅₀ values were strongly correlated ($r^2 > 0.9$; data not shown). In every case, the in vitro-to-in vivo correlation using K_i was stronger than the corresponding correlation using GTP γ S EC₅₀ (data not shown). However, the strengths of the various in vitro-to-in vivo correlations were the same relative to each other whether K_i or GTP γ S EC₅₀ was used. Therefore, only the in vitro-to-in vivo relationships with K_i are reported. In vitro-to-in vivo relationships (Fig. 6) revealed that mouse serum EC₅₀ and brain EC₅₀ correlated poorly with in vitro K_i ($r^2 < 0.5$). A modest improvement was observed for the correlation between unbound serum EC_{50,u} and K_i ($r^2 = 0.583$). The strongest relationship observed was between unbound brain EC_{50,u} and K_i ($r^2 < 0.799$).

TABLE 1

Parameters used for correlation of in vitro, preclinical, and clinical data

Parameter	Alfentanil	Fentanyl	Loperamide	Meperidine	Methadone	Morphine	Sufentanil
In vitro K_i (nM) ^a	19	1.6	0.50 ^b	193	2.2	5.7	0.10
Mouse ED ₅₀ (mg/kg)	0.079	0.020	36	3.2	2.2	0.94	0.00041
Mouse $f_{u, \text{plasma}}$	0.26	0.17 ^c	0.029	0.36	0.15 ^c	0.50 ^c	0.054 ^c
Mouse $f_{u, \text{brain}}$	0.33	0.070 ^c	0.0047	0.13	0.029 ^c	0.41 ^c	0.034 ^c
Human plasma EC ₅₀ (nM) ^d	1337	24	N.A.	6034 ^e	1151	195	1.8
Human equipotent i.v. dose (mg) ^f	0.75	0.10	N.A.	90	8.75	10	0.015
Human $t_{1/2, \text{Ke0}}$ (min) ^d	1.0	5.8	N.A.		12	178	6.2
Human $f_{u, \text{plasma}}$	0.079 ^g	0.156 ^g		0.35 ^e	0.125 ^h	0.75 ⁱ	0.075 ^g

N.A., not available.

^a Leysen et al. (1983).

^b Terenius (1975).

^c Kalvass et al. (2007a).

^d Average from EEG power spectrum analysis, analgesia (cancer pain), pain tolerance, and/or postoperative analgesia (Lotsch, 2005).

^e Unbound potency of meperidine is 20 time less than alfentanil (Kurz et al., 1997).

^f Wood and Alastair (1990).

^g Meuldermans et al. (1982).

^h Inturrisi et al. (1987).

ⁱ Glare and Walsh (1991).

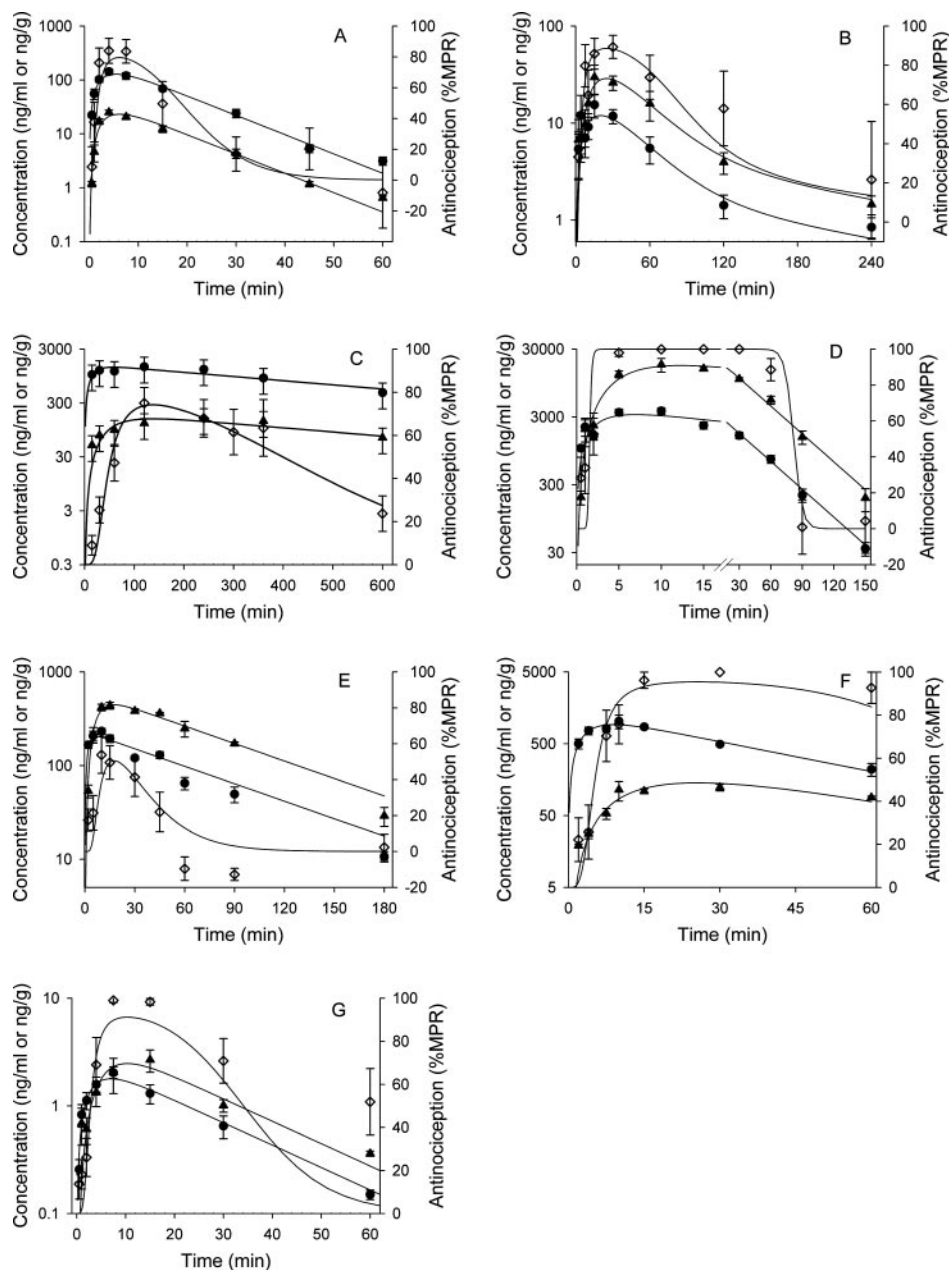


Fig. 2. Time course of antinociception (\diamond) and serum (\bullet) and brain (\blacktriangle) concentrations after 0.2 mg/kg s.c. dose of alfentanil (A), 0.9 mg/kg s.c. dose of fentanyl (B), 50 mg/kg s.c. dose of loperamide (C), 25 mg/kg s.c. dose of meperidine (D), 2 mg/kg s.c. dose of methadone (E), 3.6 mg/kg s.c. dose of morphine (F), or 0.001-mg/kg s.c. dose of sufentanil (G) in CF-1 *mdr1a*(+/+) mice. Data are presented as mean \pm S.E. [concentration data ($n \geq 3$); antinociception ($n = 4-36$)]. Lines represent the fit of the PK/PD model to the antinociception and concentration data.

Preclinical-to-clinical relationships indicated that mouse serum and human plasma EC_{50} estimates were well correlated ($r^2 = 0.949$). Despite the strength of this relationship, the correlation was improved when the EC_{50} values were corrected for binding to plasma proteins ($r^2 = 0.995$) (Fig. 7). Correlations also were explored between human equipotent clinical i.v. dose of the opioids and various estimates of in vitro and in vivo potency (Fig. 8). The relationship between equipotent clinical dose and K_i was relatively poor ($r^2 = 0.677$). Equipotent clinical i.v. dose correlated more strongly with mouse ED_{50} ($r^2 = 0.932$). Equipotent clinical i.v. dose also correlated equally well with total and unbound mouse serum EC_{50} ($r^2 = 0.922$ and $r^2 = 0.937$, respectively). Somewhat surprisingly, equipotent clinical i.v. dose did not correlate as well with total brain EC_{50} ($r^2 = 0.878$) compared with the relationships with metrics obtained from plasma. However, the equipotent clinical i.v. dose correlated most strongly with unbound brain $EC_{50,u}$ values in the mouse ($r^2 = 0.982$).

The brain equilibration half-life in mouse determined in the present study ($t_{1/2eq,brain}$) was compared with the apparent plasma-biophase equilibration half-life in humans ($t_{1/2,Ke0}$), obtained from the literature, among the opioids examined in this study. The correlation between clinical $t_{1/2,Ke0}$ and mouse $t_{1/2eq,brain}$ was excellent ($r^2 = 0.988$). With the exception of morphine, all of the opioids fell within a factor of 1.4-fold relative to the line of identity (Fig. 9).

Discussion

An integrated pharmacokinetic/pharmacodynamic modeling strategy yielded an adequate description of the time course of antinociception, serum concentrations, and brain tissue concentrations for each of the opioids examined in this study. Differences in systemic pharmacokinetics among the opioids were relatively small (~ 10 -fold for Cl) compared with differences in vivo potency measures ($\sim 30,000$ -fold) and

TABLE 2

Parameter estimates from PK/PD modeling

Parameter estimate \pm S.E. from nonlinear regression analysis of pooled *mdr1a*(+/+) mouse data.

Parameter	Alfentanil	Fentanyl	Loperamide	Meperidine	Methadone	Morphine	Sufentanil
K_a (min^{-1})	0.35 ± 0.03	0.07 ± 0.07	0.063 ± 0.015	0.39 ± 0.18	0.55 ± 0.13	0.27 ± 0.04	0.32 ± 0.12
Cl ($\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)	82 ± 3	90 ± 40	58 ± 7	190 ± 40	127 ± 12	95 ± 5	19 ± 3
V_c ($\text{ml} \cdot \text{kg}^{-1}$)	1000 ± 60	3000 ± 3000	$32,000 \pm 3000$	6100 ± 1200	8800 ± 600	2900 ± 180	400 ± 80
Cl_d ($\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)		40 ± 30					
V_p ($\text{ml} \cdot \text{kg}^{-1}$)		$5000 \pm 13,000$					
Cl_{up} ($\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)	4 ± 3	8 ± 3	0.04 ± 0.06	100 ± 40	27 ± 4	9.5 ± 0.5	37 ± 14
Cl_{emux} ($\text{ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$)	22 ± 17	3.4 ± 1.4	0.3 ± 0.5	8 ± 4	5.1 ± 0.9	1.9 ± 1.4	12 ± 5
EC_{50} (ng/g)	9.2 ± 1.7	6.4 ± 1.1	100 ± 6	2200 ± 110	510 ± 60	38 ± 4	1.0 ± 0.2
γ	1.8 ± 0.4	1.4 ± 0.3	2.7 ± 0.5	7.8 ± 4.2	3.7 ± 1.1	2.3 ± 0.5	2.5 ± 1.3
$K_{p,brain}$	0.195 ± 0.0087	2.3 ± 0.2	0.115 ± 0.014	6.8 ± 0.5	3.3 ± 0.2	1.1 ± 0.6	2.1 ± 0.3
k_{eq} (min^{-1})	0.64 ± 0.09	0.14 ± 0.04	0.025 ± 0.010	0.13 ± 0.03	0.073 ± 0.014	0.009 ± 0.006	0.16 ± 0.06
$t_{1/2eq,brain}$ (min) ^a	1.08 ± 0.16	4.9 ± 1.3	27 ± 11	5.4 ± 1.1	9.6 ± 1.8	74 ± 45	4.3 ± 1.6

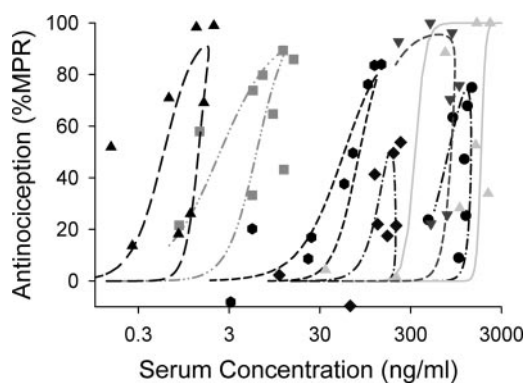
^a $t_{1/2eq,brain}$ were calculated from $0.693/k_{eq,brain}$.

Fig. 3. Relationship between plasma concentration and antinociception for μ -opioid agonists. Symbols represent data from CF-1 *mdr1a*(+/+) mice. Lines represent the fit of the PK/PD model to the antinociception-serum concentration data. Symbols are as follows: sufentanil (▲), fentanyl (■), alfentanil (●), methadone (◆), meperidine (△), morphine (▼), and loperamide (●). All opioids exhibited a counter-clockwise hysteresis in the antinociception versus serum concentration relationship. Data are presented as means [serum concentration ($n = 2-4$); antinociception ($n = 4-36$)].

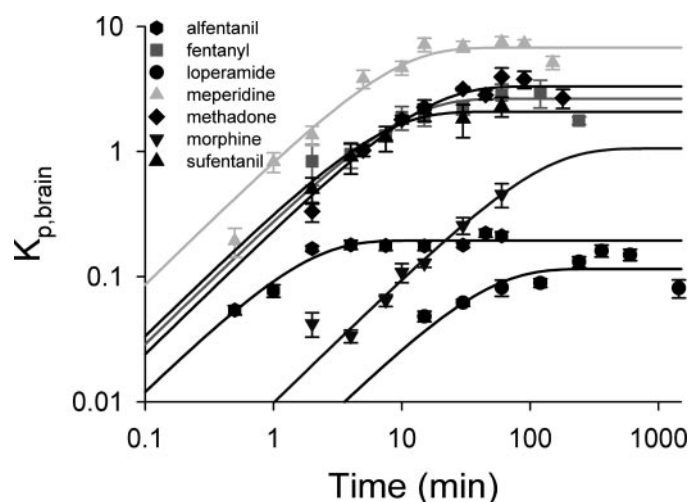


Fig. 5. Time course of opioid $K_{p,brain}$ in CF-1 *mdr1a*(+/+). Solid lines represent the fit of a kinetic model to the data. Data are presented as mean \pm S.E. ($n \geq 3$).

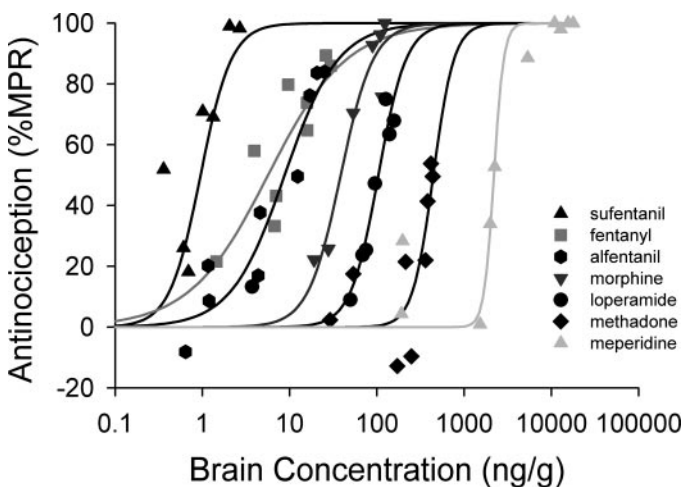


Fig. 4. Relationship between brain concentration and antinociception for μ -opioid agonists. Symbols represent data from CF-1 *mdr1a*(+/+) mice. Lines represent the fit of a sigmoidal E_{max} model to the antinociception-brain concentration data. Data are presented as means [brain concentration ($n = 2-4$); antinociception ($n = 4-36$)].

brain pharmacokinetics (>70 -fold for $K_{p,brain}$, $t_{1/2eq,brain}$, and Cl_{up}).

In Vitro-to-Preclinical Correlations. Previous studies have established in vitro-to-preclinical correlations for opioid potency using K_i . Leysen et al. (1983) reported a correlation ($r^2 = 0.81$) between in vitro K_i (displacement of sufentanil in rat forebrain membranes) and ED_{50} (rat tail withdrawal reflex) for 35 opioids from five structural classes, with $>100,000$ -fold difference in receptor affinity. However, no correlation existed between K_i and ED_{50} if only the seven opioids from this study were included in their analysis ($r^2 < 0.15$) (Niemegeers et al., 1979; Leysen et al., 1983). Results from this study show a similarly poor correlation ($r^2 = 0.167$) between in vitro K_i and mouse ED_{50} . This lack of correlation can be largely attributed to differences in pharmacokinetics and biophase distribution characteristics for the seven opioids. The opioids selected for this study come from four different structural classes and several are known P-gp substrates (Dagenais et al., 2004). Consequently, these opioids have different physiochemical properties and CNS (biophase) distribution characteristics. One would anticipate that K_i would be a better predictor of biophase EC_{50} , because the confounding influences of pharmacokinetics and biophase distribution are removed.

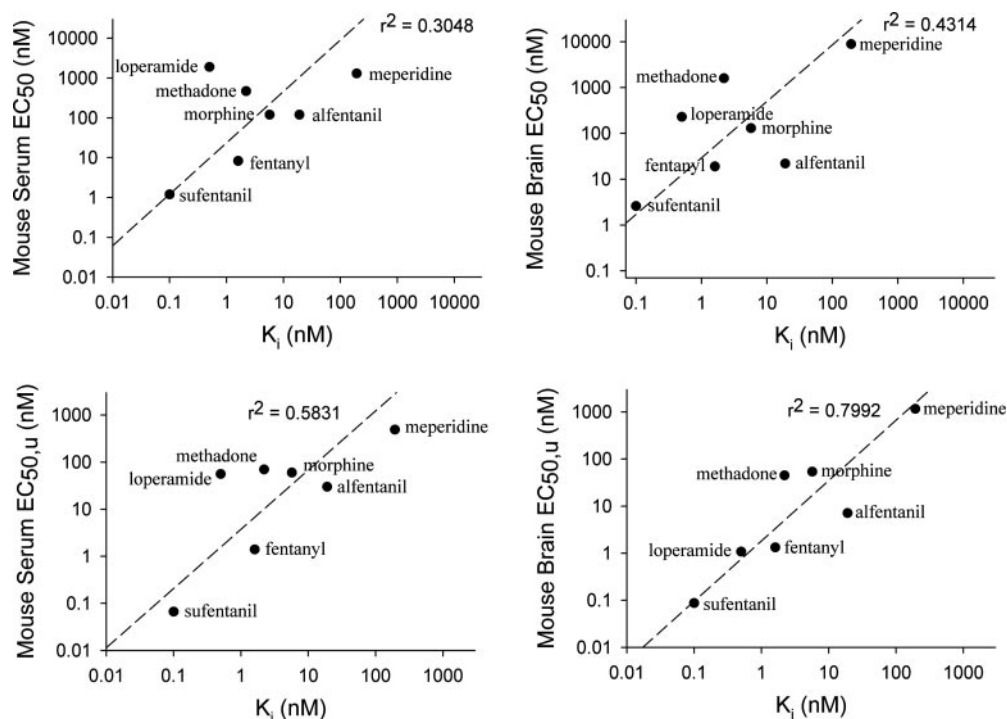


Fig. 6. Correlation analysis for various in vivo measures relative to in vitro potency. The dashed line represents the line from log-log orthogonal regression analysis.

Total plasma, unbound plasma, and CSF concentrations have been used to estimate CNS biophase concentration for in vitro-to-in vivo correlations. Visser et al. (2003) demonstrated that, for nine GABA_A modulators, in vitro-to-in vivo correlations could be made between K_i and either total or unbound plasma EC_{50} ; however, the unbound plasma EC_{50} correlated better with K_i than total plasma EC_{50} . Another study demonstrated the in vitro K_i of alfentanil, fentanyl, and sufentanil correlated well with the in vivo EC_{50} determined from CSF concentrations (Cox et al., 1998).

In the present study, total plasma, total brain, unbound plasma, and unbound brain EC_{50} estimates were used to express opioid potency, and they were evaluated as potential surrogates for biophase EC_{50} . Unbound EC_{50} values were calculated by multiplying the total EC_{50} by the appropriate unbound fraction value ($f_{u,plasma}$ or $f_{u,brain}$) determined from equilibrium dialysis experiments conducted in accordance with the methods of Kalvass and Maurer (2002). The total serum and total brain EC_{50} values were weakly related to in vitro K_i ($r^2 < 0.5$). A modest improvement was observed with unbound serum $EC_{50,u}$ ($r^2 = 0.583$). However, the strongest relationship was observed between unbound brain $EC_{50,u}$ and K_i ($r^2 = 0.799$). These results, along with the hysteresis

in the antinociception versus serum concentration relationship and the sigmoidal relationship between antinociception and brain concentration, suggest that unbound brain concentrations determined with $f_{u,brain}$, estimated from brain-homogenate equilibrium-dialysis approach, represent an optimal surrogate for CNS biophase concentrations. Furthermore, these results highlight the importance of using an appropriate surrogate for biophase concentration, presumably unbound concentration at the site of action, when making in vitro-to-in vivo correlations.

In this study, the in vitro parameter GTP γ S EC_{50} was also used to construct in vitro-to-in vivo correlations. In every case, the correlation using K_i was stronger than the corresponding correlation using GTP γ S EC_{50} (data not shown). It is unclear why in vivo EC_{50} values correlated better with receptor binding K_i values than with EC_{50} values obtained from [³⁵S]GTP γ S experiments. Intuitively, one might expect an improved correlation between two functional measures of receptor activation than between in vivo function and receptor binding. However, differences in the intrinsic efficacy of agonists in the [³⁵S]GTP γ S assay are generally observed as differences in maximal effect because receptor reserve for G protein activation in this assay is low, especially in native

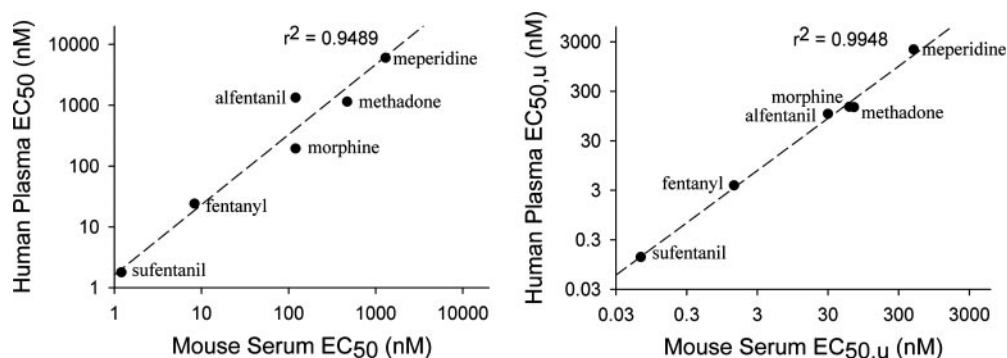


Fig. 7. Correlation between mouse serum and human plasma EC_{50} values. The dashed line represents the line from log-log orthogonal regression analysis.

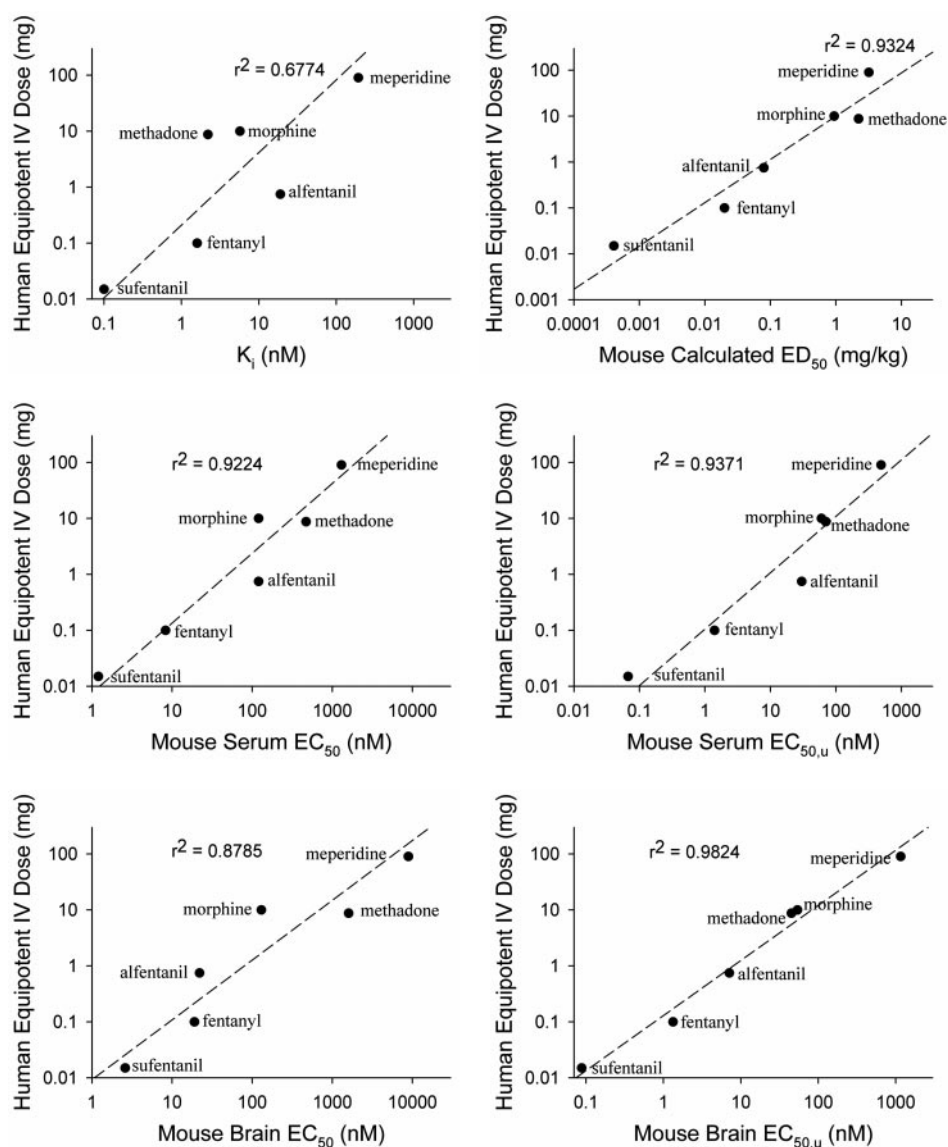


Fig. 8. Correlation of equipotent clinical dose with in vitro K_i values, mouse ED_{50} values, and mouse EC_{50} values. The dashed line represents the line from log-log orthogonal regression analysis.

tissues such as brain (Selley et al., 1998). However, an attempt was made to adjust assay conditions to minimize E_{max} differences between full and partial agonists by decreasing both sodium and GDP concentrations relative to standard assay conditions (Selley et al., 1997, 2000) so that differences in intrinsic efficacy would be observed as potency differences. Nonetheless, under these optimized conditions, there were still slight E_{max} differences, which ranged from 73 to 85% of the full agonist [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (data not shown). It should be noted that measurement of high-affinity agonist binding is also dependent on the formation of functional receptor-G protein complexes, so that these measurements when made in the absence of sodium and guanine nucleotides apparently provided slightly better correlation than EC_{50} values obtained from direct measurement of G protein activation with the [³⁵S]GTP γ S assay.

Preclinical-to-Clinical Correlations. Human efficacious plasma concentrations are often predicted from total plasma concentrations in preclinical efficacy models (Danhof et al., 1993; Ito et al., 1993). Assuming the drug has similar pharmacology and plasma protein binding in humans and

preclinical models, this prediction should be valid. In the present study, mouse total serum EC_{50} correlated well with human total plasma EC_{50} ($r^2 = 0.949$), consistent with similarities between mouse and human opioid pharmacology and plasma protein binding. However, the correlation improved when EC_{50} values were corrected for protein binding ($r^2 = 0.995$). On average, mouse unbound serum $EC_{50,u}$ overpredicted human unbound human plasma $EC_{50,u}$ by 2.8-fold. The murine model seems to be a remarkably effective predictor of relative human efficacious serum concentrations among a set of μ -opioids, even when those compounds are derived from different chemical classes.

The ability to predict human dose from in vitro and preclinical data are important. Clinical dose often correlates with ED_{50} obtained from animal models. For example, Niemegeers et al. (1979) demonstrated that rat ED_{50} determined for antidiarrheal and analgesic activity strongly correlated with clinical dose for 12 opioids. In the present study, the equipotent clinical i.v. dose and mouse ED_{50} correlated well for the seven opioids examined ($r^2 = 0.932$). Although efficacious dose is dependent on many factors, including sys-

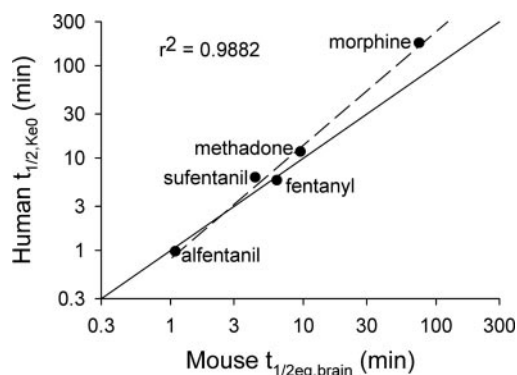


Fig. 9. The correlation between human plasma-biophase equilibration half-life ($t_{1/2,Ke0}$) and mouse brain equilibration half-life ($t_{1/2eq,brain}$). The solid line represents the line of unity; the dashed line represents the line from log-log orthogonal regression analysis. With the exception of morphine, all of the opioids fell within 1.4-fold of the line of unity.

temic disposition, target-site pharmacokinetics, and intrinsic potency, the observed differences in efficacious dose for the examined opioids seem to be dominated by intrinsic potency. Therefore, any reliable measure of opioid in vivo intrinsic potency should correlate well with the clinical equipotent i.v. dose for these opioids.

Various in vitro and in vivo measures of opioid potency were correlated with clinical equipotent i.v. dose to evaluate predictive potential. The in vitro K_i was a weak predictor of equipotent clinical i.v. dose ($r^2 = 0.677$). Equipotent clinical dose correlated better with in vivo measures, including mouse ED_{50} ($r^2 = 0.932$) and total or unbound mouse serum EC_{50} ($r^2 = 0.922$ and $r^2 = 0.937$, respectively). It was surprising that equipotent clinical dose did not correlate as well with total brain EC_{50} values ($r^2 = 0.879$) as with either serum concentration measure or murine effective dose, suggesting that nonspecific opioid binding in brain is substantial. As expected, the equipotent clinical dose correlated best with unbound brain $EC_{50,u}$ ($r^2 = 0.982$), consistent with unbound brain $EC_{50,u}$ being the best measure of in vivo intrinsic opioid potency.

It is interesting to note that the $t_{1/2eq,brain}$ determined in mice, derived from the kinetics of drug equilibration between brain tissue and serum (Fig. 5), was almost identical to the clinical $t_{1/2,Ke0}$, an inferred value from the kinetics of pharmacological response in the context of kinetics of systemic disposition (Fig. 9). Morphine evidenced the largest discrepancy between these metrics, with $t_{1/2,Ke0}$ being 2.4-fold larger than mouse $t_{1/2eq,brain}$. Morphine-6-glucuronide, an active metabolite of morphine in humans (Glare and Walsh, 1991) that is not formed extensively in mice, possesses a long $t_{1/2,Ke0}$ (>3600 min; Löttsch, 2005). Because morphine and its active metabolite exist in combination after morphine administration in humans, the $t_{1/2,Ke0}$ estimate obtained will reflect the contribution of both parent drug and metabolite. The remarkable relationship between $t_{1/2,Ke0}$ and $t_{1/2eq,brain}$, together with the strong interspecies correlation between unbound serum/plasma EC_{50} , suggests that the brain distribution characteristics are similar between humans and mice.

Relative Potency and $K_{p,brain}$. Even though all seven opioids examined are μ -agonists, they do not have the same primary indication. The primary indication of a given opioid seems not to be related mainly to intrinsic opioid potency, but rather to differences in brain distribution characteristics and

systemic pharmacokinetics. For example, the two anesthetic opioids, alfentanil and sufentanil, have the shortest mouse systemic half-life (8 and 14 min, respectively) and $t_{1/2eq,brain}$ (1 and 4 min, respectively) of the opioids examined. The short systemic half-life, combined with the short $t_{1/2eq,brain}$, results in a rapid onset and offset of action, allowing for rapid adjustment of response during i.v. infusion. Fentanyl, methadone, meperidine, and morphine, in contrast, have longer half-lives and $t_{1/2eq,brain}$, rendering them more suitable as analgesics. The brain distribution of loperamide is limited due to P-gp-mediated efflux (Schinkel et al., 1996), allowing it to act selectively on μ -opioid receptors in the intestinal tract versus the CNS and conferring ideal antidiarrheal properties. These relationships indicate that systemic pharmacokinetics and biophase distribution characteristics are more important than intrinsic potency for optimizing an opioid action toward a given indication (anesthetic, analgesic, or antidiarrheal). Undoubtedly, potency and biophase distribution characteristics must be balanced with other properties and considerations (i.e., solubility, permeability, bioavailability, systemic clearance, and half-life) to achieve compounds that will be useful clinical agents. However, the current data clearly indicate the importance of target-site pharmacokinetics and activity in determining qualitative, as well as quantitative, clinical response.

$K_{p,brain}$ often is used as a measure of CNS exposure, under the assumption that larger values of $K_{p,brain}$ equate with higher CNS exposure. CNS drug discovery programs have devoted much effort and resources to predicting and maximizing the $K_{p,brain}$ of drug candidates. Although not the main intent of this work, the results presented herein can be used to illustrate the fallacy of pursuing this strategy. As demonstrated previously, K_i , ED_{50} , EC_{50} , and equipotent i.v. clinical doses are appropriate parameters for constructing in vitro-to-in vivo and preclinical-to-clinical correlations. The same cannot be said for $K_{p,brain}$. Even though $K_{p,brain}$ values differed by more than 50-fold among the opioids examined, there was no correlation between $K_{p,brain}$ and any relevant pharmacodynamic parameter ($r^2 < 0.2$) for K_i , EC_{50} , $EC_{50,u}$, ED_{50} , equipotent i.v. clinical dose, $f_{u,plasma}$, $f_{u,brain}$, $t_{1/2eq,brain}$, and $t_{1/2,Ke0}$. With the exception of loperamide, all of the opioids examined are marketed as CNS-active drugs exhibiting $K_{p,brain}$ values ranging between 0.19 and 6.8. This large range of $K_{p,brain}$ values, which was not meaningfully correlated with any relevant measure of opioid action, indicates that $K_{p,brain}$ is not a useful parameter. In contrast to $K_{p,brain}$, the unbound brain and unbound plasma concentrations were useful for in vitro-to-in vivo and preclinical-to-clinical predictions, and the ratio of unbound brain to unbound plasma concentrations better reflects pharmacologically relevant brain exposure.

In summary, these results suggest that the mouse is a good model for opioid brain disposition and pharmacology and that superior in vitro-to-preclinical and preclinical-to-clinical correlations can be established when making comparisons between relevant unbound concentrations.

References

- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Chen C and Pollack GM (1997) Blood-brain disposition and antinociceptive effects of μ -penicillamine-2,5-enkephalin in the mouse. *J Pharmacol Exp Ther* 283:1151–1159.

- Cox EH, Kerbusch T, Van der Graaf PH, and Danhof M (1998) Pharmacokinetic-pharmacodynamic modeling of the electroencephalogram effect of synthetic opioids in the rat: correlation with the interaction at the μ -opioid receptor. *J Pharmacol Exp Ther* **284**:1095–1103.
- Dagenais C, Graff CL, and Pollack GM (2004) Variable modulation of opioid brain uptake by P-glycoprotein in mice. *Biochem Pharmacol* **67**:269–276.
- Danhof M, Mandema JW, Hoogerkamp A, and Mathot RA (1993) Pharmacokinetic-pharmacodynamic modelling in pre-clinical investigations: principles and perspectives. *Eur J Drug Metab Pharmacokinet* **18**:41–47.
- Glare PA and Walsh TD (1991) Clinical pharmacokinetics of morphine. *Ther Drug Monit* **13**:1–23.
- Inturrisi CE, Colburn WA, Kaiko RF, Houde RW, and Foley KM (1987) Pharmacokinetics and pharmacodynamics of methadone in patients with chronic pain. *Clin Pharmacol Ther* **41**:392–401.
- Ito K, Yajima N, Ohtani H, Yamada Y, Nakamura K, Sawada Y, and Iga T (1993) Prediction of the therapeutic dose for beta-stimulants based on preclinical data: application of oral dosage forms and aerosols to asthmatic patients. *J Pharmacokinetic Biopharm* **21**:133–144.
- Kalvass JC and Maurer TS (2002) Influence of nonspecific brain and plasma binding on CNS exposure: implications for rational drug discovery. *Biopharm Drug Dispos* **23**:327–338.
- Kalvass JC, Maurer TS, and Pollack GM (2007a) Use of plasma and brain unbound fractions to assess the extent of brain distribution of 34 drugs: comparison of unbound concentration ratios to in vivo p-glycoprotein efflux ratios. *Drug Metab Dispos* **35**:660–666.
- Kalvass JC, Olson ER, and Pollack GM (2007b) Pharmacokinetics and pharmacodynamics of alfentanil in P-glycoprotein-competent and P-glycoprotein-deficient mice: P-glycoprotein efflux alters alfentanil brain disposition and antinociception. *Drug Metab Dispos* **35**:455–459.
- Kurz A, Ikeda T, Sessler DI, Larson MD, Bjorksten AR, Dechert M, and Christensen R (1997) Meperidine decreases the shivering threshold twice as much as the vasoconstriction threshold. *Anesthesiology* **86**:1046–1054.
- Leysen JE, Gommeren W, and Niemegeers CJ (1983) [³H]Sufentanil, a superior ligand for mu-opiate receptors: binding properties and regional distribution in rat brain and spinal cord. *Eur J Pharmacol* **87**:209–225.
- Lötsch J (2005) Pharmacokinetic-pharmacodynamic modeling of opioids. *J Pain Symptom Manage* **29**:S90–S103.
- Meuldermans WE, Hurkmans RM, and Heykants JJ (1982) Plasma protein binding and distribution of fentanyl, sufentanil, alfentanil and lofentanil in blood. *Arch Int Pharmacodyn Ther* **257**:4–19.
- Niemegeers CJ, McGuire JL, Heykants JJ, and Janssen PA (1979) Dissociation between opiate-like and antidiarrheal activities of antidiarrheal drugs. *J Pharmacol Exp Ther* **210**:327–333.
- Schinkel AH, Wagenaar E, Mol CA, and van Deemter L (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J Clin Invest* **97**:2517–2524.
- Selley DE, Cao CC, Liu Q, and Childers SR (2000) Effects of sodium on agonist efficacy for G-protein activation in mu-opioid receptor-transfected CHO cells and rat thalamus. *Br J Pharmacol* **130**:987–996.
- Selley DE, Liu Q, and Childers SR (1998) Signal transduction correlates of μ opioid agonist intrinsic efficacy: receptor-stimulated [³⁵S]GTP γ S binding in mMOR-CHO cells and rat thalamus. *J Pharmacol Exp Ther* **285**:496–505.
- Selley DE, Sim LJ, Xiao R, Liu Q, and Childers SR (1997) μ -Opioid receptor-stimulated guanosine-5'-O-(γ -thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. *Mol Pharmacol* **51**:87–96.
- Terenius L (1975) Comparison between narcotic "receptors" in the guinea-pig ileum and the rat brain. *Acta Pharmacol Toxicol (Copenh)* **37**:211–221.
- Visser SA, Wolters FL, Gubbens-Stibbe JM, Tukker E, Van Der Graaf PH, Peletier LA, and Danhof M (2003) Mechanism-based pharmacokinetic/pharmacodynamic modeling of the electroencephalogram effects of GABAA receptor modulators: in vitro-in vivo correlations. *J Pharmacol Exp Ther* **304**:88–101.
- Wood M and Alastair JJ (1990) *Drugs and Anesthesia: Pharmacology for Anesthesiologist*, Williams & Wilkins, Baltimore, MD.

Address correspondence to: Dr. J. Cory Kalvass, Department of Drug Disposition, Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46285. E-mail: kalvassjo@lilly.com
