Co-administration of P-glycoprotein modulators on loperamide pharmacokinetics and brain distribution

Rita Nieto Montesinos, Brice Moulari, Jessica Gromand, Arnaud Beduneau, Alf Lamprecht and Yann Pellequer.

Laboratory of Pharmaceutical Engineering, EA 4267, University of Franche-Comté, Besançon, France.
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Corresponding author: Rita Nieto Montesinos
Laboratory of Pharmaceutical Engineering, University of Franche-Comté,
19, rue Ambroise Paré, 25000 Besançon, France.
Tel: +33 3 63 08 23 38
Fax: +33 3 81 66 52 90
E-mail address: milynm@gmail.com

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Abbreviations: P-gp, P-glycoprotein; ABC, ATP binding cassette; MDR, multidrug resistance; BBB, blood-brain barrier; CNS, central nervous system; I.V, intravenous; PEG$_{600}$, polyethylene glycol 600; t-BME, tert-butyl methyl ether; MeOH, methanol; ACN, acetonitrile; ED$_{50}$, half-maximum effective dose; LC-MS, liquid chromatography-mass spectrometry; LLOQ, lower limit of quantification; ESI, electrospray ionization; SIM, single ion monitoring; CDL, curved desolvation line; AUC, area under the concentration-time curve; $t_{1/2}$, half-life of elimination; K, terminal elimination rate constant; MRT, mean residence time AUMC, partial area under the moment curve; CL, clearance; AUC$_{inf}$, AUC from time zero to infinity; Vd$_{ss}$, apparent volume of distribution at steady-state; Kp, brain-to-plasma partition coefficient; B/P, brain-to-plasma concentration ratio and BCRP, breast cancer resistance protein.
Abstract

The efflux transporter P-glycoprotein, expressed at high levels at the blood-brain barrier, exerts a profound effect on the disposition of various therapeutic compounds in the brain. A rapid and efficient modulation of this efflux transporter could enhance the distribution of its substrates and thereby improve central nervous system pharmacotherapies. This study explored the impact of the intravenous co-administration of two P-glycoprotein modulators, tariquidar and elacridar, on the pharmacokinetics and brain distribution of loperamide, a P-glycoprotein substrate probe, in rats. After one hour post-dosing, tariquidar and elacridar, both at a dose of 1.0 mg/kg, increased loperamide levels in the brain by 2.3- and 3.5-fold, respectively. However, the concurrent administration of both P-glycoprotein modulators, each at a dose of 0.5 mg/kg, increased loperamide levels in the brain by 5.8-fold and resulted in the most pronounced opioid-induced clinical signs. This phenomenon may be the result of a combined non-competitive modulation by tariquidar and elacridar. Besides, the simultaneous administration of elacridar and tariquidar did not significantly modify the pharmacokinetic parameters of loperamide. This observation potentially allows the concurrent use of low but therapeutic doses of P-gp modulators to achieve full inhibitory effects.
1 Introduction

Since its discovery in 1976 (Juliano and Ling, 1976), P-glycoprotein (P-gp) has been the most extensively studied ATP-binding cassette (ABC)-dependent efflux transporter. This protein is often regarded as a model to understand the biochemical mechanism of some ABC transport proteins. Two factors make P-gp the most critical efflux transporter: (1) its broad substrate specificity which results in multidrug resistance (MDR) (Ambudkar et al., 1999) and (2) the prominent expression of P-gp in most excretory and barrier-function tissues (Loscher and Potschka, 2005). The relevant expression of P-gp at the blood brain barrier (BBB) exerts a profound effect on the brain distribution of HIV protease inhibitors, anticancer drugs, opioids, some psychotropics and other drugs, which leads to the failure of various clinical treatments for brain diseases (Loscher and Potschka, 2005; Linnet and Ejsing, 2008; Varatharajan and Thomas, 2009). The inhibition of P-gp-mediated efflux could enhance the distribution of these substrates into the brain and therefore improve central nervous system (CNS) pharmacotherapies.

The identification of some P-gp substrates that also had the ability to block the P-gp-mediated efflux led to the synthesis of their analogs in order to minimize effects not related to their inhibition of P-gp-mediated efflux. Unfortunately, these compounds, known as first- and second-generation P-gp modulators, caused undesirable pharmacokinetic profiles due to their non-specificity towards the P-gp (Ecker and Chiba, 1995). With the purpose of avoiding these limitations, third-generation P-gp modulators have been developed. To be therapeutically effective, these compounds should be non-competitive and sufficiently potent to achieve inhibitory effects at non-toxic plasma concentrations and sufficiently selective for P-gp to minimize effects on overall drug pharmacokinetics (Anderson et al., 2006). In vivo studies demonstrated that elacridar and tariquidar, third-generation P-gp modulators, significantly increased the brain distribution of several P-gp substrates without pharmacokinetic interactions (Choo et al., 2006; Hubensack et al., 2008). In contrast, recent studies promote the use of significantly high doses of these P-gp modulators to efficiently modulate the P-gp-mediated efflux at the BBB (Bauer et al., 2013). However, when co-administered with P-gp substrates, these doses may be associated with pharmacokinetic interactions and toxic profiles, thus limiting the use of these agents. This escalating doses approach could reflect the same drawbacks of the first- and second-generation P-gp modulators.
Unnecessary exposure to P-gp modulators could be minimized and potential drug-related side effects might be reduced if, instead of using one P-gp modulator at a high dose, a combination of P-gp modulators with different drug binding sites were used at lower and safe doses. Martin et al. described the presence of at least four distinct interaction sites on P-gp and the binding of tariquidar to site II (a transport and regulatory site) and elacridar to site IV (an exclusive regulatory site) (Martin et al., 2000). In the presence of a P-gp substrate, elacridar and tariquidar, complex allosteric communication between the binding sites may result in synergistic interactions, thus improving the pharmacokinetics and pharmacodynamics of the P-gp substrate. The goal of this work was to evaluate the concomitant administration of tariquidar and elacridar and the subsequent impact on the pharmacokinetics and brain distribution of loperamide in rats after simultaneous intravenous (I.V) administration of the three compounds. After effective modulation of the P-gp activity by elacridar and/or tariquidar, loperamide, a μ-opioid agonist without central effects, can become a drug that produces substantial antinociception.

2 Materials and Methods

2.1 Materials

Loperamide hydrochloride and tetracyclol were obtained from Sigma Aldrich (France), elacridar was synthesized by the Laboratory of Pharmaceutical Chemistry at the University of Bonn in Germany and tariquidar was purchased from API Services Inc. (USA). Polyethylene glycol 600 (PEG600) was obtained from Interchimie (France). Trisodium citrate solution was purchased from BD Vacutainer®80. Ketoconazole (internal standard for loperamide) and chlorpromazine hydrochloride (internal standard for elacridar and tariquidar), tert-Butyl methyl ether (t-BME), analytical grade ammonium acetate and glacial acetic acid were obtained from Sigma Aldrich (France). HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from Carlo Erba (France). Ultrapure water was freshly obtained before use from a Purelab Prima 7/15/20 - Purelab Ultra Mk 2 from Elga (France).
2.2 Animals
Behavioral observation, pharmacokinetic and brain distribution studies were conducted in male Sprague Dawley rats (Janvier, France). All animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, USA). All the animals were allowed to acclimate for one week and were seven weeks old (230 – 280 g) at the time of the experiment. The animals were maintained under a 12-h light/dark cycle and a temperature-controlled environment. Food and water were provided ad libitum. The studies were approved by the Institutional Animal Care and Use Committee of the University of Franche-Comte.

2.3 Drug solutions
The drug solutions were prepared on the day of the experiment. Elacridar was dissolved in tetraglycol at an initial concentration of 20 mg/mL. Loperamide and tariquidar were dissolved separately in a mixture of saline and PEG₆₀₀ (3:1) at concentrations of 2 mg/mL. For each treatment, loperamide, elacridar and tariquidar solutions were diluted with saline and PEG₆₀₀ (3:1). All the solutions were completely transparent indicating the full solubility of loperamide and both P-gp modulators in the vehicle.

2.4 Study design
This study was carried out using a rat model, which is a promising model to predict P-gp-based drug-drug interactions at the human BBB (Hsiao and Unadkat, 2012). The choice of loperamide as a P-gp substrate and its dose was based on its opiate-like behavior, which provides an efficient means with which to ascertain the blockage of the P-gp (Elkiweri et al., 2009). Because the reported half-maximum effective dose (ED₅₀) for tariquidar and elacridar in rats (Kuntner et al., 2010) were lethal in co-administration with loperamide in our pilot study, the doses of the P-gp modulators were reduced to 0.5 or 1.0 mg/kg.

The animals were randomly divided into five experimental groups, each of which received loperamide at a dose of 0.5 mg/kg. The co-administration of P-gp modulators was carried out as follows: Group I, elacridar 1.0 mg/kg; group II, tariquidar 1.0 mg/kg; group III, elacridar 1.0 mg/kg plus tariquidar 1.0 mg/kg; group IV, elacridar 0.5 mg/kg plus tariquidar 0.5 mg/kg.
and group V (control group), no P-gp modulator. The different treatments were administered via the jugular vein by a single I.V bolus. Groups I, II and III were used to study the influence of the co-administration of elacridar and tariquidar on their own plasma and brain distribution. Groups I, II, IV and V were used to evaluate the influence of the concurrent administration of elacridar and tariquidar on the pharmacokinetics and brain distribution of loperamide.

Prior to the pharmacokinetic and tissue distribution studies, all the animals were observed for one hour to determine the clinical signs induced by central opiate effects of loperamide. Observations were limited to two to three rats at a time to maximize visibility (n=12). The clinical signs were established according to previous data (Pinelli and Trivulzio, 1997; Zamek-Gliszczynski et al., 2012) and our pilot study. The degrees of the clinical signs were scored on a 0 to 3 scale according to the intensity of each clinical sign, where 0 = none, 1 = mild, 2 = moderate and 3 = severe (Table 3). Rats that displayed a score of 1 or higher on three or more signs were considered to display opioid-induced behavior.

In the pharmacokinetic study, blood (0.25 mL) was serially sampled from the tail vein at 1, 6, 12 and 24 hours after administration of the different treatments. The blood was collected in tubes containing trisodium citrate solution. The plasma was obtained by centrifugation of the blood at 2500 x g for 5 minutes and stored frozen at -20°C until analysis. Three rats were used at each time point (n=3).

In the tissue distribution study, animals were sacrificed at 1, 6, 12 and 24 hours after administration of the different treatments, following deep anesthesia with sodium pentobarbital (50 mg/kg; intraperitoneal), cardiac perfusion with saline and exsanguination. After sacrifice, the whole brain was immediately frozen at -20°C until analysis. Three rats were used at each time point (n=3).

The blood and brains were first sampled at 1 hour post-dosing because according to the literature, loperamide reaches a pseudoequilibrium between the brain and the plasma at this time (Hsiao and Unadkat, 2012). The subsequent time points up to 24 hours were selected to determine possible drug-drug interactions and a possible extension of the P-gp modulation at the BBB.
2.5 Analysis of loperamide, elacridar and tariquidar by Liquid Chromatography-Mass Spectrometry (LC-MS)

Loperamide, elacridar and tariquidar in plasma and brain samples were determined by an LC-MS method that has been validated for specificity, calibration curve, lower limit of quantification (LLOQ), accuracy, precision, and recovery according to the FDA guidance for bioanalytical method validation (Services., 2001). Ketoconazole was used as internal standard for loperamide and chlorpromazine for elacridar and tariquidar. The chromatographic analyses were carried out on a Shimadzu High Performance Liquid Chromatography/Mass Spectrometer LCMS-2010EV equipped with a LC-20AD solvent delivery system. The analytes were well separated on a Zorbax Eclipse XDB-C18 2.1 x 150 mm, 5.0-µm column from Agilent Technologies at 50°C using a Millipore Waters oven. The mobile phase, consisting of 10 mM ammonium acetate (pH 5.5):MeOH:ACN (37.5:40:22.5 v/v/v), was delivered in isocratic mode at 0.4 mL/min. An autosampler 360 from Kontrons Instruments was set at 20 µL. The compounds were quantitated using positive electrospray ionization (ESI) in an octupole quadrupole mass analyzer with single ion monitoring (SIM) mode at m/z 477 for loperamide, m/z 531 for ketoconazole, m/z 564 for elacridar, m/z 647 for tariquidar and m/z 319 for chlorpromazine. Nitrogen was used as the nebulizing gas at 1.5 L/min. The curved desolvation line (CDL) and heat block temperatures were set at 250°C and 300°C, respectively. The detector voltage was 1.5 KV, the interface voltage was -3.5 KV and the CDL voltage was 15.0 V.

Frozen brain samples were thawed and homogenized with one volume of water using a Janke & Kunkel T45 Ultra-turrax and a Fischer Scientific Vibra-cell homogenizer. Prior to chromatographic analysis, 25 µL of the internal standard solution containing ketoconazole and chlorpromazine hydrochloride were added to 100 µL of each plasma or homogenate sample to yield final concentrations of 100 ng/mL and 25 ng/mL, respectively. After deproteinization by addition of 800 µL of a mixture of ACN and t-BME (1:1), the samples were vortexed for 3 minutes and centrifuged at 5000 x g for 10 minutes. The upper organic layer was decanted and evaporated to dryness and the residue was reconstituted in 100 µL of mobile phase. A volume of 20 µL was injected onto the analytical column.
2.6 Pharmacokinetic calculations

The pharmacokinetic parameters were calculated by non-compartmental analysis using Kinetica™ version 4.0 (Inna Phase Corp., 2001). The area under the concentration-time curves (AUC) values were determined using the trapezoidal rule. The half-lives of elimination (t_{1/2}) were calculated as ln(2)/K, where K represents the terminal elimination rate constant obtained from the slopes of the semilogarithmic plots of the concentration-time profile. The mean residence time (MRT) was estimated from AUMC/AUC, where AUMC is the partial area under the moment curve. The plasma clearance (CL) was calculated as dose/AUC_{inf}, where AUC_{inf} is the AUC from time zero to infinity. The apparent volume of distribution at steady-state (V_{dss}) was calculated from dose x MRT/AUC_{inf}. The brain-to-plasma partition coefficient (K_{p}) was calculated as AUC_{inf-brain}/AUC_{inf-plasma}.

2.7 Statistical Analysis

The statistical analysis was carried out using SigmaStat 3.5 software. Analyses of statistical significance between two groups were examined by Student's t-test and between many groups by one-way analysis of variance (ANOVA) with the Holm-Sidak post-hoc test. A P < 0.05 was considered to be significant. Moreover, the variance of the AUC_{inf} in each treatment group was estimated according to the Bailer method (Bailer, 1988; Yuan, 1993), which is based on the variability of the concentrations at each sampling time. A Z-test was used for pairwise comparison of AUCs. A P < 0.05 was considered to be significant.

3 Results

3.1 Analysis of loperamide, elacridar and tariquidar by Liquid Chromatography-Mass Spectrometry (LC-MS)

Based on structural similarities, solubility, recovery efficiency and previous successful data (Kemper et al., 2001; Yu et al., 2004), ketoconazole was a satisfactory internal standard for loperamide, as chlorpromazine was for elacridar and tariquidar. The developed LC-MS method described in this manuscript was linear over the concentration range 5.0 ng/mL – 1000 ng/mL for all the three analytes, loperamide, elacridar and tariquidar (r^2 ≥ 0.9990). Using 100 µL of rat plasma or tissue homogenate, the validated lower limit of quantification (LLOQ) for each compound was the lowest concentration of standard on the calibration
Intra- and inter-day accuracy and precision were within 15% for the three analytes. The specificity of the method was confirmed by the absence of interferences from endogenous compounds. In this study, the sample preparation procedure using ACN and t-BME (1:1) demonstrated absolute recovery values from rat plasma and brain samples higher than 90%, for loperamide, ketoconazole, elacridar, tariquidar and chlorpromazine. Furthermore, stability tests demonstrated that the analytes were stable under the storage conditions. The current validated method (data not shown) was then used for the simultaneous quantitation of loperamide, elacridar and tariquidar in plasma and brain samples.

3.2 Influence of the co-administration of elacridar and tariquidar on their plasma and brain levels

At a first stage, the groups which received elacridar at 1.0 mg/kg and/or tariquidar at 1.0 mg/kg (groups I, II and III) were used to compare whether the concurrent administration of both P-gp modulators influenced their own plasma and brain distributions (Fig 1 and 2).

No modification in the plasma AUC_{inf} of elacridar alone or co-administered with tariquidar (31.9 ± 2.7 vs 32.2 ± 3.4 nmol.h/mL) and in the plasma AUC_{inf} of tariquidar alone or co-administered with elacridar (37.8 ± 1.9 vs 37.0 ± 2.8 nmol.h/mL) were observed (Table 1).

These values indicate that the co-administration of these P-gp modulators at 1.0 mg/kg each had no observable effects on each other plasma concentrations.

The elacridar AUC_{inf} for the brain remained unchanged after concurrent administration with tariquidar (3.1 ± 0.1 vs 3.6 ± 0.4 nmol.h/g). Vice versa, the tariquidar AUC_{inf} for the brain increased from 0.8 ± 0.1 to 1.6 ± 0.1 nmol.h/g (2.0-fold) in the presence of elacridar (Table 1). This increase was associated with a 2-fold higher K_{p} for tariquidar. These findings suggest that when both P-gp modulators are co-administered, elacridar could interfere with the active transport of tariquidar at the BBB.

3.3 Influence of the co-administration of elacridar and tariquidar on loperamide plasma levels

To evaluate the effects of the co-administration of both P-gp modulators on loperamide pharmacokinetics, the groups which received a total dose of 1.0 mg/kg of P-gp modulators (groups I, II, and IV) were compared. Group V served as a control.
In rats receiving 0.5 mg/kg loperamide alone, the concentration of loperamide in plasma ($C_{\text{max}}$) after 1 hour ($T_{\text{max}}$) was approximately 4.0 %/mL of the administered dose, which reflects a rapid metabolism of loperamide during this first hour (Fig. 3, Table 2). The mean elimination half-life of a single loperamide administration in this study was 3.6 ± 0.3 hours and it was not significantly altered in presence of elacridar and/or tariquidar. Likewise, the AUC, MRT, CL and Vdss were not significantly different in any of the treatments using one or two P-gp modulators. These results confirmed that neither elacridar nor tariquidar altered the pharmacokinetic parameters of loperamide.

### 3.4 Influence of the co-administration of elacridar and tariquidar on loperamide concentrations in the CNS

To evaluate the effects of the co-administration of both P-gp modulators on the brain distribution of loperamide, the groups which received a total dose of 1.0 mg/kg of P-gp modulators (groups I, II, and IV) were compared. Group V served as a control. Previous studies (Elkiweri et al., 2009; Kawamura et al., 2011) showed that low doses of P-gp modulators and loperamide were taken up into the brain. In agreement with these results, in the present study, these doses demonstrated sufficient degree of P-gp inhibition at the BBB (Table 4). Immediately after administration, a few animals from the loperamide-treated groups which received tariquidar at 1.0 mg/kg or elacridar at 1.0 mg/kg showed lethargy, piloerection and shallow breathing. However, these animals were able to respond if handled, and by 15 minutes post-treatment they recovered normal activity. According to our clinical score, 1.0 mg/kg of tariquidar (total score = 13) and 1.0 mg/kg of elacridar (total score = 26) slightly promoted the central effects of the opiate. More than 50% of the animals from the group that received loperamide co-administered with elacridar and tariquidar, each at 0.5 mg/kg not only immediately exhibited the same clinical signs but also demonstrated whole body tetany and eye protrusion. In addition, two of the 12 animals of this group showed the Straub reaction which is characterized by the rigidity of the tail, held in an S-shaped curve across the back of the animal (Bilbey et al., 1960). These animals recovered normal activity approximately 30 minutes later. These clinical signs indicate that the co-administration of the two P-gp modulators (total score = 102) at a total dose of 1.0 mg/kg significantly potentiated the opioid brain effects of loperamide.
The administration of loperamide alone resulted in very low levels (10.53 ± 0.51 ng/g) in the brain after 1 hour (Fig. 3). However, the co-administration of the P-gp modulators significantly increased the concentration of loperamide in the brain at this time point. Tariquidar and elacridar, each at 1.0 mg/kg increased loperamide levels in the brain by 2.3- (22.48 ± 2.93 ng/g) and 3.5-fold (33.84 ± 3.95 ng/g), respectively. However, the concurrent administration of both P-gp modulators at half doses increased the concentration of the opiate in the brain by 5.8-fold (47.26 ± 6.09 ng/g). After 6 hours, loperamide was undetectable in brains from animals that had not received either P-gp modulator and close to 10.0 ng/g in the other three groups. After 12 and 24 hours, loperamide was not detectable in any group. The differences in the loperamide AUC_{inf} for the brains and the Kp values were even more marked than the effects at the individual times (Table 3). All these results suggest a greater inhibition of the P-gp-mediated efflux by elacridar than by tariquidar and a possible synergistic effect of both P-gp modulators when they are co-administered.

4 Discussion

Given that the use of relatively high doses of the third-generation P-gp modulators (Salama et al., 2005) may be limited by the same drawbacks of the first- and second-generation P-gp modulators, this study evaluated the potential of combining the administration of two P-gp modulators to see the influence on the efflux activity of the P-gp at the BBB.

The co-administration of elacridar and tariquidar did not significantly increase the plasma concentrations of each agent relative to the values obtained for administration of the single agents. Their respective AUCs suggest that at the doses used in the current work, neither P-gp modulator interferes with the elimination pathway of the other. The Kp obtained for individual doses of elacridar and tariquidar are low due to the low concentrations of either P-gp modulator in the brain. These results contrast with prior studies that showed that the levels of tariquidar and elacridar were much higher in the brain than in the plasma (Bankstahl et al., 2008; Kuntner et al., 2010), but those experiments utilized between 3.0- and 15-fold higher doses of P-gp modulators compared to the current study. These differences were properly explained before (Kawamura et al., 2011; Sane et al., 2013), where murine models revealed that at higher doses of elacridar, a higher distribution of the P-gp modulator was attained in the brain. For instance, at 1 hour post-injection, the B/P ratio of elacridar increased from 0.4 at 0.5 mg/kg to 5.2 at 2.5 mg/kg (Sane et al., 2013). This dose-dependent distribution
relationship was also observed for tariquidar (Bankstahl et al., 2013). A supplementary explanation for the low brain distribution of elacridar and tariquidar in this study is based on the pharmacokinetic behavior of these compounds at low doses. At nanomolar doses, both P-gp modulators are actively transported not only by the P-gp but also by the breast cancer resistance protein (BCRP) at the BBB (Bankstahl et al., 2013). Thus, the amount transported by these two proteins would be higher than the amount that arrives to the brain by passive diffusion, resulting in increased plasma concentrations and decreased brain concentrations of these compounds. Nevertheless, when the two compounds are co-administered, elacridar may reduce or delay the active transport of tariquidar by both proteins (Kannan et al., 2011), thus significantly increasing the $K_p$ of tariquidar. An important issue to consider when comparing the distribution of low doses of P-gp modulators in the brain is the species differences in P-gp transport activity, which appear to be substrate-dependent (Lin and Yamazaki, 2003). It was found that several chemical entities which were P-gp substrates in mice were also P-gp substrates in rats, but the brain distribution of these compounds is not always the same in both species. In one clear example, while the $K_p$ of $N$-desmethyl-venlafaxine was the same in mice and rats, the $K_p$ of risperidone was 2.36-fold higher in mice than in rats (Bundgaard et al., 2011). These data can also account for the higher brain distribution of relative low doses of P-gp modulators in mice compared to our rat model.

To evaluate the effects of the co-administration of both P-gp modulators at a total dose of 1.0 mg/kg on P-gp activity, loperamide was chosen as a P-gp substrate probe. The mean half-life of loperamide was $3.6 \pm 0.3$ h, which is different from a previous study (Zamek-Gliszczynski et al., 2012), where less than 1.0 %/mL of the intravenous administered dose of loperamide was monitored at 5 minutes post-dosing. This difference can be attributed to the low solubility of loperamide in the vehicle used in that study. However, our $K_p$ values of loperamide are more in agreement with another study, where the $K_p(0-1h)$ of the opiate was 0.006 (Elkiweri et al., 2009). In the current investigation, the half-life as well as the AUC, MRT, CL and $V_{dss}$ were not significantly modified when loperamide was co-administered with elacridar or tariquidar or both P-gp modulators at a total dose of 1.0 mg/kg. The lack of alterations in the pharmacokinetic parameters of loperamide in the different groups confirms the minimal modulation on the cytochrome P4503A4 by elacridar and tariquidar (Choo et al., 2006). This cytochrome plays a predominant role in the metabolism of loperamide (Kalgutkar and Nguyen, 2004; Kim et al., 2004). Previously, different models showed that even higher doses
of tariquidar and elacridar did not significantly change the pharmacokinetics of the P-gp substrates (Choo et al., 2006; Hubensack et al., 2008). Therefore, the increase of loperamide levels in the brain could not be explained by the modest increase of loperamide in plasma. Instead, it was likely due to the efficient modulation of the P-gp at the BBB by tariquidar and elacridar. The clinical signs noticed in the observation phase are in line with the opiate effects (Bilbey et al., 1960) when loperamide at an oral dose of 10 mg/kg was administered to Mdr1a knockout rats (SAGE Mdr1a) (Zamek-Gliszczynski et al., 2012), a standard for the complete blockage of the P-gp at the BBB. These observations suggest an important and extremely rapid distribution of the P-gp modulators in the brain and an immediate modulation of the P-gp at the BBB. After one hour of administration, tariquidar or elacridar, each at 1.0 mg/kg, increased loperamide levels in the brain by 2.3- and 3.5-fold, respectively, thus showing that even at relatively low doses, elacridar is more potent than tariquidar. These results are consistent with precedent studies (Choo et al., 2006; Kuntner et al., 2010), where the authors used BBB mice and rat models to show that the ED_{50} of elacridar is between 2.0- and 3.0-fold lower than the ED_{50} of tariquidar. Nevertheless, the most significant finding in this investigation was that the concurrent administration of both P-gp modulators at half doses increased the concentration of loperamide in the brain by 5.8-fold. A suitable explanation for this lies on the non-competitive activity of tariquidar and elacridar towards the P-gp, which means that both P-gp modulators can independently and simultaneously bind the P-gp on distinct drug binding sites (Martin et al., 2000). Equilibrium and kinetic radioligand binding assays allowed to determine the presence of at least four distinct drug interaction sites on P-gp. Sites I, II and III were classified as sites for transport because they interacted with P-gp substrates such as vinblastine, paclitaxel, rhodamine 123 and Hoechst 33342. Site II could also interact with some P-gp modulators such as tariquidar. In contrast, site IV was classified as a regulatory site because only P-gp modulators such as elacridar could interact with this site (Martin et al., 2000). Although site IV could allosterically communicate in a negative heterotropic manner with the site II, the dissociation rate of [3H]XR9576, an analog of tariquidar, was significantly slower than that of the P-gp substrate [3H]vinblastine (Martin et al., 1999). Thus, despite the active transport, it appears that both P-gp modulators were able to bind the P-gp at their corresponding drug binding sites and the complex allosteric communication resulted in a possible synergistic interaction. Nevertheless, the dose-limiting opioid effects of loperamide preclude assessing this strategy with higher doses of loperamide co-administered with higher doses of the P-gp modulators.
Taking into account that synergism can be different at different dose levels (Chou, 2006); these preliminary synergistic effects should be further confirmed using radiolabelled [3H or 14C] loperamide associated to higher doses of P-gp modulators. Moreover, many other P-gp substrates with different therapeutic effects and several doses of elacridar and tariquidar remain to be explored and extrapolated to different species to define the synergistic interaction between both P-gp modulators. The synergistic phenomenon observed herein resulted in strong pharmacodynamic effects by loperamide, a potent CNS agent. However, this approach could be restricted in infectious or cancer diseases, where high brain concentrations of the therapeutic agents are needed and a synergism at high effect levels is more relevant than at low effect levels (Chou, 2006). Other P-gp modulators can also be used for these studies, provided that the steric hindrance of one does not affect the binding of the other.

Since this preliminary study support the synergistic modulation of P-gp using low doses of elacridar and tariquidar, this approach may represent a potential step forward to avoid the use of high, nearly toxic doses of P-gp modulators without significant pharmacokinetics interactions. Furthermore, because the distribution and the permanence of these P-gp modulators in the brain are dose-dependent, the rapid decrease of the P-gp modulators in the brain as observed in this work represents an advantage. Our approach could avoid the entry of harmful compounds after a long-lasting P-gp inhibition at the BBB.

**Autorship contributions**

*Participated in research design:* Nieto Montesinos, Beduneau, Lamprecht, and Pellequer.

*Conducted experiments:* Nieto Montesinos, and Gromand.

*Analytical tools:* Nieto Montesinos, Moulari, and Pellequer.

*Performed data analysis:* Nieto Montesinos, Lamprecht, and Pellequer.

*Wrote or contributed to the writing of the manuscript:* Nieto Montesinos, Lamprecht, and Pellequer.
References


Footnotes

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Figures legend:

Figure 1:
Brain concentrations (left axis) and plasma concentrations (right axis) of elacridar after intravenous administration of elacridar alone at 1.0 mg/kg (white bars/empty squares) or concurrently administered with tariquidar at 1.0 mg/kg (gray bars/full squares). Concentrations are expressed as mean ± standard deviation. Bars represent the standard deviation. n = 3.
# Student’s t-test: Significantly different compared to the group which received elacridar at 1.0 mg/kg alone.

Figure 2:
Brain concentrations (left axis) and plasma concentrations (right axis) of tariquidar after intravenous administration of tariquidar alone at 1.0 mg/kg (white bars/empty triangles) or concurrently administered with elacridar at 1.0 mg/kg (gray bars/full triangles). Concentrations are expressed as mean ± standard deviation. Bars represent the standard deviation. n = 3.
# Student’s t-test: Significantly different compared to the group which received tariquidar at 1.0 mg/kg alone.

Figure 3:
Brain concentrations (left axis) and plasma concentrations (right axis) of loperamide after intravenous administration of loperamide alone at 0.5 mg/kg (white bars/rhombus) or concurrently administered with tariquidar at 1.0 mg/kg (light gray bars/triangles), or with elacridar at 1.0 mg/kg (dark gray bars/squares) or with tariquidar at 0.5 mg/kg plus elacridar at 0.5 mg/kg (black bars/circles). Bars represent the standard deviation. n = 3.
#ANOVA: Significantly different compared to the concentration of loperamide in the brain (ng/g) from the group which received no P-gp modulator.
##ANOVA: Significantly different compared to the concentration of loperamide in the brain (ng/g) from the group which received elacridar or tariquidar at 1.0 mg/kg.
Table 1. Area under the concentration-time curves (AUC\textsubscript{inf}) and $K_p$ of elacridar and tariquidar

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Elacridar 1.0 mg/kg (1773.05 nmol/kg)</th>
<th>Elacridar 1.0 mg/kg (+ Tariquidar 1.0 mg/kg)</th>
<th>Tariquidar 1.0 mg/kg (1545.60 nmol/kg)</th>
<th>Tariquidar 1.0 mg/kg (+ Elacridar 1.0 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean 31.9 (2.7)</td>
<td>Mean 32.2 (3.4)</td>
<td>Mean 37.8 (1.9)</td>
<td>Mean 37.0 (2.8)</td>
</tr>
<tr>
<td></td>
<td>S.D. 2.7</td>
<td>S.D. 3.4</td>
<td>S.D. 1.9</td>
<td>S.D. 2.8</td>
</tr>
<tr>
<td>AUC\textsubscript{inf}-plasma</td>
<td>3.1 (0.1)</td>
<td>3.6 (0.4)</td>
<td>0.8 (0.1)</td>
<td>1.6* (0.1)</td>
</tr>
<tr>
<td>AUC\textsubscript{inf}-brain</td>
<td>0.098 (0.011)</td>
<td>0.115 (0.021)</td>
<td>0.022 (0.003)</td>
<td>0.043* (0.007)</td>
</tr>
</tbody>
</table>

* Bailler method: Significantly different compared to the group which only received one P-gp modulator, whether elacridar at 1.0 mg/kg or tariquidar at 1.0 mg/kg

# Student's t-test: Significantly different compared to the group which only received one P-gp modulator, whether elacridar at 1.0 mg/kg or tariquidar at 1.0 mg/kg
## Table 2. Pharmacokinetic parameter of loperamide

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>No P-gp modulator</th>
<th>Tariquidar 1.0 mg/kg</th>
<th>Elacridar 1.0 mg/kg</th>
<th>Elacridar 0.5 mg/kg + Tariquidar 0.5 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>---</td>
<td>1.0</td>
<td>---</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>5052</td>
<td>800</td>
<td>5371</td>
<td>660</td>
</tr>
<tr>
<td>$AUC_{\text{inf}}$ (ng.h/mL)</td>
<td>28900</td>
<td>4554</td>
<td>34365</td>
<td>4800</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>3.6</td>
<td>0.3</td>
<td>3.9</td>
<td>0.5</td>
</tr>
<tr>
<td>$MRT$ (h)</td>
<td>5.0</td>
<td>0.4</td>
<td>5.5</td>
<td>0.3</td>
</tr>
<tr>
<td>$Cl$ (mL/h /Kg)</td>
<td>4.4</td>
<td>0.7</td>
<td>3.7</td>
<td>0.5</td>
</tr>
<tr>
<td>$Vd$ (mL/Kg)</td>
<td>22.1</td>
<td>2.6</td>
<td>20.6</td>
<td>3.5</td>
</tr>
</tbody>
</table>

# ANOVA: Significantly different compared to the group which received no P-gp modulator.

## ANOVA: Significantly different compared to the group which received elacridar or tariquidar at 1.0 mg/kg.
### Table 3. Brain distribution of loperamide

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>No P-gp modulator</th>
<th>Tariquidar 1.0 mg/kg</th>
<th>Elacridar 1.0 mg/kg</th>
<th>Elacridar 0.5 mg/kg + Tariquidar 0.5 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td><strong>AUC&lt;sub&gt;inf&lt;/sub&gt;</strong> (ng·h/g)</td>
<td>10.5</td>
<td>0.5</td>
<td>124.5*</td>
<td>20.1</td>
</tr>
<tr>
<td><em>Bailer method: Significantly different compared to the group which received no P-gp modulator.</em>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>K&lt;sub&gt;p&lt;/sub&gt;</strong></td>
<td>0.0004</td>
<td>0.0001</td>
<td>0.0037*</td>
<td>0.0009</td>
</tr>
</tbody>
</table>
| **ANOVA: Significantly different compared to the group which received elacridar or tariquidar at 1.0 mg/kg.**

*Bailer method: Significantly different compared to the group which received no P-gp modulator.

**Bailer method: Significantly different compared the group which received elacridar or tariquidar at 1.0 mg/kg.

#ANOVA: Significantly different compared to the group which received no P-gp modulator.

##ANOVA: Significantly different compared to the group which received elacridar or tariquidar at 1.0 mg/kg.
Table 4. Opioid-induced clinical signs

<table>
<thead>
<tr>
<th>Clinical signs (1 hour post-dose)</th>
<th>No P-gp modulator</th>
<th>Elacridar 1.0 mg/kg</th>
<th>Tariquidar 1.0 mg/kg</th>
<th>Elacridar 0.5 mg/kg + Tariquidar 0.5 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N/n degree</td>
<td>N/n degree</td>
<td>N/n degree</td>
<td>N/n degree</td>
</tr>
<tr>
<td>CNS :</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lethargy</td>
<td>0/12 0</td>
<td>6/12 2</td>
<td>4/12 2</td>
<td>9/12 3</td>
</tr>
<tr>
<td>Whole body tetany</td>
<td>0/12 0</td>
<td>0/12 0</td>
<td>0/12 0</td>
<td>0/12 0</td>
</tr>
<tr>
<td>Straub tail</td>
<td>0/12 0</td>
<td>0/12 0</td>
<td>0/12 0</td>
<td>0/12 0</td>
</tr>
<tr>
<td>Piloerection</td>
<td>0/12 0</td>
<td>4/12 1</td>
<td>3/12 1</td>
<td>7/12 2</td>
</tr>
<tr>
<td>Pulmonary :</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shallow breathing</td>
<td>0/12 0</td>
<td>5/12 2</td>
<td>2/12 1</td>
<td>9/12 3</td>
</tr>
<tr>
<td>Eyes :</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eye protrusion</td>
<td>0/12 0</td>
<td>0/12 0</td>
<td>0/12 0</td>
<td>5/12 2</td>
</tr>
<tr>
<td>Total score</td>
<td>0</td>
<td>26</td>
<td>13</td>
<td>102</td>
</tr>
</tbody>
</table>

N/n = number of rats displaying these clinical signs/number of rats per group. (n=12).
The degrees of the clinical signs are scored as 0 = none, 1 = mild, 2 = moderate and 3 = severe.
Values shown in the degree columns are the mean degree score for each symptom.
Total score = total sum of N x degree
Rats that displayed a score of 1 or higher on 3 or more signs were considered to display opioid-induced behavior.
Figure 1.
Figure 2.
Figure 3.