JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648

JPET #99648

DIFFERENTIAL *IN VIVO* SENSITIVITY TO INHIBITION OF P-GLYCOPROTEIN LOCATED IN LYMPHOCYTES, TESTES, AND THE BLOOD-BRAIN BARRIER

Edna F. Choo, Daniel Kurnik, Mordechai Muszkat, Tadashi Ohkubo, Sheila D. Shay, James N. Higginbotham, Hartmut Glaeser, Richard B. Kim, Alastair J.J. Wood, and Grant R. Wilkinson.

Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA.

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: P-gp inhibition in lymphocytes and the blood:brain barrier.

Corresponding author:

Grant R. Wilkinson Ph.D., D.Sc.

Department of Pharmacology

Vanderbilt University School of Medicine

Nashville, TN 37232-6602

Tel: 615-322-3033

Fax: 615-343-6634

e-mail: grant.wilkinson@vanderbilt.edu

Contents:

Number of pages – 32

Number of tables – 0

Number of figures – 4

Number of references – 39

Number of words in:

Abstract – 250

Introduction – 732

Discussion – 1,472

Recommended section: Absorption, Distribution, Metabolism and Excretion

Abbreviations: HIV-1, human immunodeficiency virus-1; CYP3A, cytochrome P450 3A; MDR1, multidrug resistance protein 1; DMSO, dimethylsulfoxide; USPHS, United States Public Health Service; HBSS, Hank's buffered salt solution; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; APC, allophycocyanin; AUEC, area-under the latency effect-curve; ANOVA, univariate analysis of variance; IC, inhibitory concentration; ED, effective dose; E_{max}, maximal effect; A→B, apical to basal; B→A, basal to apical; Bcrp, breast cancer resistance protein; Oatp, organic anion transporting polypeptide; Tc, technetium.

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

A major functional component of the blood:brain barrier is P-glycoprotein. In principle, inhibition of this efflux transporter would permit greater distribution of its substrates into the brain and increased central effects. Tariquidar and elacridar, potent and selective P-glycoprotein inhibitors, were investigated in this regard using the opioid loperamide as an *in vivo* probe in mice. Pretreatment with both inhibitors converted intravenous loperamide from a drug without central effects to one producing antinociception. Radiolabeled loperamide tissue distribution studies indicated that inhibition was associated with increased uptake into brain and testes, in the absence of changes in plasma levels, along with enhanced efflux of rhodamine - 123 from $CD3e^+$ T-lymphocytes. With tariquidar, however, the loperamide dose-response curves for testes:plasma and brain:plasma concentration ratios were shifted 6- ($p=0.07$) and 25-fold ($p<0.01$) to the right, respectively, (ED_{50} = 1.48 and 5.65 mg/kg) compared to the rhodamine - 123 efflux curve (ED_{50} = 0.25 mg/kg). Less pronounced shifts were noted with elacridar where the brain:plasma ratio was shifted only 2-fold relative to the rhodamine - 123 efflux data (ED_{50} = 2.36 versus 1.34 mg/kg, respectively; $p<0.01$). These results indicate that the P-glycoprotein localized in the blood:brain barrier, and to a lesser extent the testes:blood barrier, is more resistant to inhibition than at other tissue sites like the lymphocyte; moreover, the extent of this effect depends on the inhibitor. Such resistance can be overcome by a sufficiently high dose of an inhibitor, however, whether this is safely attainable in the clinical situation remains to be determined.

Introduction

The importance of P-glycoprotein (MDR1, ABCB1) in the disposition of its substrates is now firmly established. For example, its presence in the intestinal tract, liver and kidney tubule enhances drug elimination of both unchanged drug and metabolites. In addition, P-glycoprotein also has a barrier function and restricts the oral absorption of drugs and their distribution into the brain, testes, and fetus. Although such a protective function is teleologically highly desirable, it presents a problem from a drug delivery standpoint, since it may, for example, limit the attainment of sufficiently high drug levels in the brain in order to effectively treat central aspects of diseases like HIV-1 infection or tumors with intact brain-type vasculature.

The effectiveness of P-glycoprotein present in brain capillary endothelial cells in restricting the distribution of substrate drugs into the brain has been well-documented by studies in "knockout" animals (Schinkel *et al.*, 1994, 1995, 1996; Kim *et al.*, 1998). For well-transported drugs, like ivermectin (Schinkel *et al.*,1994) and digoxin (Schinkel *et al.*, 1995), the increase in brain concentrations may be 30- to 90-fold in the absence of the functional transporter relative to when it is present, and usually benign dosages elicit profound neurotoxicity in knockout mice (Schinkel *et al.*,1994, 1996).

Given the critical role for P-glycoprotein in the blood-brain barrier, the possibility of modulating the transporter's function to increase drug distribution into the brain is an attractive concept. In fact, a number of *in vivo* proof-of-principle studies in animals have been undertaken using several different inhibitors of P-glycoprotein and various probe drugs. For example, drug brain levels relative to those in blood/plasma of digoxin (Mayer *et al.*, 1997, Song *et al.*, 1999) and nelfinavir (Choo *et al.*, 2000) were increased in mice pretreated with valspodar (PSC-833), and the neurotoxicities of ivermectin and

cyclosporine-A were greater following administration of the modulator (Didier and Loor, 1995). Similar studies have been reported with more recently developed inhibitors with increased potency such as elacridar (GF-120918) and various drugs (Kemper *et al*., 2003; Kemper *et al*., 2004a; Letrent *et al*., 1999; Letrent *et al*., 1998). Also, zosuquidar (LY-335979) markedly enhances nelfinavir concentrations in the brain to an extent comparable to those in *mdr1* knockout animals (Choo, *et al*., 2000) and, to a lesser degree, those of paclitaxel (Kemper *et al*., 2004b). However, the logical extension of P-glycoprotein inhibition of the blood-brain barrier to humans is not straightforward.

The critical issues are the potency, selectivity and toxicity of the modulator; that is, can effective inhibition of transporter function in specific tissues actually be achieved using available inhibitors at dosages/concentrations without untoward effects? Experience with the most clinically developed inhibitor, valspodar, indicates that this is not a readily achievable goal in patients receiving cancer chemotherapy (Leonard *et al*., 2002; Thomas and Coley, 2003). Also, most first- and second-generation inhibitors affect other disposition processes by, for example, inhibiting drug metabolism (Leonard *et al*., 2002; Thomas and Coley, 2003) complicating interpretation of study findings since increased brain concentrations may simply be a reflection of a higher blood level. Finally, and in contrast to animal studies, it is difficult in humans to measure the brain levels of a drug in order to determine whether pretreatment with a P-glycoprotein inhibitor affects drug distribution into this tissue. On the other hand, centrally mediated drug effects indicate passage of drug across the blood-brain barrier. Accordingly, the requirements for *in vivo* studies of modulating P-glycoprotein function in human brain capillaries requires a sufficiently potent inhibitor that does not affect the metabolism of

the probe drug, *i.e.*, selectivity, and ideally the probe drug must, in turn, elicit a measurable and quantitative central effect, secondary to increased brain levels.

The purpose of this study, therefore, was to obtain proof-of-principle based on the above requirements in a mouse model that would provide the necessary underpinning for extension to human subjects. The primary experimental approach involved the use of the potent and selective P-glycoprotein inhibitor tariquidar (XR-9576) and loperamide as a probe drug. Selection of the latter was based on the fact that loperamide is a potent opioid with an affinity for the μ-receptor similar to morphine (Mackerer *et al*., 1976; Stahl *et al*., 1977), however, it is normally devoid of central effects because the blood-brain barrier prevents its distribution into the brain; by contrast, in *mdr1* knockout mice stereotypical opiate effects are present (Schinkel *et al*., 1996). Additional studies were also undertaken with elacridar, another third-generation inhibitor.

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Drug solutions

Tariquidar (Xenova Ltd., Slough, Bucks, UK) for injection was dissolved in propylene glycol, 5% dextrose solution and ethanol (4:5:1) at an initial concentration of 10 mg/ml; subsequently, this was diluted with 5% dextrose solution to provide a concentration of 1.56 mg/ml. For the flow cytometry studies, tariquidar (3mM) was dissolved in DMSO and diluted to 60 μM with Hank's buffered salt solution (HBSS;Gibco/Invitrogen Corp., Carlsbad, CA). In the transport studies, the stock solution was diluted with Optimem I (GibcoBRL, Gaithersburg, MD) to provide a 40 nM solution. Elacridar (GlaxoSmithKline, Research Triangle Park, North Carolina, USA) solution for injection was prepared as a propylene glycol/ethanol/water solution (3:2:5) at a concentration of 5 mg/ml; subsequent dilutions were made with water. Loperamide hydrochloride (Sigma, St. Louis, MO) was initially dissolved in 10% ethanol and saline solution (2 mg/ml) and dilutions made with saline to provide appropriate strength solutions for injection. When tritiated loperamide (specific activity 596 GBq/nmole, 95% purity, Janssen Pharmaceutica, Beerse, Belgium) was used, it was added to the unlabeled solution at a concentrate of 8.33 μCi/ml. In the *in vivo* studies, all drug solutions were injected in a total volume of 4 μl/g body weight.

In vitro inhibition of loperamide transport by tariquidar

Cultured L-MDR1 cells were grown under previously described conditions (Kim *et al.*, 1998) and plated at a density of 2 x 10^5 cells/12 mm well on porous (3.0 μm) polycarbonate membrane filters (Transwell™, Costar Corp., Cambridge, MA). Cells

were supplemented with fresh media every 2 days and used in transport studies on the fourth or fifth day after plating. Transepithelial resistance was measured in each well using a Millicell ohmmeter (model ERS, Millipore Corp., Beford, MA); wells registering a resistance of 200Ω or greater, after correcting for that measured in control blank wells, were used in the transport studies. About 1-2 hr before the start of the transport experiment, the medium in each compartment was replaced with Optimem I, a serum-free medium. Then, the transport of ^{3}H-labeled loperamide was measured after replacing the medium in each compartment with 700 µl Optimem with or without the radiolabeled drug. Radioactivity appearing in the opposite compartment after 1, 2, 3, and 4 hr was measured in 25 µl aliquots taken from each compartment, following the addition of 5 ml ScintiVerse scintillation fluid (Fisher Scientific Co., Fairlawn, NJ). Inhibition of transport was determined in a similar fashion after the addition of tariquidar (0.001 to 1 µM) to both the apical and basal compartments before the addition of radiolabeled loperamide. The same experiments were performed with parental LLC-PK1 cells which do not over express human P-glycoprotein. The apparent permeability of loperamide across LLC-PK1 cells was estimated as previously described (Mahar *et al*., 2002).

Inhibition by tariquidar of rhodamine - 123 efflux from CD3e^{+} T-lymphocytes

Studies of inhibition of efflux were performed in male FVB/NTTafcBr mice weighing 20-30 g (Taconic, Germantown, NY) following intravenous administration of tariquidar through a tail vein 30 min prior to sacrifice with isoflurane (Forane, Baxter Healthcare Corp, Deerfield, IL). Approval for these and other animal studies was obtained from the Vanderbilt University, Institutional Animal Care and Use Committee,

and the mice were cared for in accordance with USPHS guidelines. The tariquidar dose range ranged from 0.05 to 6.25 mg/kg along with a vehicle control injection, and 3 to 4 animals were used at each dose level. Similar studies were also performed following pretreatment of mice with elacridar (0.1 to 10 mg/kg; n=3 at each dose level). The time course of inhibition was determined following administration of vehicle and tariquidar (1 and 6.25 mg/kg) with splenocytes being obtained at 0, ½, 1, 2, and 4 hr. After removing the spleen, it was washed (x 3) with sterile, ice-cold HBSS containing 15 mM HEPES (EM Science, Gibbstown, NJ) and 0.1% bovine serum albumin (Sigma), and then cut and mashed. The tissue was then centrifuged at 4ºC and 250 g for 7 min and the isolated cells were washed (x 3) with supplemented HBSS, then resuspended in the same buffer to a concentration of about 10^6 cells/ml.

Inhibition of P-glycoprotein-mediated efflux from the isolated splenocytes was determined using a modified procedure of a previously described method (Robey *et al.*, 1999). Specifically, spleen cells were loaded with rhodamine - 123 (Sigma) and counter-stained with pan T-cell marker, antimouse CD3e conjugated to APC. This conjugate was chosen to maximize sensitivity and simultaneously minimize the requisite compensation, which in all experiments was less than 2%. Cell suspensions from each mouse were divided into 4 x 1 ml aliquots; to two of these was added 3 µM tariquidar in 50 µl HBSS as controls, and the same volume of buffer was added to the remaining 2 aliquots. All preparations were kept in the dark and on ice except when they were being incubated. Rhodamine - 123 was added to each preparation, 0.5 µg/ml, which was then incubated with shaking for 10 min at 37ºC in the dark. Net cellular accumulation of rhodamine - 123 was stopped by immediate cooling to 4ºC and the suspension was subsequently

centrifuged at 250 g for 5 min at 4ºC. Cells were washed (x 1) and resuspended in 1 ml ice-cold HBSS, and tariquidar (3 µM), to produce >99% inhibition of efflux, or HBSS was added to the appropriate cells. One each of the HBSS- and tariquidar-treated preparations was kept on ice with shaking, to serve as the zero-time point with respect to rhodamine efflux, whereas the other cell suspensions were incubated with shaking at 37ºC for 30 min. Rhodamine - 123 efflux was stopped by immediate cooling of the cells to 4ºC and APC-conjugated hamster, anti-mouse CD3e monoclonal antibody (BD Biosciences/Pharmingen, San Diego, CA) was added. After 30 min incubation with shaking in the dark at 4ºC, the cells were spun down at 250 g for 5 min at 4ºC and washed (x 2) before resuspension in 1 ml ice-cold HBSS. Propidium iodide (0.2 µg; Molecular Probes, Eugene, OR) was added to allow assessment of viability.

Flow cytometry (LSRII, Beckman Dickinson) with lasers at 488 nm, 407 nm and 635 nm) was performed within 1 hr of the completion of the final cell incubation. Cells were initially gated into a P1-gate for the exclusion of doublets and viability using a plot of side-scatter (SSC) *versus* forward scatter (FSC). "Bright" (dye efflux negative) propidium iodide-stained cells were excluded by a P2 gate. Viable lymphocytes were gated into P3 based on their reactivity to anti-CD3e antibody and rhodamine - 123 containing cells were plotted as a single parameter histogram gated to the zero-time point cut-off. "Dim" cells to the left of this point were considered to be effluxing cells. PMT voltages were as follows: FSC, 420; SSC, 450; rhodamine - 123, 550 (LP505, 530/30 DF); PI/7 AAD, 560 (LP 670, 595/40 DF); and APC, 580(660/20 DF).

Efflux was expressed as the reduction in "dim" cells from baseline (average of "dim" cells in vehicle-treated) in each animal according to Equation 1:

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

$$\left(1-\frac{\text{"dim"cells inhibition}}{\text{"dim"cells vehicle}}\right) \text{ x } 100\% \qquad \text{(Equation 1)}$$

In vivo effect of tariquidar on loperamide's antinociceptive response

Male *mdr1a/1b*(-/-) FVB/TacfBR-[KO] Pgy2N7 and syngeneic FVB/NTTacfBR wild-type (*mdr1a/1b*(+/+)) mice weighing 20-30g were obtained from Taconic. Antinociception was measured by a hot plate test (Analgesia Hotplate, Columbus Instruments, Columbus, OH.) Mice were individually placed onto the hot plate (surface temperature, 52 ± 0.1°C) and the time taken before they licked their hind paw was recorded. If the animal did not perform this act within 30 sec, it was removed from the hot plate to prevent tissue injury. Each mouse was used only once, and a pre-drug response time, following 3 days of training, was determined prior to the administration of any drug. In one series of studies, *mdr1a/1b*(+/+) and (-/-) mice (n = 3 at each dose level) were pre-treated with vehicle or tariquidar (6.25 mg/kg) intravenously administered into a tail vein 15 min prior to administration of loperamide (0.1 to 1mg/kg) or vehicle by the same route. The antinociceptive response of individual mice was determined at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 min. In a second series of studies the dose of tariquidar was varied (1 to 6.25 mg/kg) and that of loperamide was fixed (1 mg/kg), and studies were only undertaken in *mdr1a/1b*(+/+) mice (n=3-4 at each dose level). Studies were also undertaken in a similar fashion to determine the antinociceptive response to loperamide (1mg/kg) following pretreatment with elacridar (0.1 to 30 mg/kg; n=3-4 at each dose level). Antinociception over the first 2hr of study was measured by Equation 2:

$$\%\ \text{antinociception} = \frac{AUEC_{lop} - AUEC_{baseline}}{AUEC_{max} - AUEC_{baseline}} \text{ x } 100\%, \qquad \text{(Equation 2)}$$

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

where AUEC is the area-under the latency effect-curve estimated by the trapezoid rule under control conditions ($AUEC_{baseline,}$), following loperamide ($AUEC_{lop}$), and the 30 sec maximal effect ($AUEC_{max}$).

Determination of loperamide's plasma, testes and brain concentrations

Male mdr*1a/b(+/+)* mice weighing 20-30g were administered either tariquidar (0.1 to 30 mg/kg; n=3-4 for each dose) or elacridar (0.1 to 30 mg/kg; n=1-2 for each dose) into a tail vein. Fifteen minutes later, radiolabeled loperamide (0.083μCi/g) along with 1 mg/kg unlabeled drug was also injected intravenously. After a further 15 min, the animals were anesthetized using isoflurane, and blood was obtained from the inferior vena cava. Following sacrifice, organs were harvested, blotted with filter paper to remove blood, weighed and then homogenized with a 10-fold volume of 4% bovine serum albumin solution (Sigma). Total radioactivity was determined by liquid scintillation after the addition of 100μl plasma or 500 μl tissue homogenate to 6ml BCS reagent (Amersham, Arlington Heights, IL).

Data Analysis

Data are expressed as mean ± standard deviation (SD), and drug responses are shown as mean ± standard error (SEM). Log dose-response relationships were analyzed by non-linear regression analysis based on a log-sigmoidal model with variable slope (Prism 4.0, GraphPad Software Inc., San Diego, CA). This statistical software provides estimates of means and confidence intervals for IC_{50}/ED_{50} and E_{max} values. Univariate analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons was

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

used to compare $logED_{50}$ values for the different responses. All tests were two-tailed, and a p-value of <0.05 was considered significant. For graphic representation, the response data are expressed as a percentage of the maximum effect.

Results

Tariquidar as an inhibitor of P-glycoprotein:

LMDR1 cells are LLC-PK1 cells stably transfected with human *MDR1* cDNA and, therefore, overexpress the MDR1 gene product P-glycoprotein. Use of these two cell lines, accordingly, permits the assessment of P-glycoprotein-mediated efflux of substrate drugs in a side-by-side fashion. Consistent with the apical expression of the transporter, transfer of loperamide across cultured LMDR1 cells displayed polarization dependent on the direction of flux; basal-to-apical being more extensive than apical-to-basal with a ratio at 3 hr of 35.7 ± 9.4 (n=14). By contrast, uptake in parental LLC-PK1 cells was similar regardless of the direction of flux. Addition of tariquidar to LMDR1 cells reduced the extent of polarization and inhibition of efflux transport exhibited a dose-response relationship (Fig.1) with an IC_{50} value of 15nM (95% CI, 10.1 to 21.5 nM) ~ 10 ng/ml. The estimated B→A/A→B transport ratio for loperamide in the presence of 1μM tariquidar was 1.23 ± 0.37, and its permeability in LLC-PK1 cells was 4.00 ± 0.95 nm/sec (B→A) and 4.37 ± 1.09 nm/sec (A→B). Similar values were obtained in LMDR1 cells (4.74 ± 2.36 nm/sec (B→A) and 5.04 ± 1.13 (A→B) in the presence of 1 μM tariquidar).

Effect of tariquidar on loperamide's antinociceptive effect:

In both *mdr1a/1b*(+/+) and (-/-) mice pretreated with 6.25 mg/kg tariquidar, increasing doses of intravenous loperamide resulted in enhanced antinociception (Fig 2). At dosages of 0.5 mg or greater of loperamide in knockout animals, the maximal latency period was achieved within 5 min and maintained for almost 3 hr or longer before, in the case of the 0.5 mg/kg dose, the effect began to decline after this time. In *mdr1a/1b* (+/+) mice a more blunted response was seen at any particular dose level, but even so a maximal response was maintained for nearly 1 and 2 hr after 0.5 and 1 mg/kg loperamide, respectively. Pretreatment of *mdr1a/1b*(+/+) mice with varying doses of tariquidar, followed by 1 mg/kg loperamide resulted in a dose-related antinociceptive response especially at levels of 2 to 6.25 mg/kg (Fig 3). The effect was maximal within 5 min, and subsequently it declined towards baseline over the 4 hr study period. After 4 and 6.25 mg/kg tariquidar, the maximal plateau effect was maintained for 1½ hr before it began to decline. Similar findings were also noted after pretreating the mice with 0.1 to 30 mg/kg elacridar (data not shown).

Dose-response relationships to P-glycoprotein inhibition:

Rhodamine - 123 efflux from $CD3e^+$ lymphocytes obtained from *mdr1a*/*1b*(+/+) mice pretreated with tariquidar 30 min previously was inhibited in a dose - response fashion (Fig. 4). The ED_{50} was estimated to be 0.25 mg/kg (95% CI, 0.06 to 1.02 mg/kg), and the maximal inhibition was the same as that obtained in $CD3e^+$ lymphocytes from *mdr1a/1b* (-/-) mice. Greater than 95% inhibition was maintained over the 4hr study period following intravenous administration of 1 and 6.25 mg/kg tariquidar to wild-type

mice (data not shown). Elacridar pretreatment also inhibited rhodamine - 123 efflux, but it was about 5-fold less potent than tariquidar with an ED_{50} value of 1.34 mg/kg (95% CI 1.21 to 1.47 mg/kg). Furthermore, the dose-response curve was far steeper than that of tariquidar (Hill slope factor: 0.53 (95%CI, -0.18 to 2.03) and 3.84 (95% CI, 2.66-5.02) for tariquidar and elacridar, respectively; $p<0.05$).

Testes:plasma and brain:plasma concentration ratios of radiolabeled loperamide measured 30 min after pretreatment with tariquidar and 15 min following intravenous loperamide administration to *mdr1a/1b*(+/+) mice also demonstrated dose relationships (Fig. 4). These appeared to reflect changes in loperamide's tissue distribution since the plasma radioactivity concentration was similar regardless of the pretreatment tariquidar dose (data not shown). However, the curves were shifted markedly (6- and 25- fold) to the right relative to the rhodamine - 123 efflux data (Fig. 4) with ED_{50} values of 1.48 mg/kg (95% CI, 0.79 to 2.77 mg/kg) (testes:plasma) and 5.65 mg/kg (95% CI, 4.28 to 7.66 mg/kg) (brain:plasma), respectively ($p=0.07$ and $p<0.01$ for comparison with rhodamine-123 efflux data, respectively). With elacridar, the ED_{50} for the brain:plasma concentration ratio was only 2-fold greater, 2.36 mg/kg (95%, CI 2.11 to 2.63), than that for rhodamine - 123 efflux from $CD3e^{+}$ lymphocytes (ED_{50} = 1.34 mg/kg, 95% CI, 1.21 to 1.47 mg/kg; $p<0.01$). Also, elacridar pre-treatment (10 mg/kg) of *mdr1a/1b*(-/-) mice resulted in a similar brain:plasma ratio as seen in *mdr1a/1b*(+/+) animals administered 5 to 30 mg/kg tariquidar. The ED_{50} for elacridar distribution into the testes of 1.09 mg/kg (95% CI, 0.75 to 1.58 mg/kg) was similar to that for rhodamine - 123 efflux from lymphocytes ($p>0.05$). The brain:plasma ratio following pretreatment with 10 mg/kg elacridar was similar in both *mdr1a/1*b (+/+) and (-/-) mice.

Loperamide's antinociceptive effect at 15 min following its intravenous administration (1mg/kg) in mice pre-treated with varying doses of tariquidar was essentially dichotomous – either no effect or maximal response (Fig. 3). Accordingly, the area under the latency effect-time curve (AUEC) over 2 hr was used as a measure of loperamide's central action. (Fig. 3). The resulting dose-response relationship was somewhat steeper than that observed for rhodamine - 123 efflux from lymphocytes and also the brain:plasma concentration ratio; the estimated ED_{50} value, 2.18 mg/kg (95% CI, 1.76 to 2.70 mg/kg), being 3-fold lower than the brain:plasma distribution ratio ($p>0.05$) but about 10-fold higher than that for rhodamine - 123 efflux ($p<0.05$) (Fig. 4). With elacridar, the antinociceptive dose-response curve was also shifted to the left of the brain:plasma concentration ratio, and it was quite steep, with the no-effect to maximal effect dose range being between 1 and 2 mg/kg. However, the ED_{50} (1.65 mg/kg (95% CI 1.55 to 1.76 mg/kg) for antinociception was not significantly different from that for rhodamine - 123 efflux from lymphocytes ($p>0.05$).

Discussion

The purpose of this study was to obtain proof-of-principle that pharmacological inhibition of P-glycoprotein localized at the blood-brain barrier would convert loperamide from a drug without opioid effects on the brain to one with such a characteristic. In turn, this would provide underpinning for studies in humans based on measuring changes in the drug's central effects. The results with tariquidar, a potent and selective third-generation P-glycoprotein inhibitor (Martin *et al*., 1999; Mistry *e*t *al*., 2001, provided such proof in that antinociception in *mdr1a*/1b(+/+) mice exhibited a

dose-response relationship reflective of the extent of inhibition. Additionally, the onset of the effect was rapid indicating fast distribution into the brain followed by a decline dependent on the dose of loperamide. Similar observations were also noted with elacridar, thus confirming the generalizability of the phenomenon. In addition, elacridar, at concentrations similar to those associated with inhibition of P-glycoprotein, also inhibits Bcrp1 (Abcg2) (Allen and Schinkel, 2002); however, its administration to *mdr1a/1b* (-/-) mice did not increase the maximal brain:plasma concentration ratio relative to that seen in untreated animals. This would suggest that the observed effects at the blood:brain barrier are primarily accounted for by inhibition of P-glycoprotein, although other efflux transporters cannot be entirely excluded.

Neither tariquidar nor elacridar altered loperamide's plasma level, consistent with their minimal inhibition of cytochrome P4503A (personal communication, Xenova Ltd.; Cummins, *et al.*, 2002), which is mainly responsible for the opioid's primary metabolism, at least in humans (Kim *et al*., 2004). Thus, the observed increases in loperamide's brain:plasma concentration ratio with increasing dosages of the P-glycoprotein inhibitors reflected a change in its distribution into the brain. Such distribution of unbound drug, generally, is determined by the balance of its membrane permeability at the blood:brain barrier, influencing influx, *versus* efflux mediated by P-glycoprotein. Thus, drug delivery to the brain is likely if the drug is not a good P-glycoprotein substrate as indicated by B→A/A→B transport ratio in LMDR1 cells of < 2 at 3hr and has appropriate passive permeability (Lin and Yamazaki, 2003). Similar characteristics have been suggested using MDR1-MDCKII cells (Mahar *et al*., 2002).

In the absence of tariquidar, the B→A/A→B transport ratio of loperamide in LMDR1 cells was greater than 30 - consistent with it being a good MDR1 substrate (Schinkel *et al*., 1996). Furthermore, treatment of the cells with tariquidar resulted in concentration-dependent inhibition of transport. Thus, the observed results are entirely consistent with an increasing distribution of loperamide into the brain resulting from a decrease in the effectiveness of the blood:brain barrier, secondary to P-glycoprotein inhibition. Interestingly, loperamide's *in vitro* permeability across cultured LLC-PK1 cells was 5-fold less than the suggested critical value for distribution into the brain of 20nm/sec (Mahar et al., 2002; Lin and Yamazaki, 2003). However, *in vivo* the drug's brain levels and antinociceptive effect were rapidly achieved indicating ready passage into central tissue. Moreover, direct measure of loperamide's permeability *in vivo* is also consistent with the lack of any difficulty in the drug entering the brain in the absence of P-glycoprotein (Dagenais *et al*., 2004). This suggests the possible involvement of transport-mediated uptake at the blood:brain barrier by perhaps an Oatp transporter, which is not expressed in the cultured cells (Marzolini *et al*., 2004).

Although the major and original goal of the studies was focused on P-glycoprotein in the blood:brain barrier, inhibition was determined at other sites in order to obtain a more global perspective and, in the case of lymphocytes to relate the results to those previously obtained in humans (Stewart *et al*., 2000). Tariquidar is a potent inhibitor of rhodamine - 123 efflux from human $CD56^+$ lymphocytes with an *ex vivo* IC_{50} of 33 nM (~20 ng/ml). Furthermore, after intravenous administration in humans, this inhibition was maximal after a dose of 2 mg/kg; moreover, it was sustained for almost 24 hr (Stewart *et al*., 2000). Additionally, following an intravenous dose of 150 mg

tariquidar to cancer patients, the uptake of the P-glycoprotein substrate ^{99m}Tc-sestamibi into liver and various drug resistant tumors was substantially increased, and excretion into the gastro-intestinal tract and by the kidneys was markedly reduced (Agwaral *et al*., 2003). Such findings along with those from tissue distribution studies in animals (Investigator Brochure, Tariquidar, 2005) all indicate that tariquidar at dosages/concentrations associated with complete inhibition of lymphocyte P-glycoprotein also inhibits the transporter's function at other tissue sites including tumors. It was, therefore, unexpected to find that measures reflecting P-glycoprotein dependent drug distribution into the brain, in particular, exhibited greater resistance to inhibition than did lymphocytes. Such differential sensitivity is even more remarkable since, within the microvasculature, P-glycoprotein located in the lymphocyte and endothelial cell membrane is presumably exposed to the same luminal concentration of the inhibitor.

It would, therefore, appear that P-glycoprotein function localized in the blood:brain barrier and to a lesser extent in the blood:testes barrier is more resistant to inhibition by tariquidar than that in lymphocytes and at other sites. This makes teological sense given the critical nature of these organs and the desirability of protecting them from potentially damaging xenobiotics. However, the involved mechanism is unknown. Generally, P-glycoprotein mediated transport is considered to be associated with a 170 kDa protein, (Ambudkar *et al.*, 1999) but functional “mini P-glycoproteins” rather than the classic transporter have been reported in certain lymphocytes (Trambas *et al*., 2001). Such a difference or other structural distinctions in the transporter may account for the differential tissue sensitivity. An alternative explanation could be related to the fact that P-glycoprotein is localized in caveolae within brain microvascular endothelial cell

membranes whereas such microdomains are absent in lymphocytes (Fra *et al*., 1994; Demeule *et al*., 2000). Because caveolae are enriched in cholesterol (Smart *et al*., 1999) and P-glycoprotein activity is increased in an enhanced cholesterol micro-environment (Gayet *et al*., 2005; Troost *et al*., 2004) this could affect the transporter's interaction with inhibitors dependent on cell type. A further possible factor may be the difference in the amount/number of P-glycoprotein pumps present in the two involved cell membranes. This could be important because tariquidar apparently acts on the cytoplasmic side of the membrane rather than at the extracellular face, and in such a situation the apparent half inhibition concentration (IC_{50}) increases with the number of pumps (Litman *et al*., 2003). Thus, the more abundant expression of P-glycoprotein in the brain capillary endothelial cell, compared to lymphocytes and other cell types (Ambudkar, *et al*., 1999) would account for its greater resistance to inhibition. Additional studies will be required to investigate these possible causal differences and their relative importance.

A further unexplainable observation is the greater difference between lymphocyte and brain capillary endothelial cell inhibition following pretreatment with tariquidar (20-fold) compared to that with elacridar (2-fold). Also, despite the fact that it was not statistically significant, there was a trend for a greater differential sensitivity of the blood:testes barrier to inhibition relative to lymphocyte efflux for tariquidar (6-fold; $p=0.07$) compared to elacridar (no difference). In contrast to earlier P-glycoprotein modulators, these third-generation drugs are not substrates for the transporter rather they interact with multiple, distinct binding sites on P-glycoprotein which results in complex allosteric changes leading to altered transport function (Martin *et al*., 1999, 2000). Moreover, the binding sites for tariquidar and elacridar appear to be different (Martin *et*

al., 2000). Perhaps this difference in the interaction with the transporter accounts for the greater similarity between P-glycoprotein inhibition in lymphocytes and that in the blood:brain and blood:testes barrier following elacridar administration compared to the more marked difference with tariquidar. It is also possible that the two drugs may possess different and unknown inhibitory properties to alter uptake and/or efflux transporters that are differentially expressed in different cell types.

The described studies clearly demonstrate and confirm that pharmacologic modulation of the blood:brain barrier through inhibition of P-glycoprotein can markedly increase brain levels of a substrate for the transporter that is normally excluded from this site. In the case of loperamide, measurable central effects of the opioid become manifest. However, this situation appears to be more complex than at first sight since, in addition to considerations related to inhibitor potency, the nature of the molecular interaction between the inhibitor and P-glycoprotein also seem to be important. Thus, the greater resistance to inhibition of the transporter located in the brain compared to other sites requires doses/concentrations greater than those associated with inhibition at other tissue sites, including tumors. Moreover, the difference appears to depend on the individual inhibitor being greater for tariquidar and elacridar – whether other third-generation, non-competitive inhibitors such as zosuquidar and laniquidar or earlier used inhibitors that are substrates, *e.g*., quinidine, cyclosporine-A, or valspodar, have similar characteristics is unknown. Certainly, the fact that P-glycoprotein inhibition is present in lymphocytes and other tissue does not necessarily indicate that this situation also applies to the brain. Accordingly, strategies to increase drug delivery to the brain through inhibiting P-glycoprotein will have to take these considerations into account.

Acknowledgements: The gifts of radiolabeled loperamide, tariquidar, and elacridar used in the research and provided by Janssen Pharmaceutica, Xenova Ltd., and GSK Ltd., respectively, are gratefully acknowledged.

References:

Agrawal M, Abraham J, Balis FM, Edgerly M, Stein WD, Bates S, Fojo T, and Chen CC (2003) Increased ^{99m}Tc-sestamibi accumulation in normal liver and drug-resistant tumors after the administration of the glycoprotein inhibitor, XR9576. *Clin Cancer Res* 9:650-656.

Allen JD, Schinkel AH, (2002) Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein (BCRP/A BcG2). *Mol Cancer Therap* 1:427-434.

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, GottesmanMM (1999) Biochemical, cellular, and pharmacological aspects of the multidrugtransporter. *Annu Rev Pharmacol Toxicol* 39:361-398.

Choo EF, Leake B, Wandel C, Imamura H, Wood AJJ, Wilkinson GR and Kim RB (2000) Pharmacological inhibition of P-glycoprotein transport enhances the distribution of HIV-1 protease inhibitors into brain and testes. *Drug Metab Disp* 28:655-660.

Cummins CL, Jacobsen W and Benet LZ (2002) Unmasking the dynamic interplay between intestinal P-glycoprotein and CYP3A4. *J Pharmacol Exp Ther* 300:1036-1045.

Dagenais C, Graff CL, and Pollack GM (2004) Variable modulation of opioid brain uptake by P-glycoprotein in mice. *Biochem Pharmacol* 67:269-276.

Demeule M, Jodoin J, Gingras D and Béliveau R (2000) P-glycoprotein is localized in caveolae in resistant cells and in brain capillaries. *FEBS Letters* 466:219-224.

Didier AD and Loor F (1995) Decreased biotolerability for ivermectin and cyclosporin A in mice exposed to potent P-glycoprotein inhibitors. *Int J Cancer* 63:263-267.

Fra AM, Williamson E, Simons K, and Parton RG (1994) Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J Biol Chem* 269:30745-30748.

Gayet L, Dayan G, Barakat S, Labialle S, Michaud M, Cogne S, Mazane A, Coleman A, Rigal D and Baggetto, LG (2005) Control of P-glycoprotein activity by membrane cholesterol amounts and their relation to multidrug resistance in human CEM leukemia cells. *Biochemistry* 44:4499-4509.

Investigator Brochure, Tariquidar (XR-9576) – P-glycoprotein Pump Inhibitor (2005), Xenova Ltd, Slough, U.K.

Kemper EM, van Zandbergen AE, Cleypool C, Mos HA, Boogerd W, Beijnen JH and van Tellingen O (2003) Increased penetration of paclitaxel into the brain by inhibition of P-glycoprotein. *Clin Cancer Res* 9:2849-2855.

Kemper EM, Verheij M, Boogerd W, Beijnen JH, van Tellingen O (2004a) Improved penetration of docetaxel into the brain by co-administration of inhibitors of P-glycoprotein. *Eur J Cancer* 40:1269-1274.

Kemper EM, Cleypool C, Boogerd W, Beijnen JH and van Tellingen O (2004b) The influence of the P-glycoprotein inhibitor zosuquidar trihydrochloride (LY335979) on the brain penetration of paclitaxel in mice. *Cancer Chemother Pharmacol* 53:173-178.

Kim KA, Chung J, Jung, DH, and Park JY (2004) Identification of cytochrome P450 isoforms involved in the metabolism of loperamide in human liver microsomes. *Eur J Clin Pharmacol* 60:575-581.

Kim RB, Fromm MF, Wandel C, Leake B, Wood AJJ, Roden DM, Wilkinson GR (1998) The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J Clin Invest* 101: 289-294.

Leonard GD, Polgar O, and Bates SE (2002) ABC transporters and inhibitors: new targets, new agents. *Curr Opin Invest Drugs* 3:1652-1659.

Letrent SP, Pollack GM, Brouwer KR and Brouwer KLR (1998) Effect of GF120918, a potent P-glycoprotein inhibitor, on morphine pharmacokinetics and pharmacodynamics in the rat. *Pharmaceut Res* 15:599-605.

Letrent SP, Pollack GM, Brouwer KR and Brouwer KLR (1999) Effects of a potent and specific P-glycoprotein inhibitor on the blood-brain barrier distribution and antinociceptive effect of morphine in the rat. *Drug Metab Disp* 27:827-834.

Litman T, Skovgaard T, Stein WD (2003) Pumping of drugs by P-glycoprotein: a two-step process? *J Pharmacol Exp Ther* 307:846-853.

Lin JH and Yamazaki M (2003) Clinical relevance of P-glycoprotein in drug therapy. *Drug Metab Rev* 35: 417-454.

Mackerer CR, Clay GA and Dajani EZ (1976) Loperamide binding to opiate receptor sites of brain and myenteric plexus. *J Pharmacol Exp Ther* 199:131-140.

Mahar Doan KM, Humphreys J, Webster LO, Wring SA, Shampine LJ, Serabjit-Singh CJ, Adkison KK, Polli JW (2002) Passive permeability and P-glycoprotein-

mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. *J Pharmacol Exp Ther* 303:1029-1037.

Martin C, Berridge G, Mistry P, Higgins C, Charlton P and Callaghan R (1999) The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein. *Br J Pharmacology*. 128:403-411.

Martin C, Berridge G, Higgins CF, Prakash M, Charlton P and Callaghan R (2000) Communication between multiple drug binding sites on P-glycoprotein. *Mol Pharmacol*: 58:624-632.

Marzolini C, Tirona R, and Kim, RB (2004) Pharmacogenomics of the OATP and OAT families. *Pharmacogenomics* 5:273-282.

Mayer U, Wagenaar E, Dorobet B, Beijnen JH, Borst P and Schinkel AH (1997) Full blockade of intestinal P-glycoprotein and extensive inhibition of blood-brain barrier P-glycoprotein by oral treatment of mice with PSC 833. *J Clin Invest* 10:2430-2436.

Mistry P, Stewart AJ, Dangerfield W, Okiji S, Liddle C, Bootle D, Plumb J, Templeton D and Charlton P (2001) *In vivo* and *in vivo* reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. *Cancer Res* 61:749-758.

Robey R, Bakke S, Stein W, Meadows B, Litman T, Patil S, Smith T, Fojo T, and Bates S (1999) Efflux of rhodamine from $CD56^+$ cells as a surrogate marker for reversal of P-glycoprotein-mediated drug efflux by PSC 833. *Blood* 93:306-314.

Schinkel AH, Smit JJM, van Tellingen O, Beijnen JH, Wagenaar E, van Deemter L, Mol CAAM, van der Valk MA, Robanus-Maandag EC, te Riele HPJ, Berns AJM and

Borst P (1994) Disruption of the mouse *mdr1a* P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* 77:491-502.

Schinkel AH, Wagenaar E, van Deemter L, Mol CAAM and Borst P (1995) Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* 96:1698-1705.

Schinkel AH, Wagenaar E, Mol CAAM 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.

Smart EJ, Graf GA, McNiven MA, Sessa WC, Engelman JA, Scherer PI, Okamoto T and Lisanti MP (1999) Caveolins, liquid-ordered domains, and signal transduction. *Mol Cell Biol* 19:7289-7304.

Song S, Suzuki H, Kawai R and Sugiyama Y (1999) Effect of PSC 833, a P-glycoprotein modulator, on the disposition of vincristine and digoxin in rats. *Drug Metab Disp* 27:689-694.

Stahl KD, van Bever W, Janssen P, and Simon EJ (1977) Receptor affinity and pharmacological potency of a series of narcotic analgesic, anti-diarrheal and neuroleptic drugs. *Eur. J. Pharmacol.* 46:199-205.

Stewart A, Steiner J, Mellows G, Laguda B, Norris D, and Bevan P (2000) Phase I trial of XR9576 in healthy volunteers demonstrates modulation of P-glycoprotein in CD56+ lymphocytes after oral and intravenous administration. *Clin Cancer Res* 6:4186-4191.

Thomas H and Coley, HM (2003) Overcoming multidrug resistance in cancer: an update on the clinical strategy of inhibiting P-glycoprotein. *Cancer Control* 10:159-165.

Trambas C, Wang Z, Cianfriglia M, and Woods G (2001) Evidence that natural killer cells express mini P-glycoproteins but not classic 170 kDa P-glycoprotein. *Br J Haematol* 114:177-184.

Troost J, Albermann N, Emil HW, Weiss J (2004) Cholesterol modulates P-glycoprotein activity in human peripheral blood mononuclear cells. *Biochem Biophys Res Commun* 316:705-711.

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Supported in part by USPHS grants GM31304 and CA68485, and Merck Sharp & Dohme International Fellowships in Clinical Pharmacology (DK and MM).

EFC, DK, and MM contributed equally to this work.

Reprint requests to:

Dr. Grant R. Wilkinson
Department of Pharmacology
Room 538, RRB
Vanderbilt University
Nashville, TN 37232-6602

grant.wilkinson@vanderbilt.edu

JPET Fast Forward. Published on March 14, 2006 as DOI: 10.1124/jpet.105.099648
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures:

Fig. 1. Inhibition of loperamide (40nM) transport in LMDR1 cells by tariquidar.

Fig. 2. Effect of increasing intravenous loperamide dose on antinociception in *mdr1a/1b* (+/+) mice (■) and (-/-) mice (▲) pretreated 15 min earlier with a fixed 6.25 mg/kg dose of intravenous tariquidar. The control study involved loperamide vehicle plus 6.25 mg/kg tariquidar (gray ▲) and tariquidar vehicle (gray ■) in *mdr1a/1b* (+/+) mice, and loperamide vehicle plus 6.25 mg/kg tariquidar in *mdr1a/1b* (-/-) mice (black ▲).

Fig. 3. Effect of intravenous tariquidar pretreatment dose on antinociception in *mdr1a/1b* (+/+) mice administered 1mg/kg loperamide intravenously 15 min later.

Fig. 4. Dose-response relationships of P-glycoprotein inhibition of rhodamine-123 efflux (green, ■), antinociceptive response to loperamide (red, ●), and loperamide brain:plasma (blue, ▼) and testes:plasma (brown, ▲) concentration ratios in *mdr1a/1b* (+/+) mice administered tariquidar intravenously (upper panel) and elacridar intravenously (lower panel).

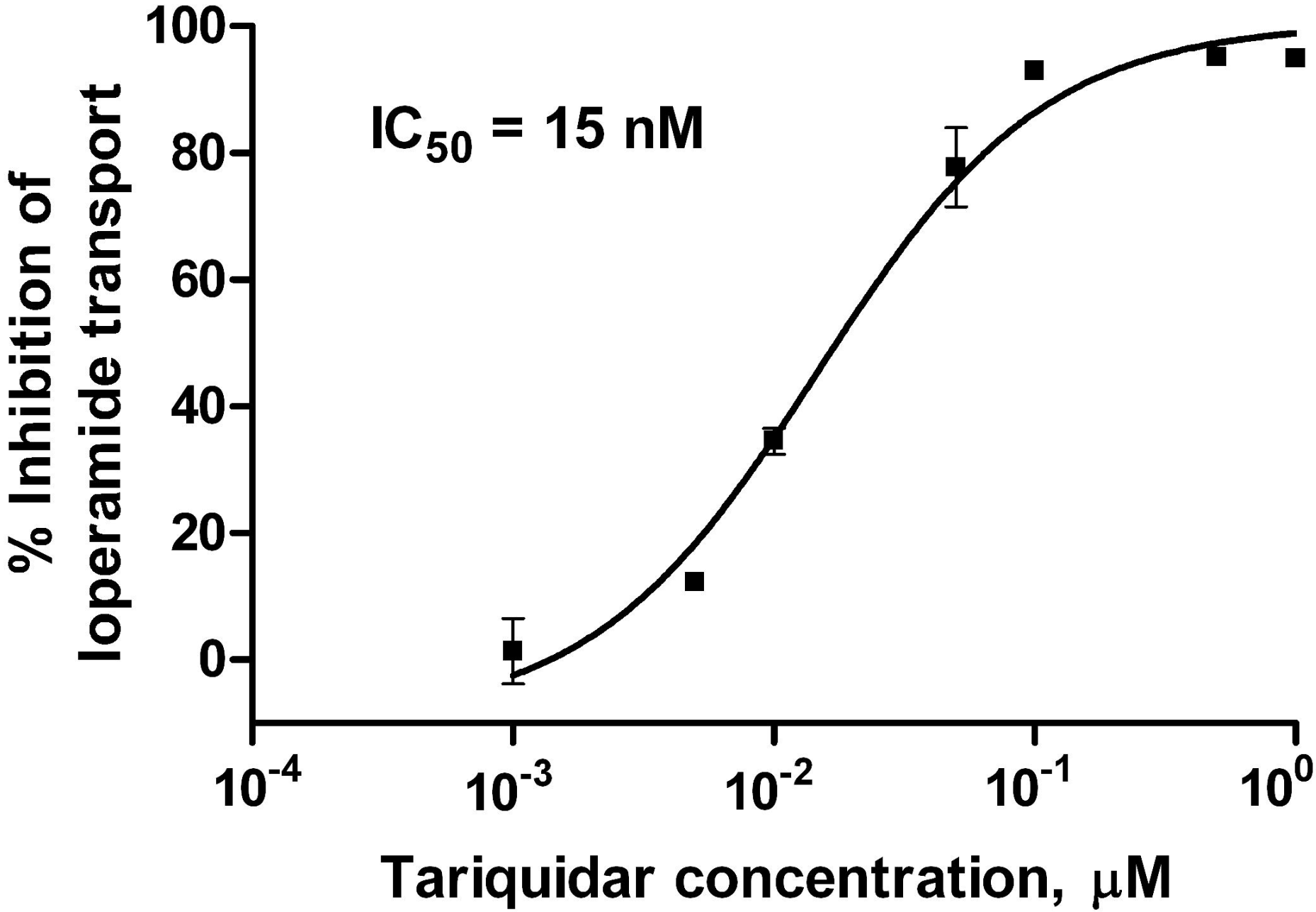

Figure 1

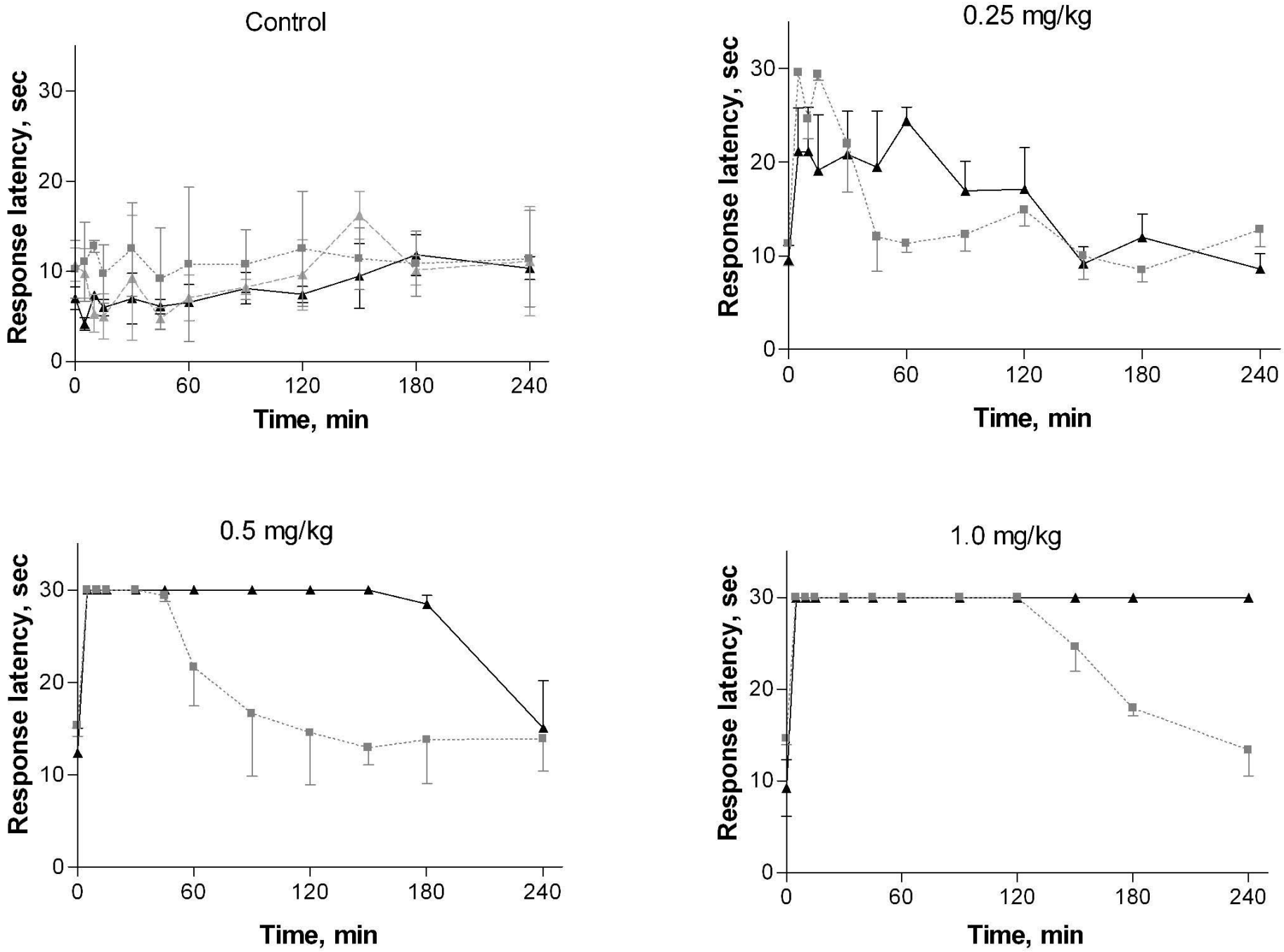

Figure 2

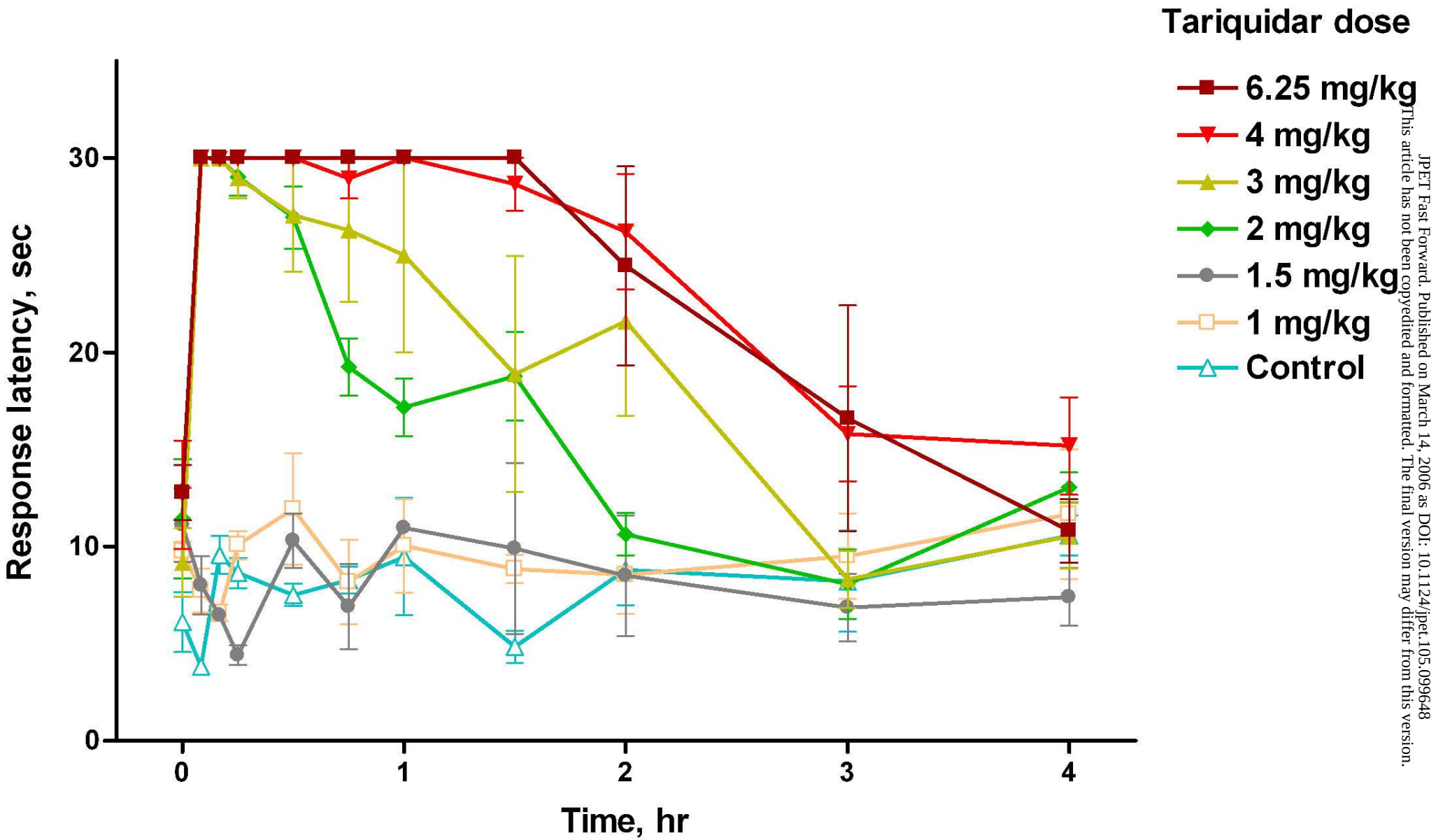

Figure 3

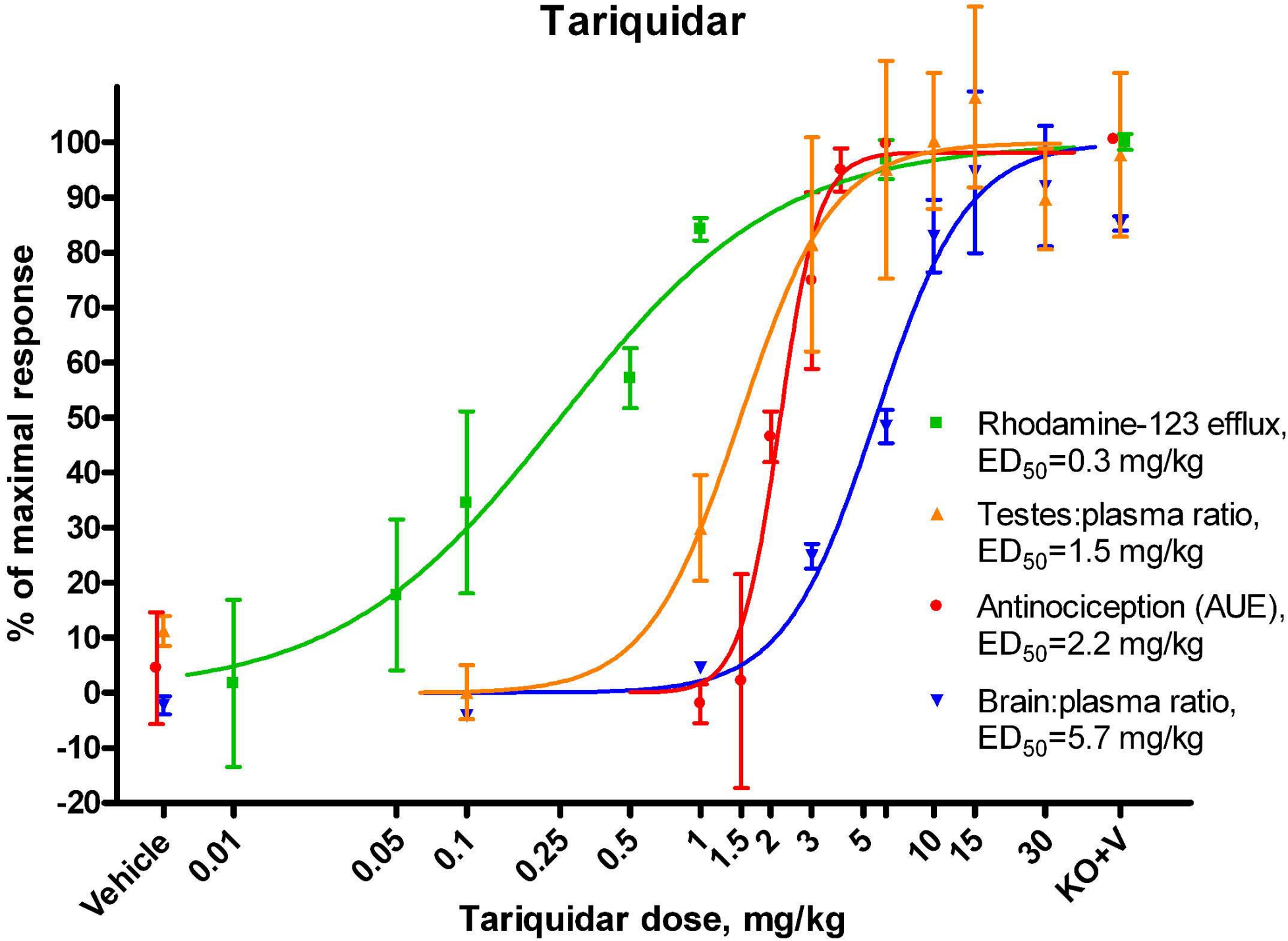

Figure 4a

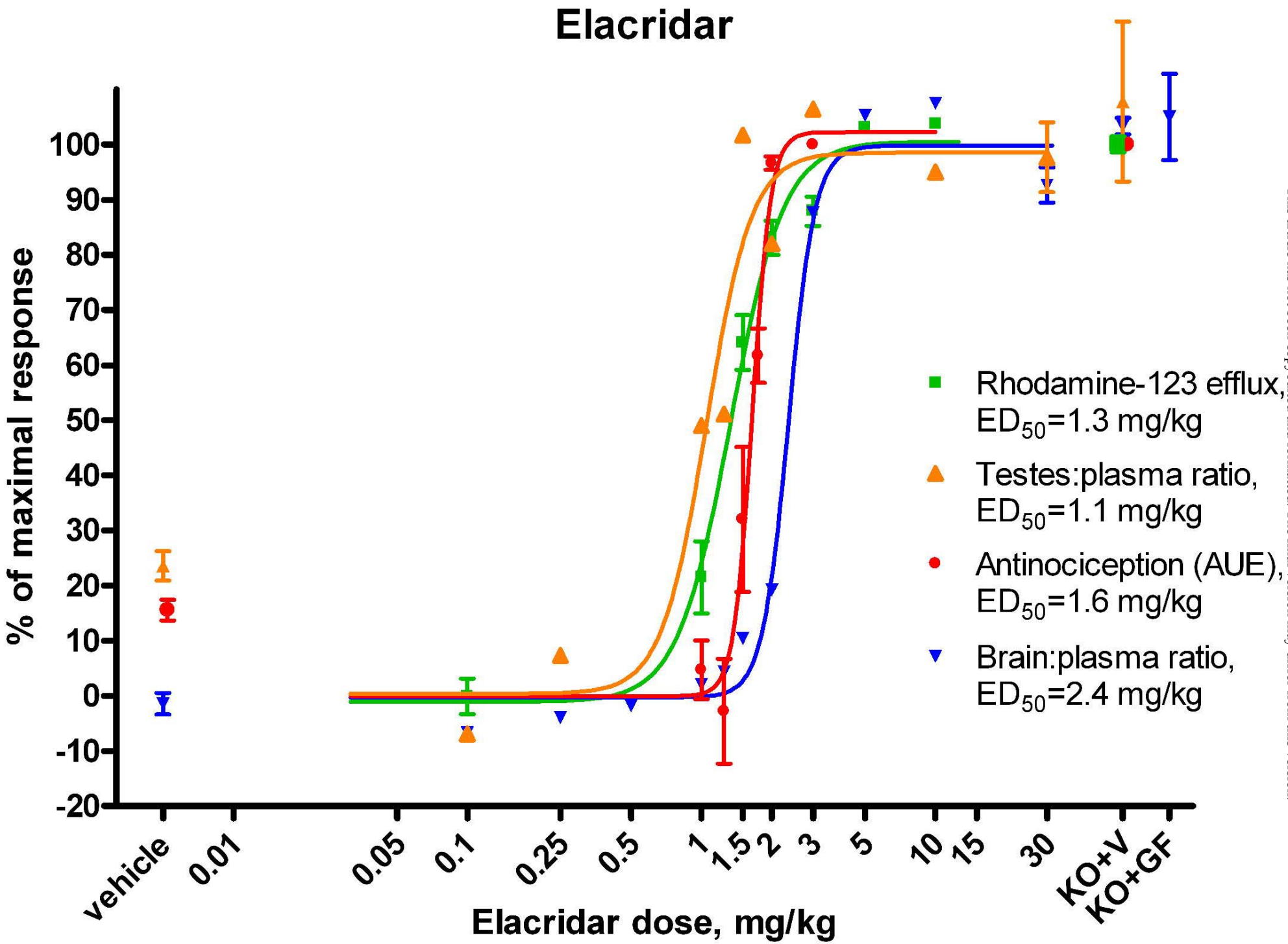

Figure 4b