The role of P-glycoprotein in limiting brain penetration of the peripherally acting anticholinergic OAB drug trospium chloride

Joachim Geyer, Olga Gavrilova, Ernst Petzinger

Institute of Pharmacology and Toxicology, Justus Liebig University of Giessen, Frankfurter Str. 107, D-35392 Giessen, Germany (J.M., O.G., E.P.)
Running title: Trospium Chloride Brain Penetration in mdr1a,b-/- Knockout Mice

Correspondence and reprints:
Prof. Dr. Joachim Geyer
Institute of Pharmacology and Toxicology
Justus Liebig University of Giessen
Frankfurter Str. 107
35392 Giessen, Germany
Tel: +49 641 9938404
Fax: +49 641 9938419
E-mail: Joachim.M.Geyer@vetmed.uni-giessen.de

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Abbreviations: BBB, blood-brain barrier; CNS, central nervous system; OAB, overactive bladder; P-gp, P-glycoprotein; b.w., body weight; i.v., intravenous; p.o., per oral; PBS, phosphate buffered saline.
Abstract
The aim of the present study was to characterize the role of the drug-efflux transporter P-glycoprotein (P-gp) for the disposition of trospium chloride, a widely used anticholinergic drug for the treatment of overactive bladder (OAB). P-gp deficient mdr1a,b<sup>−/−</sup> knockout mice were given either 1mg/kg trospium chloride orally or 1mg/kg intravenously to analyze brain penetration, intestinal secretion and hepatobiliary excretion of the drug. The concentrations of trospium chloride in the brain were up to 7 times higher in the mdr1a,b<sup>−/−</sup> knockout mice compared to wild-type mice (p<0.05) making P-gp a limiting factor for the blood-brain barrier penetration of this drug. Moreover, the residence time of the drug in the CNS was significantly prolonged in mdr1a,b<sup>−/−</sup> knockout mice. Apart from the blood-brain barrier, P-gp also had significant effects on the overall pharmacokinetics of trospium chloride. In the mdr1a,b<sup>−/−</sup> knockout mice, hepatobiliary excretion and intestinal secretion were significantly reduced compared to the wild-type mice. Our study indicates that the multidrug resistance transporter P-gp is a major determinant for the distribution of trospium chloride in the body and highly restricts its entry into the brain.
Introduction

Antagonists of the acetylcholine muscarinic receptors, such as trospium chloride, oxybutynin, tolterodine, fesoterodine, darifenacin, and solifenacin are the cornerstone of pharmacotherapy for the symptoms of overactive bladder (OAB) (Andersson, 2005). A potential problem in OAB therapy with such drugs are the undesirable side effects involving the central nervous system (CNS) including dizziness, nervousness, sleep disorders, cognitive impairment, memory impairment, hallucination, and confusion (Scheife and Takeda, 2005; Kay and Ebinger, 2008). The occurrence of these CNS side effects is greatly dependent on the ability of the individual drug to pass the blood-brain barrier (BBB) (Andersson, 2005; Staskin and MacDiarmid, 2006). While most of the aforementioned antimuscarinic drugs are tertiary amines which are quite lipophilic and can easily penetrate into the brain, trospium chloride is a highly polar quaternary amine that exhibits low lipophilicity (Singh-Franco et al., 2005) (for chemical structure see Schladitz-Keil et al., 1986). Therefore, trospium chloride can be expected to show much lower penetration through the BBB of patients than other more lipophilic and uncharged antimuscarinic drugs (Wiedemann and Schwantes, 2007).

Apart from their physicochemical properties, brain penetration of many drugs is also affected by drug transporters expressed at the BBB (Löscher and Potschka, 2005). Here, in particular the drug transporting P-glycoprotein (P-gp) limits the entry of many drugs and xenobiotics into the brain (Schinkel et al., 1996) by an efflux-based transport mechanism. Outside of the brain, P-gp is also expressed in tissues with secretory/excretory functions such as the liver (canalicular membrane of hepatocytes), kidney (luminal membrane of proximal tubules) and intestine (brush border membrane of enterocytes) (Thiebaut et al., 1987). Apical/luminal expression of P-gp in these organs diminishes oral drug bioavailability and promotes drug elimination into bile and urine (Fromm, 2004).

P-gp transports a wide range of structurally unrelated drugs, toxins, and xenobiotics of which many are amphiphilic and/or positively charged (Marzolini et al., 2004). This led us to
hypothesize that trospium chloride might also be a substrate of P-gp. A previous in vitro study showed that trospium chloride slightly inhibited the P-gp mediated digoxin transport across LLC-PK₁ cell monolayers which over-expressed the human P-gp (Sandage et al., 2006). Another in vitro study with P-gp expressing Caco-2 monolayers showed that trospium chloride was transported from the basolateral to the apical direction in a verapamil-sensitive manner (Langguth et al., 1997). Both findings therefore supported our assumption.

The present study aimed to characterize the role of P-gp in the disposition of trospium chloride using the P-gp deficient mdr1a,b⁻/⁻ knockout mouse model, which to our knowledge has not previously been applied to any other OAB anticholinergic drug. We found that brain penetration of trospium chloride is restricted by P-gp, and thus increased up to 7-fold in mdr1a,b⁻/⁻ knockout mice. Furthermore, P-gp is significantly involved in the intestinal secretion and hepatobiliary elimination of trospium chloride in mice.

Materials and Methods

Animals. Whereas humans only have one gene encoding the drug-transporting P-gp (MDR1), in the mouse genome, two P-gp coding genes, mdr1a and mdr1b have been identified. The tissue distribution of mouse mdr1a and mdr1b P-gps suggests that both proteins together fulfill the same function as the single MDR1 P-gp in humans (Borst and Schinkel, 1997). We therefore used mdr1a/b⁻/⁻ double knockout mice (further referred to as mdr1a,b⁻/⁻ knockout mice) of a FVB genetic background together with wild-type FVB mice (wild-type mice) in our studies. Male wild-type mice, and mdr1a,b⁻/⁻ knockout mice were obtained from Taconic Farms Inc. (Germantown, NY, USA). All mice were housed in isolated ventilated cages under controlled temperature with a 12h/12h light/dark cycle and provided with sterilized food and water ad libitum. The mice were between 12 and 19 weeks of age. All animal experiments were registered and approved by the local administration and were conducted in accordance...
with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised in 1985).

Drug Preparation and Application. $[^3]$H]Trospium trifluoracetate (70 Ci/mmol) was purchased from RC TRITEC AG (Teufen, Switzerland) and unlabeled trospium chloride was kindly provided by Dr. R. Pfleger GmbH (Bamberg, Germany). For drug application, a mixture of $[^3]$H]trospium trifluoracetate (2 µCi to 2.5 µCi, representing 0.06 – 0.07 % of the total dose) and unlabeled trospium chloride were prepared in 50 µl phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 7.3 mM Na$_2$HPO$_4$, pH 7.4) for i.v. administration or in 200 µl 0.9% NaCl for p.o. application. Due to the high excess of chloride in relation to trifluoracetate in the drug preparation, this is further referred to as $[^3]$H]trospium chloride. For oral drug administration, the animals were fasted overnight, until food was made available 3 h after drug application.

Gall-Bladder Cannulation. Wild-type mice and mdr1a,b$^{-/-}$ knockout mice were anesthetized with a combination of ketamine and xylazine at a final dose of 116 mg/kg ketamine and 8 mg/kg xylazine. The anesthetic solution was injected intraperitoneally. After laparatomy and distal ligation of the common bile duct, a polyethylene catheter with an inner diameter of 0.25 mm was inserted into the incised gall bladder and was fixed to the gall bladder with an additional ligation. After i.v. injection of $[^3]$H]trospium chloride into the tail vein, bile was collected over a 120 min time period, during which the mice were placed in a temperature-controlled hood. At the end of the experiments, blood was collected by cardiac puncture, and the organs were removed and homogenized in 100-7000 µl 0.05 M NaOH (depending on the tissue weight). The levels of radioactivity in serum, bile, urine, faeces, and tissue homogenates were quantified by a Wallac 1409 liquid scintillation counter.
**Tissue distribution.** For analysis of the tissue distribution, $[^3]$Htrospium chloride was applied i.v. and p.o. at 1 mg/kg b.w. to wild-type and $mdr1a,b^{-/-}$ knockout mice. After 12 h the animals were euthanized by cervical dislocation. The organs were removed and processed as described above.

**Statistical Analysis.** All data is presented as the mean ± SD of three to four animals. Student’s two-tailed unpaired $t$-test and one-way ANOVA followed by Bonferroni’s post hoc test were used to identify significant differences between groups.

**Results**

**Hepatobiliary and urinary excretion of $[^3]$Htrospium chloride.** Due to the significance of P-gp in determining overall body distribution and drug elimination in general, under an intravenous application regimen we analyzed the role of P-gp for the hepatobiliary elimination, urinary excretion and intestinal secretion of trospium chloride using gall-bladder cannulated wild-type mice and $mdr1a,b^{-/-}$ knockout mice. $[^3]$HTrospium chloride was applied i.v. at a dosage of 1 mg/kg b.w. into the tail vein, and bile and urine samples were collected by catheterization. After 2 h, mice were euthanized and the intestinal content was recovered from the small intestine and the colon. Hepatobiliary excretion of $[^3]$Htrospium chloride was rapidly detected within a few minutes following i.v. application and tended to be higher in the wild-type mice over 50 min (Fig. 1A). The maximum drug concentrations in the bile were 12.1 ± 2.7 µg/ml and 9.5 ± 2.6 µg/ml in the wild-type mice and $mdr1a,b^{-/-}$ knockout mice, respectively. In contrast to the bile flux data, which did not reach a level of significance, the overall hepatobiliary excretion of $[^3]$Htrospium chloride during 120 min was significantly lower in the $mdr1a,b^{-/-}$ knockout mice: whereas the wild-type mice excreted 26.0 ± 0.5 % of the applied dose into bile, the $mdr1a,b^{-/-}$ knockout mice only excreted 20.0 ± 1.4 % of the applied dose (Fig. 1B).
Significant differences between the wild-type and the mdr1a,b<sup>−/−</sup> knockout mice also occurred in the liver content of [³H]trospium chloride: 120 min after drug application only 3% of the applied drug was recovered in the liver tissue of the wild-type mice whereas 26% passed the liver and were detected in the bile. In contrast, 11% of the applied dose were detected in the liver of the mdr1a,b<sup>−/−</sup> knockout mice pointing to an accumulation in the liver due to a reduced biliary efflux of the drug. Supporting this conclusion, the sum of drug recovered in liver and bile were nearly identical between wild-type and mdr1a,b<sup>−/−</sup> knockout mice, at 29% and 31%, respectively. By balancing their reduced hepatobiliary elimination the mdr1a,b<sup>−/−</sup> knockout mice showed higher excretion of the drug into the urine compared to the wild-type mice, namely 39% vs. 29% of the total dose within 2 h.

**Intestinal secretion of [³H]trospium chloride.** Two hours after i.v. [³H]trospium chloride application, the intestinal content was recovered from the small intestine and colon and analyzed for intestinal drug secretion. As we used gall-bladder cannulated and bile duct ligated mice for this study, biliary excretion of [³H]trospium chloride could be excluded, and all gut radioactivity must has been derived solely from intestinal secretion. In both samples, i.e. from the small intestine and colon, significantly lower drug amounts were detected in the mdr1a,b<sup>−/−</sup> knockout mice compared to wild-type mice pointing to an important role of P-gp for intestinal secretion of trospium chloride (Fig. 2A). The overall amount of trospium chloride in the intestinal content was 0.7% of the applied dose in the mdr1a,b<sup>−/−</sup> knockout mice, and 1.7% in the wild-type mice within a 2 h investigation period. The overall amount of intestinal excretion of [³H]trospium chloride was also analyzed in mdr1a,b<sup>−/−</sup> knockout mice and wild-type mice without bile fistula 12 h after application of 1 mg/kg b.w. [³H]trospium chloride (Fig. 2A). This study showed that the intestinal drug content tended to be lower in mdr1a,b<sup>−/−</sup> knockout mice compared to wild-type mice, but this difference did not reach the level of significance (p < 0.12).
Brain penetration and organ distribution of \([3H]\)trospium chloride. In both studies involving i.v. \([3H]\)trospium chloride application to gall-bladder cannulated mice (2 h), and mice without bile fistula (12 h), we also analyzed drug penetration into the brain. Additionally, \(mdr1a,b^-/-\) knockout mice and wild-type mice received 1 mg/kg b.w. \([3H]\)trospium chloride orally as this is the approved route of application for this drug in humans. The overall drug concentrations in the brain were highest 2 h after i.v. application, and extremely low 12 h after p.o. application. In the \(mdr1a,b^-/-\) knockout mice, \([3H]\)trospium chloride brain concentrations were 2 times higher (36.5 ± 7.3 ng/g vs. 20.6 ± 3.0 ng/g) in the 2 h/i.v. group, 7 times higher (26.2 ± 3.3 ng/g vs. 3.8 ± 2.1 ng/g) in the 12 h/i.v. group, and 4 times higher (2.3 ± 1.6 ng/g vs. 0.6 ± 0.6 ng/g) in the 12 h/p.o. application group compared with wild-type mice (Fig. 2B).

Additionally, the brain-to-plasma ratio 2 h after i.v. application was higher for the \(mdr1a,b^-/-\) knockout mice (0.27 ± 0.03) compared to the wild-type mice (0.17 ± 0.05) (Table 1). Unfortunately, \([3H]\)trospium chloride concentrations in the plasma were below the detection level 12 h after i.v. and p.o. applications. Therefore, brain-to-plasma ratios could not be calculated from these application groups.

Discussion

Brain penetration of trospium chloride. Trospium chloride, a quaternary ammonium compound, which is hydrophilic and highly polar, is considered to have low ability to diffuse passively across the BBB (Scheife and Takeda, 2005; Wiedemann and Schwantes, 2007). These physicochemical properties may therefore limit the extent of CNS side effects of trospium chloride which are expected to occur as a result of the nonselective interaction of anticholinergics with CNS muscarinic receptors (Kay et al., 2005). In the present study we confirmed the very low ability of trospium chloride to penetrate the BBB and demonstrate that in mice, as an additional and biological protective barrier, the P-gp mediated drug efflux...
at the BBB highly limits brain penetration of this drug. Since the transport data for many
drugs closely correlate between the human and mouse P-gp (Feng et al., 2008), the same
protective effect will most likely occur also at the human BBB. This is clearly consistent with
data from clinical studies in man that did not find any undesirable CNS side effects under
OAB treatment with trospium chloride (Singh-Franco et al., 2005).

The role of P-gp for overall trospium chloride pharmacokinetics. Apart from the prominent
role of P-gp in limiting brain penetration of trospium chloride, we found significant effects of
the P-gp efflux pump on the intestinal secretion and hepatobiliary elimination, and hence on
the overall pharmacokinetics of this drug. A comparable effect of P-gp has also been
previously shown for other P-gp transported drugs such as cyclosporine A, digoxin,
paclitaxel, and loperamide (Schinkel et al., 1996; Sparreboom et al., 1997). Regarding
trospium chloride clearance from the brain and liver, clear differences were found: 2 h after
application, \[^{3}H\text{trospium chloride}\] accumulated by a greater extent in the liver (ratio 3.2) than
in the brain of the \(m_{dr1a,b}^{-/-}\) knockout mice (ratio 1.8). This is likely to be due to the impaired
hepatobiliary elimination route in the absence of P-gp. This order was reversed 12 h after
application, where 6.9-fold higher drug concentrations were detected in the brain of the
\(m_{dr1a,b}^{-/-}\) knockout mice compared to the wild-type mice. Liver concentrations were no
longer different between the mouse strains at this later time point. This effect can be
explained by a slow rate of elimination of trospium chloride from the brain in the absence of
P-gp which caused a decline from 37 ng/g to 26 ng/g from 2 h to 12 h, respectively. Such
reduced clearance from the brain in the absence of P-gp has been described before for other P-
gp drugs such as vinblastine and digoxin (Schinkel et al., 1994; Mayer et al., 1996). In
contrast, \[^{3}H\text{trospium chloride}\] was quite rapidly cleared from the brain of the wild-type mice
(21 ng/g to 4 ng/g from 2 h to 12 h, respectively) (Table 1).
Drug-drug interactions at the level of P-gp transport. Because the prevalence of OAB increases markedly with age (Stewart et al., 2003), patients treated with antimuscarinic drugs are likely to take several concomitant medications and thus it is important to consider the potential for drug-drug interactions in this situation. Most of the available antimuscarinic drugs such as oxybutynin, tolterodine, and darifenacin are extensively metabolized by the cytochrome P450 enzymes CYP3A4 and/or CYP2D6 (Michel and Hegde, 2006). In contrast, trospium chloride is not a substrate of the cytochrome P450 isoenzymes and is almost entirely excreted into the urine as the active drug (Schladitz-Keil et al., 1986; Singh-Franco et al., 2005). As apart from trospium chloride, many other drugs often used in elderly patients are substrates or inhibitors of P-gp, drug-drug interactions with trospium chloride could occur at the level of transport (Pal and Mitra, 2006). However, in recent clinical studies it has been shown that trospium chloride co-medication did not significantly alter the pharmacokinetics of the P-gp substrate digoxin, and interactions with other drugs have also not been identified in vivo (Sandage et al., 2006; Singh-Franco et al., 2005). Therefore, from our study of the available literature we postulate that drug-drug interactions with trospium chloride via P-gp transport probably are of minor clinical importance, if at all.

In conclusion. This study adds a new drug to the list of P-gp substrates investigated in the mdr1a,b<sup>−/−</sup> knockout mouse model. Our results have shown that the drug efflux transporter P-gp at the BBB highly restricts the entry of trospium chloride into the brain. This mechanism is likely to be at least part of the reason for the reduced CNS-side effect problems for trospium chloride during OAB treatment in humans.
References


Footnote

This study was kindly supported by Dr. R. Pfleger GmbH, Bamberg, Germany.
Legends for figures

**Figure 1:** Biliary excretion-time profiles (A) and cumulative biliary excretion (B) of [³H]trospium chloride given intravenously at a dose of 1.0 mg/kg b.w. to gall-bladder cannulated wild-type mice and mdr1a,b⁻/⁻ knockout mice. Within 120 min wild-type mice and mdr1a,b⁻/⁻ knockout mice excreted 202 ± 23 µl and 206 ± 36 µl of bile, respectively. Data represent means ± SD of three animals. * Significantly lower levels in mdr1a,b⁻/⁻ knockout mice compared with wild-type mice (p < 0.05).

**Figure 2:** Intestinal secretion (A) and brain penetration (B) of [³H]trospium chloride in wild-type FVB mice and mdr1a,b⁻/⁻ knockout mice. [³H]trospium chloride was applied at a dosage of 1.0 mg/kg b.w. either to gall-bladder cannulated mice (2 h, i.v.) or mice without bile fistula (12 h, i.v. and 12 h, p.o.). (A) The intestinal contents of the small intestine (S. intestine) and colon were recovered by three rinse cycles each with 2 ml PBS. (B) Whole brain samples were isolated and drug concentrations were determined by liquid scintillation counting. Data represent means ± SD of three to four animals per group. * Significantly different concentrations of mdr1a,b⁻/⁻ knockout mice compared with wild-type mice (p < 0.05).
Table 1. Concentrations of [3H]trospium chloride in tissues (ng/g) and plasma (ng/ml) of wild-type and mdr1a,b−/− knockout mice 2 h and 12 h after i.v. application of 1 mg/kg b.w.. Data are presented as means ± SD of n = 3 (2 h) or n = 4 (12 h) animals per group. Ratios are obtained by dividing the drug concentration in mdr1a,b−/− knockout mice by the concentration in wild-type mice (* p ≤ 0.05); n.d. = not detectable.

<table>
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<th>Tissue</th>
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<th>12 h, i.v., without bile fistula</th>
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<tbody>
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<td></td>
<td>Wild-type</td>
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</tr>
<tr>
<td>Brain</td>
<td>21±3</td>
<td>37±7</td>
</tr>
<tr>
<td>Liver</td>
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<td>3028±2054</td>
</tr>
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<td>3086±1001</td>
<td>2662±1236</td>
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