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Influence of efflux transporters on liver, bile and brain disposition of amitriptyline in mice

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ABSTRACT

The aim of this work was to clarify the role of Abcb1 and the possible involvement of Abcc2 and Abcg2 in liver, bile and brain disposition of amitriptyline (AMI).

AMI was administrated to Abcb1a deficient mice ($n = 36$): CF1 (−/−) and CF1 (+/+) mice received via intraperitoneal route (i.p.) 5 mg/kg AMI and CF1 (+/+) mice received i.p. 5 mg/kg AMI + 100 mg/kg quinidine (Abcb1 inhibitor). Then, Swiss mice ($n = 24$) received i.p. 5 mg/kg AMI alone and in association with 200 mg/kg novobiocin (Abcg2 inhibitor), 20 mg/kg probenecid (Abcc2 inhibitor) and 100 mg/kg quinidine.

Plasma concentrations of AMI were not influenced by novobiocin, probenecid and the lack of Abcb1, but were significantly increased by quinidine, resulting from the inhibition of hydroxylation mediated by CYP2D6. Brain distribution of AMI was not influenced by the lack of Abcb1 but was slightly significant with quinidine and not with novobiocin and probenecid. At the hepato-biliary interface, we showed the involvement of Abcb1, Abcc2 and Abcg2; indeed, AMI concentration was increased in liver and decreased in bile, where quinidine is the strongest inhibitor, followed by probenecid and novobiocin.

These results show that in brain the effect of Abcb1, Abcc2 and Abcg2 should be negligible and that at the hepato-biliary level, Abcb1 plays a predominant role compared to Abcc2 and Abcg2.

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1. Introduction

Amitriptyline (AMI) is a tricyclic antidepressant which remains one of the major antidepressants despite the introduction of newer drugs such as selective serotonin reuptake inhibitors (Barbui and Hotopf, 2001). AMI has also analgesic properties and is commonly used in the treatment of chronic pain where it is still the mainstay of front-line therapy of diabetic neuropathy, postherpetic neuralgia, fibromyalgia, central pain and peripheral neuropathy of different etiology (Bryson and Wilde, 1996). The oral bioavailability of AMI is highly variable, ranging from 33% to 62% in humans (Schultz et al., 1985), and is responsible for large interindividual variations in the therapeutic effects. AMI is usually classified as a BCS Class I drug but has been regarded as lying at the interface of BCS Class I/II given its solubility pattern (Manzo et al., 2006) so that transporter effects may affect the oral bioavailability of AMI (Wu and Benet, 2005).

The ATP binding cassette (ABC) transporters are the largest superfamily of transmembrane proteins that transport various substances over cell membrane and influence the intestinal absorption and urinary or biliary excretion of many drugs (Ho and Kim, 2005).

Among them, ABCB1 is considered to be one of the most important factors involved in the multidrug resistance (MDR) to cancer chemotherapy, by leading to the efflux of anti-cancer drugs from tumor cells (Harris and Hochhauser, 1992). The range of substrates transported by P-gp is broad including anticancer agents (vinblastine, vincristine, doxorubicin), antiarrhythmics (quinidine), immunosuppressants (cyclosporine), antidepressants (paroxetine, sertraline) (Marzolini et al., 2004). AMI is known to be a substrate of ABCB1 transporter. Indeed, Uhr et al. (2000) showed that central nervous system concentrations of AMI and its metabolites in knockout mice lacking a functional Abcb1 transporter were higher than in control mice after a single intraperitoneal administration of AMI. However, in studies performed at steady state, Grauer and Uhr (2004) did not confirm these initial findings for AMI but only for its metabolites and in more recent studies, they showed that AMI was similarly metabolized and that there were no significant differences in the pharmacokinetics of AMI in double-knockout and in control mice (Uhr et al., 2007). Finally, ex vivo experiments (using intestinal segments and in situ recirculating intestinal perfusion model) and bioavailability studies in animals in our laboratory (Abaut et al., 2007) have suggested that other transporters may be involved and that Abcb1 may not be a major player.

Hence, it appears that the role of Abcb1 in AMI disposition should be clarified and that the potential role of other efflux transporters such as Abcc2 and Abcg2 should be investigated since this

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has not been studied before. Given the location of these efflux transporters at the blood–brain-barrier and hepato-biliary levels, we performed a study of the brain, liver and bile distribution of AMI and its metabolites in Abcb1a deficient CF1 mice with and without quinidine (QUI) as Abcb1 inhibitor, and in normal mice with and without novobiocin (NOVO) and probenecid (PROB) as Abcg2 and Abcc2 inhibitors, respectively (Shiozawa et al., 2004; Horikawa et al., 2002).

2. Material and methods

2.1. Chemicals

Amitriptyline hydrochloride, clomipramine hydrochloride used as internal standard, quinidine sulfate dihydrate, novobiocin sodium salt and probenecid were purchased from Sigma Chemical Co. (St Louis, USA). Amitriptyline metabolites (nortriptyline (NOR), E and Z-hydroxy-amitriptyline (E and Z-OH-AMI), E and Z-hydroxy-nortriptyline (E and Z-OH-NOR) were kindly given by Lundbeck (Copenhagen, Denmark). All other reagents were of analytical or HPLC grade.

2.2. Animals

The study was approved by the Committee of Laboratory Investigation and Animal Care of our institution and performed in accordance with French Ministry of Agricultures laws and guidelines for laboratory animal experiments (agreement no. B35-238-21).

Experiments were carried out on female Swiss mice (28.2 ± 1.8 g) obtained from Janvier Laboratories (Le Genest Saint Isle, France) and on female CF1 mice Abcb1a (+/+) and Abcb1a (−/−) (26.7 ± 1.2 g and 10 weeks old), obtained from Charles River Laboratories (Wilmington, MA, USA). CF1 mice are deficient in Abcb1a gene and have a deficiency in functional Abcb1 in intestinal epithelium and brain capillary endothelial cells; they represent a naturally occurring analog of the transgenic Abcb1a (−/−) knockout mice (Lankas et al., 1997).

The animals were housed individually and maintained in animal care facilities for at least one week before use. They received food and water *ad libitum* and were fasted 12 h before each experiment.

2.3. Study design

The impact of Abcb1 on AMI distribution was carried out on Abcb1a (+/+) and Abcb1a (−/−) CF1 mice. The protocol included three groups:

- CF1 (+/+) mice (n = 12) received i.p. 5 mg/kg AMI alone
- CF1 (−/−) mice (n = 12) received i.p. 5 mg/kg AMI alone
- CF1 (+/+) mice (n = 12) received i.p. 5 mg/kg AMI + 100 mg/kg QUI administered 1 h before AMI dosing

The impact of Abcg2 and Abcc2 on AMI distribution was carried out on 24 Swiss mice divided in four groups which were administrated i.p. 5 mg/kg AMI alone and in association with 200 mg/kg NOVO, 20 mg/kg PROB and 100 mg/kg QUI, as a comparator.

In both protocols, plasma and tissue samples were collected at a pseudo equilibrium distribution time, i.e. 1 h after i.p. injection, because in previous experiments (data not shown) we shown that after 1 h, 2 h and 4 h tissue to plasma ratio (brain and liver) were similar.

Mice were anesthetised with isofurane inhalation (Forene®, Abbott, Rungis, France) and decapitated. Trunk blood was collected and centrifuged at 3000 × g for 5 min to determine the plasma concentration of AMI and its main metabolites. Brain and liver were dissected and rinsed with 0.9% (m/v) saline. They were weighed and then homogenized with, respectively, 2.0 ml and 5.0 ml of a phosphate buffer saline (PBS, pH 7.2) containing 8.0 g/l NaCl, 0.20 g/l KCl, 1.80 g/l Na_2HPO_4 and 0.20 g/l KH_2PO_4, with a polytron PT-MR 3000 from Kinematica (Littau, Switzerland). Bile was collected from gallbladder, weighed and diluted with 0.50 ml of PBS. Plasma samples and homogenates were frozen at −20 °C until analysis by HPLC.

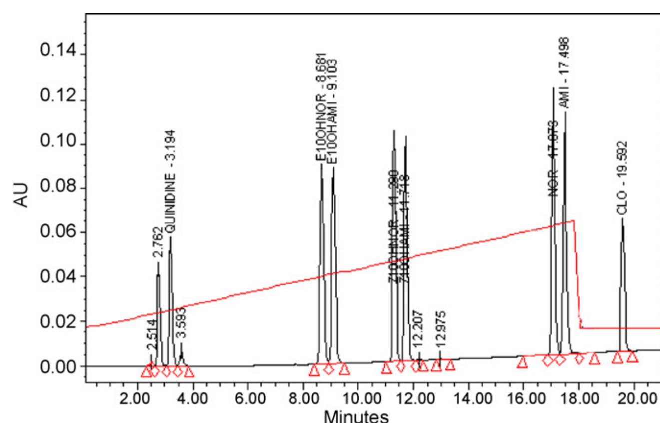

Fig. 1. Representative chromatogram of mouse plasma obtained one hour after i.p injection of 5 mg AMI/kg mouse.

2.4. Extraction procedure

After thawing, the plasma samples and homogenates (0.50 ml) were homogenized and 100 μl of clomipramine (10 mg/L) used as internal standard, were added. A total of 50 μl of 1N NaOH and 2 ml of heptane with ethyl acetate (80/20) were added and the samples were mixed for 10 min at room temperature. After centrifugation for 5 min at 4000 × g, the organic layer was transferred to a tube containing 50 μl of 0.05 M sulfuric acid, mixed for 10 min and centrifuged at 3000 × g for 5 min. The organic layer was then discarded and a 50 μl aliquot of the aqueous phase was mixed to 10 μl of 0.5 M dipotassium hydrogen phosphate. Finally 20 μl of the aqueous phase were injected for chromatographic separation. The separation and quantification of AMI and its metabolites in plasma samples were carried out using a HPLC method with UV absorbance detection (λ = 205 nm). The chromatographic system consisted of a Waters model 600A pump (Waters, Milford, USA) equipped with a Waters model 717 automatic injector, a Waters model 996 photodiode array detector and a Waters model Empower integration software. The analytical chromatographic column was a Supelco 5 μ C18 250 mm × 4.6 mm (Sigma–Aldrich, Bellefont, USA) maintained at 45 °C and the flow rate of the mobile phase was 1 ml/min. The mobile phase A (acetonitrile–water 10/90, 900 μl of 85% phosphoric acid, 1.22 g of potassium dihydrogen phosphate) and B (acetonitrile) were filtered through a 0.45 μm PTFE membrane (Millipore, St Quentin, France) and degassed immediately before use. A 20–50% mobile phase B gradient in 21 min was used for chromatographic analysis. Plasma samples were calibrated by using seven different concentrations ranging from 5 ng/ml to 1000 ng/ml. Quantification was performed by calculating the analyte/internal standard peak-area ratio. Representative chromatogram of mouse plasma obtained 1 h after i.p injection of 5 μg AMI/g mouse is shown in Fig. 1.

2.5. Data analysis

- For distribution analysis, we calculated the organ:plasma ratio (for liver and brain samples) and the bile:liver ratio (for bile samples) and then we compared them with the corresponding ratio in control mice:

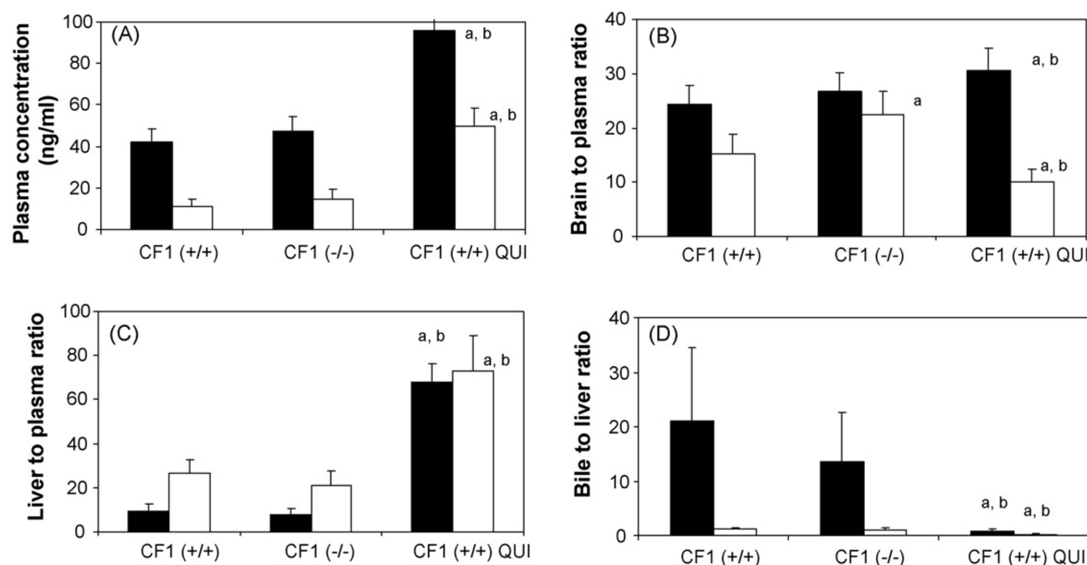

Fig. 2. Plasma concentration (A), brain-to-plasma (B), liver-to-plasma (C) and bile-to-liver (D) concentration ratio of AMI (black bar) and of NOR (white bar) in Abcb1a (+/+) CF1 mice, in Abcb1a (−/−) CF1 mice and in Abcb1a (+/+) CF1 mice in presence of QUI (a) significantly different versus control (Abcb1a (+/+) CF1 mice) (b) significantly different versus control (Abcb1a (−/−) CF1 mice).

- Brain to plasma ratio = brain concentration/plasma concentration
- Liver to plasma ratio = liver concentration/plasma concentration
- Bile to liver ratio = bile concentration/liver concentration
- The extraction ratio calculated: sum of metabolites hepatic concentrations/(AMI hepatic concentration + sum of metabolites hepatic concentrations)
- For the statistical analysis, all data are presented as mean ± SD. Analysis of variance followed by multiple comparison testing was used. A *p*-value less than 0.05 was considered as statistically significant.

3. Results and discussion

To evaluate the impact of Abcb1 on AMI disposition, we used a model of Abcb1a deficient CF1 mice. In mice, the Abcb1 transporter is encoded by two genes, Abcb1a and Abcb1b. In Abcb1 deficient subpopulations of CF1 mice, only Abcb1a gene is lacking but CF1 (−/−) mice have non-detectable levels of Abcb1 transporter in liver, in brain and heart (Lankas et al., 1997).

Plasma concentrations of AMI and NOR in CF1 (−/−) and CF1 (+/+) mice with and without QUI are shown in Fig. 2. In plasma, there was no difference in AMI and NOR concentrations in Abcb1a deficient mice, while inhibition of Abcb1 by QUI showed a significant increase in both AMI and NOR levels (Fig. 2A) that should result from an inhibition of the hydroxylation pathway. Indeed QUI is known to be an inhibitor of CYP2D6, and in vitro and in vivo studies in humans have shown that CYP2D6 is the major determinant of the hydroxylation leading to E/Z-OHAMI and E/Z-OHNOR (Fig. 3) (Venkatakrishnan et al., 2001). Plasma concentrations of AMI, NOR and pooled hydroxylated metabolites in Swiss mice in the presence of inhibitors of Abcb1, Abcc2 and Abcg2 showed that PROB and NOVO had no significant effect on AMI hydroxylation while QUI led to a significant increase in AMI and NOR concentra-

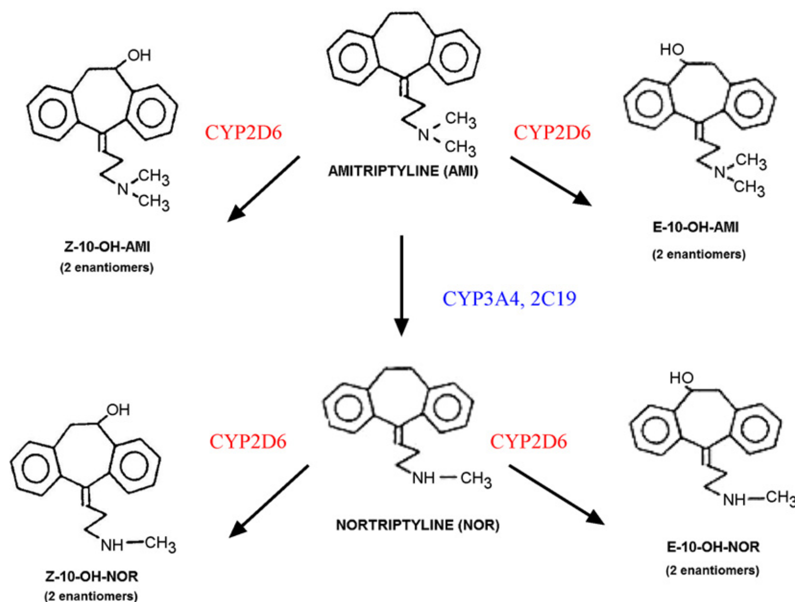

Fig. 3. Principal amitriptyline metabolic pathways.

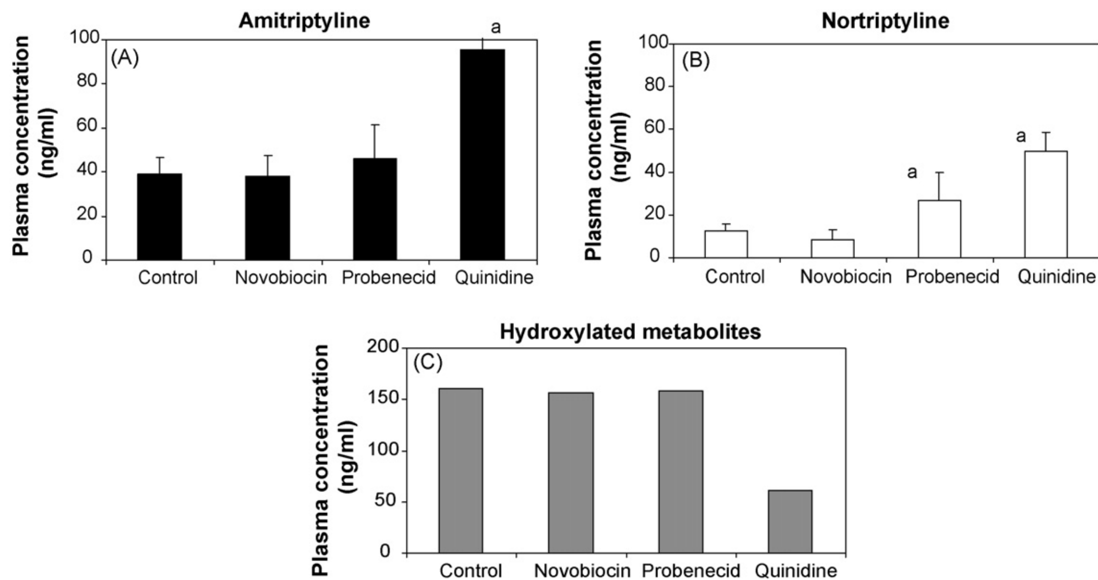

Fig. 4. Plasma concentrations of AMI (A), NOR (B) and of their hydroxylated metabolites (C) in Swiss mice treated with NOVO, PROB and QUI (a) significantly different versus control.

Table 1
Brain-to-plasma concentration ratio of the hydroxylated metabolites of amitriptyline in Abcb1a (+/+) CF1 mice and in Abcb1a (−/−) CF1 mice.

	AMI	NOR	Z-OH-AMI	Z-OH-NOR	E-OH-AMI	E-OH-NOR
Brain-to-plasma ratio in CF1 (+/+)	24.4 ± 3.4	15.2 ± 3.7	3.5 ± 0.7	0.2 ± 0.1	2.6 ± 0.4	0.3 ± 0.2
Brain-to-plasma ratio in CF1 (−/−)	26.7 ± 3.5	22.3 ± 4.3	12.4 ± 4.3[a]	1.3 ± 0.3[a]	4.4 ± 1.6[a]	2.2 ± 0.4[a]
Ratio (−/−)/(+/+)	1.1	1.5	3.5	4.3	1.7	7.3

[a] Significantly different versus control.

tions and a decrease in hydroxylated metabolites concentrations (Fig. 4).

Brain distribution of AMI was unchanged in CF1 (−/−) mice but slightly and significantly increased in CF1 (+/+) mice in the presence of QUI. The brain distribution of NOR was slightly but significantly increased in CF1 (−/−) mice but unexpectedly decreased in CF1 (+/+) mice with QUI (Fig. 2B). These results suggest a rather limited effect of Abcb1 on brain distribution of AMI. This should not be attributed to the mice study model since in similar studies in CF1 (−/−) mice, a 2-fold and 20-fold increase in brain-to-plasma ratio was observed for riluzole and indinavir, respectively (Milane et al., 2007; Pereira de Oliveira et al., 2005). Indeed, although in CF1 mice only Abcb1a gene is lacking and compensatory mechanisms involving an upregulation of the expression of the Abcb1b gene products have been reported, results of western blot analysis confirm that Abcb1a (−/−) CF1 mice have non detectable levels of Abcb1 transporter in brain, liver and heart. Moreover, these findings confirm results recently published by Uhr et al. (2007) that showed in Abcb1ab double knockout mice that AMI was not so strong a substrate of Abcb1. Indeed Grauer and Uhr (2004) investigated whether a significant difference of the cerebral concentrations of AMI and its metabolites between wild and knockout mice was observed after a repeated i.p. administration of AMI. They showed that the cerebral concentrations were significantly increased in the Abcb1 knockout mice for all metabolites except for AMI. Finally, they compared the pharmacokinetics and metabolism of AMI in knockout mice and in controls (Uhr et al., 2007) and showed that AMI there were no significant differences in the pharmacokinetics of AMI in knockout and in control mice. The brain distribution of hydroxylated Z and E metabolites of AMI and NOR was much lower than that of the parent compound, especially for the hydroxylated metabolites of NOR. This should result from their higher hydrophilicity. However, their brain distribution in CF1 (−/−) mice was more affected than that of AMI and NOR displaying a 2–7-fold increase in distribution. Moreover, these data show that Abcb1 did not display stereoselectivity for E and Z enantiomers of AMI and NOR since the ratios in CF1 (+/+) mice were very close (Table 1).

Table 2
Brain-to-plasma concentration ratio of AMI and NOR in Swiss mice treated with treated with NOVO, PROB and QUI.

	Control	NOVO	PROB	QUI
AMI	25.2 ± 3.8	27.4 ± 6.5	18.6 ± 4.2[a]	30.6 ± 4.2[a]
NOR	16 ± 3.5	16.7 ± 8.2	7.2 ± 2.7[a]	9.9 ± 2.5[a]

[a] Significantly different versus control.

The study of the influence of Abcc2 and Abcg2 on the brain distribution has been evaluated by the transporter inhibition in Swiss mice, in comparison with the Abcb1 inhibition by QUI. These results showed that the effect of QUI was similar as in CF1 mice with a slight but significant increase in brain distribution for AMI and with a decrease for NOR (Table 2). The results suggest that the impact of both Abcc2 and Abcg2 transporters on the brain distribution of AMI should be considered as negligible. On the whole, these data suggest that the brain distribution of AMI and NOR is minimally affected by the lack or the inhibition of Abcb1 so that drug interaction at the Abcb1 level should not have significant effect on the central effects of AMI and NOR. However, attention should be paid to the effect on the hydroxylated metabolites since they have a pharmacological activity. Furthermore, drug inhibition of Abcc2 and Abcg2 transporters at the brain level should be unlikely.

At the hepatocyte level, the effect of Abcb1a deficiency and of the different inhibitions of ABC transporters showed interesting effects on AMI disposition at the hepato-biliary interface where these transporters are located. CF1 (−/−) mice displayed a very slight decrease in liver distribution while the QUI inhibition led to a very

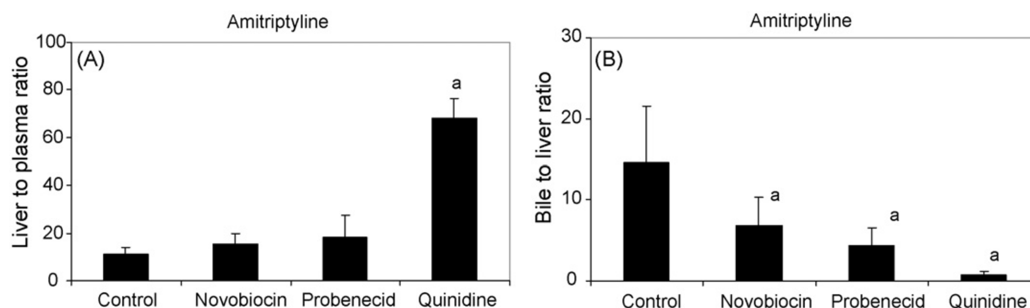

Fig. 5. Liver-to-plasma concentration ratio (A) and bile-to-liver concentration ratio (B) of AMI in Swiss mice treated with NOVO, PROB and QUI.

Table 3
Hepatic AMI metabolism: extraction ratio (ER) for total metabolism and for hydroxylation and demethylation pathways in CF1 (+/+) mice, in CF1 (−/−) mice, in Swiss mice with NOVO, PROB and QUI.

	ER hydroxylation	ER demethylation	ER total
Control	0.79 ± 0.02	0.10 ± 0.01	0.89 ± 0.01
ABCB1–CF1 (−/−)	0.81 ± 0.03	0.09 ± 0.03	0.90 ± 0.03
ABCG2–NOVO	0.82 ± 0.02[a]	0.07 ± 0.01[a]	0.89 ± 0.02
ABCC2–PROB	0.70 ± 0.13	0.14 ± 0.06[a]	0.85 ± 0.07
ABCB1–QUI	0.18 ± 0.02[a]	0.29 ± 0.04[a]	0.47 ± 0.05[a]

[a] Significantly different versus control.

significant increase in both AMI and NOR ratios (Fig. 2C). These data may appear inconsistent since both interventions should lead to the same effect. Alternatively, the more pronounced effect of the chemical inhibition may result from effect of QUI on other transporters Abcc2 and Abcg2. However, these data should be analyzed together with the data on the bile distribution since the concentration in the hepatocytes depends partly on the export from hepatocytes to the bile which can be modulated by the activity or inhibition of ABC transporters at the canalicular level. Indeed, the bile distribution of AMI was decreased, while non-significantly as a result of a large variability (c.v. around 60%) in Abcb1a deficient mice, and more profoundly with the QUI inhibition (Fig. 2D). Such a pattern was also observed for NOR. This suggested that the decrease in bile excretion may result in an increased accumulation in the hepatocytes, highlighting the significant role of Abcb1 in the hepato-biliary disposition of AMI.

The role of the ABC transporters on the hepato-biliary disposition of AMI was clearly suggested by the experiment achieved in Swiss mice (Fig. 5 and Table 3). The inhibition of the bile excretion of AMI where QUI was the strongest inhibitor followed by PROB and NOVO, was clearly consistent with the influence noticed on the liver to plasma ratios (mirror images of Fig. 5A and B). This experiment confirmed the significant role of Abcb1 in the hepatocyte-to-bile transport of AMI, and clearly suggested that Abcc2 and Abcg2 were implicated since PROB and NOVO may affect Abcb1. The effect of transporter inhibition at the hepato-biliary level could potentially influence drug metabolism since it increased the drug availability to the metabolic enzymes. However, such effect may be apparent only for drugs with a low extraction ratio, which have a rate-limited elimination. Indeed, drugs with a high extraction ratio have a clearance witch is insensitive to the cellular metabolic activity. Since AMI is a flow-limited clearance drug with a high extraction ratio, its metabolism should not be altered by transporter modulation. The hepatic metabolism, estimated by the hepatic extraction ratio, was altered neither in CF1 (−/−) mice nor in the PROB and NOVO experiments (Table 3). Conversely, the metabolic pattern was altered after QUI treatment where the ER decreased from 0.89 to 0.47 (Table 3). This decrease in ER was associated with a decrease in the hydroxylation ER (0.79–0.18) resulting from CYP 2D6 inhibition, and with an increase in the demethylation ER (0.10–0.29), probably resulting from a shift in the metabolic pathway. Using QUI, the overlap in inhibitor selectivity for metabolic enzyme versus transporter makes it difficult to differentiate the roles of efflux transporter and enzyme in hepatic disposition of AMI because inhibition of either can lead to an increase in hepatic exposure. However, the lack of role of Abcb1 inhibition at the hepato-biliary interface on AMI metabolism was confirmed since the ER's were unchanged in CF1 (−/−) mice. Given the magnitude of the hepatic ER of AMI (0.90), the impact of an efflux inhibition is unlikely. For those reasons, the inhibition of the other ABC transporters had no effect on the ER of AMI. Hence, inhibition of ABC transporters at the hepato-biliary level may alter the hepatic clearance of AMI by decreasing the biliary clearance and not by altering the metabolic elimination. It should be noticed that the disposition of AMI at the hepatocyte level could be much more complex since other tricyclic antidepressants with a close chemical structure (i.e., desipramine and imipramine) have been shown to interact with influx transporters such as OCTN1 (Wu et al., 2000).

In conclusion, AMI is known to be a substrate of Abcb1, and these results suggest that other ABC transporters (i.e., Abcc2 and Abcg2) may affect its disposition. At the brain level, inhibition of these transporters did not display significant modifications. At the hepato-biliary level, the inhibition of these transporters induced a decrease in the biliary secretion where QUI was the strongest inhibitor followed by PROB and NOVO. The high extraction ratio of the drug may explain the lack of influence of the efflux inhibition on the metabolism. Given the possible involvement of Abcc2 and Abcg2 in the disposition of AMI, it should be interesting to study the effect of their inhibition at the intestinal level on the systemic bioavailability. Indeed, we have previously shown (Abaut et al., 2007) that the inhibition of Abcb1 at the intestinal level increased the oral bioavailability of AMI.

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