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Effects of Ketoconazole on the Biodistribution and Metabolism of [¹¹C]Loperamide and [¹¹C]*N*-Desmethyl-loperamide in Wild-type and P-gp Knockout Mice

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Abstract

Introduction—[¹¹C]Loperamide and [¹¹C]*N*-desmethyl-loperamide ([¹¹C]dLop) have been proposed as radiotracers for imaging brain P-glycoprotein (P-gp) function. A major route of [¹¹C]loperamide metabolism is *N*-demethylation to [¹¹C]dLop. We aimed to test whether inhibition of CYP3A4 with ketoconazole might reduce the metabolism of [¹¹C]loperamide and [¹¹C]dLop in mice, and thereby improve the quality of these radiotracers.

Methods—Studies were performed in wild-type and P-gp knockout (*mdr-1a/b* ^{-/-}) mice. During each of seven study sessions, one pair of mice, comprising one wild-type and one knockout mouse, was pretreated with ketoconazole (50 mg/kg, i.p.) while another such pair was left untreated. Mice were sacrificed at 30 min after injection of [¹¹C]loperamide or [¹¹C]dLop. Whole brain and plasma samples were measured for radioactivity and analyzed with radio-HPLC.

Results—Ketoconazole increased the plasma concentrations of [¹¹C]loperamide and its main radiometabolite, [¹¹C]dLop, by about two-fold in both wild-type and knockout mice, whereas the most polar radiometabolite was decreased three-fold. Furthermore, ketoconazole increased the brain concentrations of [¹¹C]loperamide and the radiometabolite [¹¹C]dLop by about two-fold in knockout mice, and decreased the brain concentrations of the major and most polar radiometabolite in wild-type and knockout mice by 82 and 49%, respectively. In contrast, ketoconazole had no effect on plasma and brain distribution of administered [¹¹C]dLop and its radiometabolites in either wild-type or knockout mice, except to increase the low plasma [¹¹C]dLop concentration. The least polar radiometabolite of [¹¹C]dLop was identified with LC-MSⁿ as the *N*-hydroxymethyl analog of [¹¹C]dLop and this also behaved as a P-gp substrate.

Conclusion—In this study, ketoconazole (50 mg/kg, i.p.) proved partially effective for inhibiting the *N*-demethylation of [¹¹C]loperamide in mouse *in vivo* but had relatively smaller or no effect on [¹¹C]dLop.

Keywords

[¹¹C]Loperamide; [¹¹C]dLop; P-glycoprotein; Ketoconazole; Biodistribution; Metabolism

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1. Introduction

The antidiarrheal drug, loperamide, is a potent opiate receptor agonist that acts on the gastrointestinal tract [1,2]. Loperamide lacks undesirable or dangerous central nervous system effects because it is excluded from brain by the efflux transporter, P-glycoprotein (P-gp) [3, 4]. Since loperamide is an avid substrate for P-gp, the radiolabeling of loperamide in its *N*-methyl position with carbon-11 ($\beta^+ = 100\%$; $t_{1/2} = 20.4$ min) gave a potential radiotracer for evaluating brain P-gp function in vivo with positron emission tomography (PET) [5–7]. However, a major route of metabolism for loperamide is demethylation to *N*-desmethyl-loperamide (dLop) [8,9]. Thus, [*N*-methyl- ^{11}C]loperamide ([^{11}C]loperamide; Fig. 1A) is metabolized to [*N*-methyl- ^{11}C]N-desmethyl-loperamide ([^{11}C]dLop; Fig. 1B) in mouse and monkey in vivo [7]. Metabolism also produces less lipophilic radiometabolites. These radiometabolites may be regarded as troublesome [10] in the sense that they may enter brain and severely hamper accurate quantification of P-gp function [7].

Cytochrome P450 (CYP) enzymes, especially CYP3A4, likely play a crucial role in the metabolism of loperamide to dLop [9]. Among more than fifty human cytochrome P450 enzymes, CYP3A4 is involved in the metabolism of the majority of clinically useful drugs [11]. In fact, CYP3A4 is the most highly expressed CYP enzyme in the liver and small intestine [11]. Inhibitors of CYP3A4, such as ketoconazole [9,12,13], ritonavir [14] and constituents of some fruit juices [15,16], can perturb the pharmacokinetics of drugs that are substrates of CYP3A4 [17,18]. For example, administration of ketoconazole with either of two benzodiazepine drugs, triazolam or alprazolam, prolongs their eliminations, increases areas-under-curves and decreases clearances [12]. Ketoconazole is also capable of 90% inhibition of the *N*-demethylation of loperamide by cDNA-expressed CYP3A4 in vitro [9]. Thus, we considered that inhibition of CYP3A4 with ketoconazole in vivo might improve the survival and effectiveness of [^{11}C]loperamide as a radiotracer of P-gp function. The first aim of this study was to test this hypothesis in mice in vivo. We were encouraged in this aim in that we had recently used a similar strategy to annul the undesirable radiodefluorination of the PET radioligand, [^{18}F]FCWAY, with selective inhibitors of CYP2E1 [19,20].

The primary radiometabolite of [^{11}C]loperamide (Fig. 1A), [^{11}C]dLop (Fig. 1B), has itself been found to be an avid substrate for P-gp [7] and to be a superior radiotracer of P-gp function in mouse and monkey [21]. [^{11}C]dLop is now being evaluated in human subjects [22]. In mouse and monkey [7], unknown radiometabolites of [^{11}C]dLop were discovered but were found to be much less troublesome to quantification of P-gp function than those of [^{11}C]loperamide. Nonetheless, we hypothesized that [^{11}C]dLop might also be metabolized by CYP3A4, with demethylation a strong possibility, since di-desmethyl-loperamide is produced as a metabolite of loperamide by rat and human liver microsomes in vitro [23]. In fact, many P-gp substrates are also substrates for CYP3A4 [24]. Therefore, we considered that the pharmacokinetics of [^{11}C]dLop might also be improved for imaging P-gp function by inhibition of CYP3A4 with ketoconazole. The second aim of this study was to test this hypothesis in mice in vivo.

2. Materials and methods

2.1. Materials

Solvents used for high-performance liquid chromatography (HPLC) and LC-MSⁿ analysis were from Sigma-Aldrich (St. Louis, MO). Reference di-desmethyl-loperamide and dLop were prepared as described previously [21]. Loperamide was obtained from Sigma-Aldrich (St. Louis, MO). Ketoconazole was also obtained from Sigma-Aldrich and prepared for i.p. administration by dissolution in ethanol and Tween 80 and then further dilution with saline.

2.2. Radiotracers

[¹¹C]Loperamide [25] and [¹¹C]dLop [21] were prepared as solutions in sterile saline (0.9% w/v) containing ethanol (0.5% w/v), as described previously [7,21]. The radiochemical purities of [¹¹C]loperamide and [¹¹C]dLop were $99.9 \pm 0.09\%$ ($n = 5$) and $98.6 \pm 0.64\%$ ($n = 4$), respectively, and specific activities at the times of injection into mice were 31 ± 14 GBq/ μ mol ($n = 5$) and 214 ± 154 GBq/ μ mol ($n = 4$), respectively.

2.3. Animals

All mice were used in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, U.S., 1996) with approval by the National Institute of Mental Health (NIMH) Animal Care and Use Committee [26]. All mice were purchased from Taconic (Germantown, NY). Thirteen knockout mice (29 ± 4.1 g; *mdr-1a/b* $-/-$; model 001487-MM, double homozygous; FVB) and thirteen wild-type mice (26 ± 4.3 g; *mdr-1a/b* $+/+$; model FVB) were used (Table 1). For all experiments, mice anesthesia was induced with 5% isoflurane and maintained throughout with 1–1.5% isoflurane administered via a nose cone. The doses of radiotracers plus doses of accompanying loperamide and dLop that were injected into mice are shown in Table 1.

2.4. Statistics

Group data are expressed as mean $\pm$ SD. Group data from mice were compared with independent *t*-tests, with significance evaluated at $p < 0.5$ in a two tailed test.

2.5. Ex vivo analyses of mouse plasma and brain

For each experimental session, two mice (1 wild-type and 1 knockout) were pretreated with ketoconazole (50 mg/kg, i.p.) while two control mice (1 wild-type and 1 knockout) were left untreated. Ketoconazole (50 mg/kg, i.p.) was injected about 30 min before the radiotracer in order to achieve maximal concentration of ketoconazole in brain and liver [27]. A blood sample (~ 1 mL) was removed from each mouse by cardiac puncture at 30 min after injection of radiotracer and just before mouse decapitation. Whole brains were immediately removed, weighed, and placed on ice before measurement of their radioactivity contents by γ -counting and processing for reversed phase radio-HPLC analysis. Plasma and whole brain samples were prepared and then analyzed as previously described [7,21,28]. Percentage recoveries of radioactivity were measured. The HPLC column (Novapak C18, 4 μ m, 100 mm $\times$ 8 mm; Waters Corp.) was housed in a radial compression module (RCM-100) and eluted at 2 mL/min with either methanol: water: triethylamine (75: 25: 0.1 by vol.) for analyses of [¹¹C]loperamide (t_R , 7.33 ± 0.51 min, $n = 15$, for brain samples and 7.52 ± 0.27 min, $n = 15$, for plasma samples) and its radiometabolites, or with methanol: water: triethylamine (70: 30: 0.1 by vol.) for analyses of [¹¹C]dLop (t_R , 7.53 ± 0.27 min, $n = 14$, for brain samples and 7.64 ± 0.15 min, $n = 14$, for plasma samples) and its radiometabolites. Complete recovery of radioactivity from the HPLC column was checked by showing that methanol eluted no further radioactivity from the column after a few injections of radioactive analytes. The concentrations of radioactivity in plasma and brain were normalized for injected radioactive dose and body weight by expression as percent standardized uptake value (%SUV), calculated as:

$$100 \times [(\text{activity per gram of tissue})/(\text{injected activity})] \times \text{gram of body weight}$$

2.6. Identification of radiometabolites by LC-MSⁿ

For these experiments, wild-type rather than knockout mice were selected for injection of carrier-added radiotracer to avoid the entry of high amounts of either loperamide or dLop into the animal brain. In P-gp knockout mice, the ingress of a potent opiate receptor antagonist,

such as loperamide or dLop, into brain would risk death of the animal through respiratory depression [29]. Wild-type mice were each injected with radiotracer, either [^{11}C]loperamide or [^{11}C]dLop, along with carrier loperamide or dLop (~ 1 mg), respectively. Thirty minutes after intravenous injection of radiotracer, blood was withdrawn by cardiac puncture. The plasma was harvested from whole blood by centrifugation, and subjected to extraction and radio-HPLC analysis as described above [28]. Radiochromatographic peaks for the most lipophilic radiometabolite of each radiotracer were collected separately and stored at $-70\text{ }^{\circ}\text{C}$ until LC-MSⁿ analysis. Compounds identified with LC-MSⁿ were then matched with the radiochromatographic peaks, specifically radiometabolite peak [^{11}C]D in plasma for [^{11}C]loperamide and radiometabolite peak [^{11}C]H in plasma for [^{11}C]dLop.

To identify radiometabolites, [^{11}C]D or [^{11}C]H, an LC-MSⁿ instrument (LCQ Deca; Thermo Fisher Scientific; Waltham, MA) was equipped with a Synergi Fusion-RP column ($4\text{ }\mu\text{m}$, $150 \times 2\text{ mm}$; Phenomenex, Torrance, CA) and equilibrated with an 80: 20 (v/v) mixture of water-methanol-acetic acid (90: 10: 0.5 by vol.) (A) and methanol-acetic acid (100: 0.5 v/v) (B) at $250\text{ }\mu\text{L}/\text{min}$ for 3 min and then run at $200\text{ }\mu\text{L}/\text{min}$. For analysis, the collected radiometabolite fraction was concentrated and reconstituted in aqueous acetonitrile (50% v/v; $200\text{ }\mu\text{L}$) and an aliquot ($10\text{ }\mu\text{L}$) injected onto the column. At 1 min after sample injection, the pump ran a linear gradient of mobile phase, reaching 20% A and 80% B over 8 min and then held this composition for 4 min. At 2.5 min after sample injection, the entire column output was directed to the electrospray (sheath gas flow rate 64; spray voltage 5 kV; capillary temperature $220\text{ }^{\circ}\text{C}$). In the MS mode, ions through m/z 150 to 600 were acquired. The $[\text{M} + \text{H}]^{+}$ for the putative metabolite was isolated from the total-ion chromatogram and subjected to MS-MS, and for metabolite H also to MS³ and MS⁴ (isolation width = 1.5 amu; collision energy = 29 to 33%), generating a product ion spectrum in each case.

2.7. Stability of [^{11}C]loperamide and [^{11}C]dLop in mouse brain homogenates in vitro

The stabilities of [^{11}C]loperamide and [^{11}C]dLop in mouse brain homogenates were measured in vitro. Brains ($0.41 \pm 0.03\text{ g}$) were obtained from three wild-type mice ($32 \pm 2.2\text{ g}$) and immediately placed over ice. Each brain was homogenized in cold 0.9% NaCl (saline; 4 mL). [^{11}C]Loperamide (1 mCi) or [^{11}C]dLop (0.75 mCi) was added to each homogenate and then placed in an oscillating water bath at $37\text{ }^{\circ}\text{C}$. Homogenates for both radiotracers were sampled after 60 and 120 min of incubation. Aliquots of homogenates ($50\text{ }\mu\text{L}$) were added to acetonitrile and analyzed with radio-HPLC, as described earlier.

3. Results

3.1. Effect of ketoconazole on [^{11}C]loperamide and radiometabolite concentrations in plasma

For the analysis of radiometabolites in plasma from all mice ($n = 15$), the recoveries of radioactivity into acetonitrile from deproteinized samples were consistently high ($95.5 \pm 2.0\%$).

At 30 min after radiotracer injection under baseline conditions of no ketoconazole pretreatment, the plasma concentrations of [^{11}C]loperamide were very low in both wild-type ($10.3 \pm 2.1\%$ SUV) and P-gp knockout ($7.37 \pm 4.55\%$ SUV) mice and insignificantly different ($p = 0.30$) (Fig. 2A). Four radiometabolites were detected in plasma, each with lower lipophilicity than parent [^{11}C]loperamide (t_R , $7.52 \pm 0.14\text{ min}$), as judged by their shorter HPLC retention times. Radiometabolite retention times were similar between wild-type (1.96 ± 0.13 , 2.86 ± 0.10 , 4.09 ± 0.78 and $5.49 \pm 0.33\text{ min}$) and knockout mice (1.91 ± 0.15 , 2.64 ± 0.28 , 3.93 ± 0.87 and $5.47 \pm 0.13\text{ min}$). Therefore, radiometabolites in wild-type and knockout mice plasma were considered to have the same identities, named [^{11}C]A–[^{11}C]D, respectively. [^{11}C]D was later identified as [^{11}C]dLop (see below). Radiometabolites were at similarly low concentrations in

each of the two groups of mice. [^{11}C]A was the most prevalent radioactive species in plasma (Fig. 2A).

Under ketoconazole pretreatment conditions, the plasma of wild-type and P-gp knockout mice also contained [^{11}C]A–[^{11}C]D, plus [^{11}C]loperamide. Pretreatment caused no significant differences in the concentrations of radioactive species between wild-type and knockout mice. However, in both groups of mice, pretreatment caused major changes to the concentrations of radioactive species relative to those seen under baseline conditions. Thus, the plasma concentrations of [^{11}C]loperamide were increased about two-fold in both ketoconazole-treated wild-type and knockout mice to $17.6 \pm 5.9\%$ SUV and $17.1 \pm 9.0\%$ SUV, respectively (Fig. 2B). The increase became statistically significant when wild-type and knockout mice data were grouped ($p = 0.01$). Ketoconazole pretreatment also significantly increased the concentration of the radiometabolite, [^{11}C]D ([^{11}C]dLop), in both wild-type ($p = 0.05$) and knockout mice ($p = 0.01$), again by about two-fold. Moreover, ketoconazole pretreatment significantly decreased the high plasma concentrations of the unknown most polar radiometabolite ([^{11}C]A) by about three-fold in both wild-type ($p = 0.001$) and knockout ($p = 0.02$) mice (Fig. 2B).

3.2. Effect of ketoconazole on [^{11}C]loperamide and radiometabolite concentrations in brain

For the analysis of radiometabolites from the brains of all mice ($n = 15$, the recoveries of radioactivity into acetonitrile from deproteinized samples were consistently high $90.3 \pm 3.3\%$).

Under baseline conditions, at 30 min after [^{11}C]loperamide injection, the brain concentration of [^{11}C]loperamide was about 7-fold higher in P-gp knockout mice ($20.4 \pm 3.2\%$ SUV) than in wild-type mice ($2.86 \pm 0.97\%$ SUV) ($p = 0.0001$) (Fig. 3A). Mice brains contained four radiometabolites. In knockout mice, the retention times for these radiometabolites (1.98 ± 0.15 , 3.63 ± 0.12 , 4.21 ± 0.24 and 5.27 ± 0.86 min) were not appreciably different from those seen in plasma. Radiometabolites in brain were dominated by [^{11}C]D ([^{11}C]dLop) and [^{11}C]A. The same four radiometabolites were seen in brains of wild-type mice. Radiometabolite concentrations were generally greater in knockout mice than in wild-type mice, but were only significantly so for the two most lipophilic radiometabolites, [^{11}C]C ($p = 0.006$) and [^{11}C]D ([^{11}C]dLop) ($p = 0.003$) (Fig. 3A).

Ketoconazole pretreatment increased the brain concentrations of [^{11}C]loperamide about two-fold in P-gp knockout mice to $43.9 \pm 14.0\%$ SUV ($p = 0.04$), but had no effect on its very low brain concentration ($2.9 \pm 0.7\%$ SUV) in wild-type mice ($p = 0.98$) (c.f., Fig. 3B with Fig. 3A). There was no effect of ketoconazole pretreatment on the identities of radiometabolites seen in brain, as evidenced by HPLC retention times. Ketoconazole pretreatment significantly decreased the already low concentrations of the major and least lipophilic radiometabolite ([^{11}C]A) in the brains of wild-type mice by 82% ($p = 0.001$) and in knockout mice by 49% ($p = 0.08$) (c.f., Fig. 3B with Fig. 3A). Furthermore, ketoconazole pretreatment did not increase radiometabolite [^{11}C]D ([^{11}C]dLop) in the brains of wild-type mice ($p = 0.94$), but increased this radiometabolite two-fold in the brains of knockout mice ($p = 0.20$) (Fig. 3B).

Although ketoconazole increased the plasma concentrations of [^{11}C]loperamide in the wild-type mice, the ratio of the brain concentration of [^{11}C]loperamide to that in plasma under baseline conditions (0.30 ± 0.10) was similar to that in the ketoconazole-treated mice (0.20 ± 0.04) ($p = 0.005$).

3.3. LC-MS/MS identification of [^{11}C]loperamide metabolite, [^{11}C]D

LC-MS analysis of the carrier associated with radiometabolite [^{11}C]D showed a peak for m/z 463 ion at $t_R = 7.60$ min, consistent with formation of this metabolite by demethylation of

loperamide. MS/MS of this ion showed major product ions, m/z 445 and 252 (Fig. 4). LC-MS and LC-MS/MS analysis of reference dLop gave the same results as [^{11}C]D.

3.4. Effect of ketoconazole on [^{11}C]dLop and radiometabolite concentrations in plasma

For the analysis of radiometabolites from the plasma of all mice ($n = 11$), the recoveries of radioactivity into acetonitrile from deproteinized samples were consistently high ($95.2 \pm 2.5\%$).

Under baseline conditions at 30 min after the administration of [^{11}C]dLop, the plasma concentration of [^{11}C]dLop was very low in all mice, but about 1.5-fold higher in knockout mice ($2.26 \pm 0.54\%$ SUV) than in wild-type mice ($1.36 \pm 0.18\%$ SUV) ($p = 0.05$) (Fig. 5A). Four radiometabolites ([^{11}C]E–[^{11}C]H) were detected in plasma. Respective retention times for [^{11}C]E–[^{11}C]H did not differ significantly between wild-type (1.91 ± 0.05 , 2.80 ± 0.14 , 4.98 ± 0.73 , 6.54 ± 0.11 min) and knockout mice (1.90 ± 0.03 , 2.82 ± 0.13 , 4.37 ± 0.73 and 6.56 ± 0.22 min). These radiometabolites accounted for the vast majority of plasma radioactivity and they had similar concentrations in both wild-type and knockout mice (Fig. 5A). The two less lipophilic radiometabolites, [^{11}C]E and [^{11}C]F, were present in much greater concentrations than [^{11}C]G and [^{11}C]H in all mice (Fig. 5A).

The plasma of ketoconazole-treated wild-type and P-gp knockout mice contained [^{11}C]dLop and the same four radiometabolites as untreated mice. Ketoconazole pretreatment increased the plasma concentration of [^{11}C]dLop in both wild type and knockout mice by an average of two-fold to $3.3 \pm 0.4\%$ SUV ($p = 0.003$) and $3.4 \pm 0.4\%$ SUV ($p = 0.04$), respectively. The concentrations of the four radiometabolites were not statistically different from those in the respective untreated mice groups (c.f., Fig. 5B with Fig. 5A).

3.5. Effect of ketoconazole on [^{11}C]dLop and radiometabolite concentrations in brain

For the analysis of radiometabolites from the brains of all mice ($n = 11$), the recoveries of radioactivity into acetonitrile from deproteinized samples were consistently high ($93.9 \pm 2.1\%$).

Under baseline conditions, at 30 min after the administration of [^{11}C]dLop, the brain concentration of [^{11}C]dLop was about 19-fold higher in knockout mice ($37.1 \pm 7.3\%$ SUV) than in wild-type mice ($1.93 \pm 0.52\%$ SUV) ($p = 0.001$) (Fig. 6A). [^{11}C]dLop was by far the most prevalent radioactive species in knockout mice brain. Mouse brains contained four radiometabolites, all of which appeared to have lower lipophilicity than [^{11}C]dLop ($t_R = 7.53 \pm 0.27$ min). The retention times for the radiometabolites from knockout mouse brains (1.94 ± 0.10 , 3.07 ± 0.37 , 4.06 ± 0.29 and 6.55 ± 0.07 min, respectively) were not significantly different from those in plasma. Thus, these radiometabolites, [^{11}C]E–[^{11}C]H, occurred in both strains of mice and in both brain and plasma. The brain concentrations of the two most polar radiometabolites ([^{11}C]E and [^{11}C]F) were similar in the knockout and wild-type mice (Fig. 6A). However, the concentrations of the two least polar radiometabolites, [^{11}C]G ($p = 0.02$) and [^{11}C]H ($p = 0.003$), were significantly greater in the knockout mice than in the wild-type mice, indicating that these radiometabolites are substrates for P-gp.

Pretreatment of mice with ketoconazole had little effect on the brain concentrations of [^{11}C]dLop in either knockout ($37.9 \pm 6.7\%$ SUV) or wild-type mice ($2.2 \pm 0.5\%$ SUV) (c.f., Fig. 6B with Fig. 6A). Pretreatment with ketoconazole decreased the most polar radiometabolite, [^{11}C]E, in the brains of both wild-type and knockout mice by about 75 and 45%, respectively (Fig. 6B). The decrease was statistically insignificant.

Although ketoconazole increased plasma concentrations of [^{11}C]dLop in the wild-type mice, the ratio of the brain concentration of [^{11}C]dLop to that in plasma under ketoconazole-treated conditions (0.7 ± 0.1) was similar to that under baseline conditions (1.4 ± 0.3) ($p = 0.005$).

3.6. LC-MSⁿ identification of [¹¹C]dLop metabolite, [¹¹C]H

LC-MS of the carrier associated with the radiometabolite [¹¹C]H identified a specific ion m/z 479 eluting at $t_R = 7.3$ min in the total-ion chromatogram. The mass 479 amu would correspond to the $[M+H]^+$ ion for an hydroxylated derivative of dLop. MS/MS, MS³ and MS⁴ analyses were performed on metabolite H to discover the position of the hydroxyl group. Dissociation of m/z 479 ion generated product ions m/z 461, 449, 432, 268 and 238. These fragment ions are explained if metabolite H is formed by hydroxylation of the *N*-methyl group of dLop, giving the structure shown in Fig. 7A. The dominant ion, m/z 449, can only be formed by the neutral loss of HCHO from the proposed structure. To rule out the presence of a hydroxyl group on a phenyl ring, m/z 449 ion was further dissociated (MS³ experiment). The secondary product ions were m/z 431 and 238, in accord with the proposed *N*-hydroxymethyl structure. Another dissociation (MS⁴), involving m/z 238 ion, resulted in product ions m/z 210, 195, 182, 117 and 91. Thus, both MS³ and MS⁴ experiments verified that the hydroxyl group of metabolite H is not on an aryl group.

MS/MS and MS³ analyses of the authentic primary amide, di-*N*-desmethyl-loperamide, were further consistent with this conclusion (Fig. 7B). Dissociation of its $[M+H]^+$ ion (m/z 449) generated the same product ions m/z 431 $[M+H-H_2O]^+$ and 238 $[Ph_2C(C_2H_4)CONH_2]^+$ as the dissociation of the product m/z 449 ion of metabolite H. MS³ analysis of the reference primary amide, involving dissociation of m/z 238, also generated a product ion spectrum matching that obtained by MS⁴ analysis of metabolite H. Therefore, [¹¹C]H is the *N*-hydroxymethyl analog of [¹¹C]dLop ([¹¹C]*N*-OH-dLop).

3.7. Stability of [¹¹C]loperamide and [¹¹C]dLop in mouse brain in vitro

The radiochemical purities of [¹¹C]loperamide (99.6%) and of [¹¹C]dLop (99.1%) were virtually unchanged during incubation with brain homogenate of wild-type mice for up to 120 min at 37°C.

4. Discussion

In this study, the main findings were that pre-administration of mice with the potent CYP3A4 inhibitor, ketoconazole at 50 mg/kg, i.p. successfully increased the plasma and brain concentration of [¹¹C]loperamide but not the brain concentration of independently administered [¹¹C]dLop. The ketoconazole pretreatment did not inhibit P-gp.

For PET brain imaging radiotracers, significant brain ingress of radiometabolites may hamper quantification of outcome measures [10,29,30]. As confirmed here, demethylation of [¹¹C]loperamide in mouse gives [¹¹C]dLop as a major radiometabolite [7]. CYP3A4 was found to be the major enzyme responsible for loperamide demethylation in vitro [9]. We considered that [¹¹C]dLop may also be a substrate for demethylation by CYP3A4, since loperamide has been shown to produce di-desmethyl-loperamide in rat [8]. Ketoconazole is a potent (K_i , 0.02–0.5 μ M; [31–33]) and selective CYP3A4 inhibitor [32] that markedly decreases the metabolism and increases the plasma concentrations of some drugs prone to demethylation [34,35]. Therefore, with regard to our aim of improving the imaging qualities of [¹¹C]loperamide and [¹¹C]dLop, we chose to test ketoconazole for its ability to reduce the metabolism of these radiotracers in mice. Ketoconazole is also a weak inhibitor (K_i , 3–25 μ M) of the efflux transporter, P-gp [31,33]. We chose a ketoconazole dose of 50 mg/kg i.p., since this dose was expected to be selective for CYP3A4 versus P-gp inhibition in vivo [31,33,36]. Also, this study included both wild-type and P-gp knockout mice so that any effects of enzyme or P-gp inhibition might be disentangled (Table 1). Radiotracers were administered at no-carrier-added specific radioactivities, corresponding to injected carrier masses of 24.3–29.3 nmol/kg for [¹¹C]loperamide and 2.91–13.6 nmol/kg for [¹¹C]dLop (Table 1). These doses were expected

to be well below those required to cause any significant degree of saturation of P-gp or metabolic enzymes; their less than ten-fold variation was not therefore expected to result in any significant differences in metabolism or biodistribution.

Experiments were commenced with [^{11}C]loperamide as radiotracer in wild-type and knockout mice, with and without pretreatment with ketoconazole. Ex vivo radio-HPLC analyses of plasma and brain were performed at 30 min after radiotracer administration. This interval was chosen because intravenously administered [^{11}C]loperamide is extensively metabolized after 30 min in mice, while the level of decay-corrected radioactivity in brain becomes quite stable [7]. In untreated mice, four less lipophilic radiometabolites ([^{11}C]A–[^{11}C]D) emerged in plasma of which the two least lipophilic ([^{11}C]A and [^{11}C]B) were most prevalent (Fig. 2A). Radioactivity composition in plasma was virtually the same in knockout and wild type mice. However, pretreatment with ketoconazole dramatically altered the distribution of radioactivity in all mice, significantly decreasing the concentrations of [^{11}C]A and [^{11}C]B, while greatly enhancing the concentrations of [^{11}C]loperamide and [^{11}C]D (Fig. 2B). The identity of [^{11}C]D as [^{11}C]dLop was confirmed with LC-MSⁿ; the fragmentation pathway proposed in Fig. 4 accounts for the generation of the observed ions.

Clearly, ketoconazole, was ineffective in the desired aim of completely blocking [^{11}C]loperamide demethylation in vivo. The ineffectiveness of ketoconazole to block demethylation might have been due to any combination of different factors. Firstly, the dose of ketoconazole was set at a level to avoid P-gp inhibition, but this dose may have been too low to inhibit CYP3A4 fully. Secondly, other enzymes may have performed the demethylation reaction. In vitro studies indicate this possibility. Thus, both CYP3A4 and CYP2C8 played major roles in loperamide demethylation in human liver microsomes in vitro, and CYP2B6 and CYP2D6 minor roles [9,23]. Ketoconazole is a relatively weak inhibitor of CYP2C8, CYP2B6 and CYP2D6 [32], and hence these or other enzymes may have caused the demethylation seen in this study. In this regard, a combination of the CYP3A4 inhibitor, itraconazole, and the CYP2C8 inhibitor, gemfibrozil, was more effective in reducing the demethylation of loperamide in human subjects in vivo than either inhibitor when used alone [37]. Thirdly, there may be species differences between the distributions and activities of mouse and human CYP enzymes [38,39]. Briefly, humans express four CYP3A enzymes in which CYP3A4 and –3A5 are the most abundant in liver and are the primary enzymes involved in the metabolism of a majority of drugs [39]. By contrast, mice express eight CYP3A genes in which CYP3A11 has ~76% amino acid homology to human CYP3A4 [40,41]. Future studies employing humanized mouse models of CYP3A4 enzymes might more accurately simulate human drug metabolism [38,42].

Why does ketoconazole enhance the plasma concentrations of [^{11}C]loperamide and its desmethyl radiometabolite while suppressing the concentrations of more polar radiometabolites? A possibility is that ketoconazole may also have reduced the metabolism of [^{11}C]loperamide through other pathways. Other known but relatively minor pathways of loperamide metabolism include ring hydroxylation, *N*-oxidation, and aromatization of the piperidine ring [23]. CYP3A4 mediates all these pathways in human liver microsomes in vitro.

Analyses of the brains from mice given [^{11}C]loperamide revealed the same four radiometabolites seen in plasma (Fig. 3A). These radiometabolites originate from the periphery, since [^{11}C]loperamide was found to be stable to brain homogenates in vitro. Brains from wild-type mice had low radioactivity content with the most polar radiometabolite, [^{11}C]A, being the most abundant. Brains from knockout mice had much higher radioactivity with unchanged [^{11}C]loperamide being most abundant. The concentration of [^{11}C]D ([^{11}C]dLop) was also substantially increased in the brains of knockout mice. The ratios of the brain concentrations of [^{11}C]loperamide and the radiometabolite [^{11}C]D ([^{11}C]dLop) to their

respective concentrations in plasma were not increased but slightly decreased by ketoconazole pretreatment in knockout mice. This shows that ketoconazole was not inhibiting P-gp, but that it was enhancing radiotracer uptake into brain through increasing the plasma concentration [43]. The concentration of [^{11}C]C was also significantly greater in the knockout mice, so indicating that [^{11}C]C is also a P-gp substrate.

The same set of experiments were performed with [^{11}C]dLop as radiotracer. In all mice, four less lipophilic radiometabolites, ([^{11}C]E–[^{11}C]H), emerged in plasma, with [^{11}C]E and [^{11}C]F most abundant (Fig. 5). Ketoconazole pretreatment slightly increased the low concentrations of [^{11}C]dLop in all mice plasma, suggesting that ketoconazole decreased the extent of [^{11}C]dLop metabolism. However, plasma concentrations of radiometabolites did not vary significantly between wild-type and knockout mice.

Brains from untreated wild-type mice administered [^{11}C]dLop contained low radioactivity. The most abundant radioactive species was [^{11}C]dLop itself (Fig. 6A). Four radiometabolites were detected, corresponding to those in plasma. Indeed, the brain radiometabolites originated from the periphery, since [^{11}C]dLop was stable to incubation with mouse brain homogenates in vitro. Ketoconazole pretreatment had no effect on the level or distribution of brain radioactivity in wild-type mice (Fig. 6B). Brains from untreated knockout mice showed the expected increase in level of radioactivity [21], primarily due to a much higher level of [^{11}C]dLop (Fig. 6A). The brain concentration of radiometabolite, [^{11}C]H, was 22-fold higher in knockout mice than in wild-type mice, showing it to be a P-gp substrate. [^{11}C]G may also be a P-gp substrate since even its very low brain concentration in knockout mice was nonetheless significantly greater than in wild type mice. The two more polar radiometabolites of [^{11}C]dLop, namely [^{11}C]E and [^{11}C]F, are not P-gp substrates, since they appear in wild type and knockout brains at similar levels.

Brains from ketoconazole-treated wild type and knockout mice had similar levels of the same radioactive species as their untreated counterparts. The level of [^{11}C]dLop in plasma of ketoconazole-treated mice was very low but apparently elevated compared to that in untreated mice. However, this increase but was not reflected in a corresponding increase in the brain concentration of [^{11}C]dLop in either wild type or knockout mice. This lack of effect of ketoconazole on [^{11}C]dLop accumulation in brain contrasts starkly with that seen for [^{11}C]loperamide. However, the apparently statistically significant difference in [^{11}C]dLop in plasma between non-treated and treated mice may have been false due to small sample sizes and potentially greater errors in measurements of low radioactivity levels.

The least polar radiometabolite of [^{11}C]dLop ([^{11}C]H) was identified as the *N*-hydroxymethyl analog ([^{11}C]OH-dLop) (Fig. 7A). Interestingly, CYP3A4 converted looperamide into its *N*-hydroxymethyl analog in human and rat liver microsomes in vitro [23]. *N*-Hydroxymethyl derivatives occur along the CYP-mediated oxidative demethylation pathways of both aromatic [44–46] and aliphatic amides [45,47]. These derivatives are often unstable and show a general propensity to cleave to the next lower order amide and formaldehyde [44,46]. The *N*-hydroxymethyl analogs of tertiary amides are generally considered to be less stable than their secondary amide counterparts. This may explain the successful detection of [^{11}C]OH-dLop but not [^{11}C]OH-loperamide in this study.

In summary, ketoconazole did not inhibit P-gp at the mouse blood-brain barrier, and, in P-gp knockout mice, ketoconazole was partially effective in reducing the metabolism of [^{11}C]loperamide, resulting in over two-fold higher plasma and brain concentrations (Fig. 8A). However, ketoconazole had no appreciable effect on the brain accumulation of [^{11}C]dLop (Fig. 8B). At the administered dose, ketoconazole was ineffective in completely inhibiting [^{11}C]loperamide demethylation in vivo. In view of the inability of ketoconazole to completely block

the demethylation of [^{11}C]loperamide to [^{11}C]dLop, [^{11}C]dLop remains the superior radiotracer, because of its lower degree of conversion into troublesome radiometabolites [7, 21,22]. The most prevalent radiometabolite of [^{11}C]dLop in blood was identified as [^{11}C]OH-dLop. This radiometabolite also behaved as a substrate for P-gp.

5. Conclusions

Here, we conclude that the metabolism of [^{11}C]loperamide in mice in vivo is appreciably reduced by pre-administration of ketoconazole, resulting in increased plasma and brain concentrations of unchanged [^{11}C]loperamide in P-gp knockout mice. In contrast, pre-administration of ketoconazole has little effect on the metabolism and biodistribution of [^{11}C]dLop. An unexpected radiometabolite of [^{11}C]dLop is the *N*-hydroxymethyl analog ([^{11}C]OH-dLop), which also has potential for development as a PET radiotracer of P-gp function. Furthermore, this study indicates that the *N*-demethylation of *N*-[^{11}C]methylamido-type PET radiotracers in vivo may occur through the action of multiple enzymes, that may be difficult to inhibit completely with a single pharmacological agent.

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Fig. 1.
Chemical structures of [¹¹C]loperamide (Panel A) and [¹¹C]dLop (Panel B).

Fig. 2. Plasma concentrations of $[^{11}\text{C}]\text{loperamide}$ and its radiometabolites in wild-type and P-gp knockout mice. Radioactivity was extracted from the plasma of mice with no treatment (Panel A) and after treatment with ketoconazole at 30 min before radiotracer injection (Panel B). Four radiometabolites were detected in plasma ($[^{11}\text{C}]\text{A}$ – $[^{11}\text{C}]\text{D}$ in order of apparently increasing lipophilicity by reversed phase HPLC). Bars represent mean $\pm$ SD. $[^{11}\text{C}]\text{D}$ was later identified as $[^{11}\text{C}]\text{dLop}$.

Fig. 3. Brain concentrations of $[^{11}\text{C}]\text{loperamide}$ and its radiometabolites in wild-type and P-gp knockout mice. Radioactivity was extracted from brains of mice with no treatment (Panel A) and after treatment with ketoconazole at 30 min before radiotracer injection (Panel B). Four radiometabolites were detected in mouse brains ($[^{11}\text{C}]\text{A}$ – $[^{11}\text{C}]\text{D}$ ($[^{11}\text{C}]\text{dLop}$), in order of apparently increasing lipophilicity by reversed phase HPLC). Bars represent mean $\pm$ SD.

Fig. 4.

Proposed fragmentation pathway for ions formed by CID of $[M+H]^+$ m/z 463 of loperamide metabolite D. The fragmentation accounts for the major ions in the product-ion spectrum of loperamide.

Fig. 5. Plasma concentrations of [¹¹C]dLop and its radiometabolites in wild-type and P-gp knockout mice. Radioactivity was extracted from the plasma of mice with no treatment (Panel A) and after treatment with ketoconazole preceding radiotracer administration (Panel B). Four radiometabolites were detected in mouse plasma ([¹¹C]E–[¹¹C]H, in order of increasing lipophilicity). Bars represent mean ± SD. [¹¹C]H was later identified as [¹¹C]N-OH-dLop.

Fig. 6. Brain concentrations of $[^{11}\text{C}]\text{dLop}$ and its radiometabolites in wild-type and P-gp knockout mice. Radioactivity was extracted from brains of mice with no pretreatment (Panel A) and after treatment with ketoconazole at 30 min before radiotracer injection (Panel B). Four radiometabolites were detected ($[^{11}\text{C}]\text{E}$ – $[^{11}\text{C}]\text{H}$). Bars represent mean $\pm$ SD.

Fig. 7.

Panel A: Proposed fragmentation pathway for ions formed by CID of $[M+H]^+$ m/z 479 of dLop metabolite H. The fragmentation accounts for the major ions in the product-ion spectrum of dLop. Panel B: Proposed fragmentation pathway for ions formed by CID of $[M+H]^+$ m/z 449 of metabolite *di*-desmethyl-loperamide.

Fig. 8. Plasma and brain concentrations of [¹¹C]loperamide and parent [¹¹C]dLop at baseline and after pre-administration of ketoconazole in P-gp knockout mice. The metabolism of [¹¹C]loperamide was reduced by pre-administration of ketoconazole resulting in increased plasma and brain concentrations of parent radiotracer (Panel A), whereas pre-administration of ketoconazole caused little change to the metabolism and distribution of administered [¹¹C]dLop (Panel B).

Table 1

Summary of mice and radiotracers used in this study.

Radioligand	Mouse type	Treatment	n	Weight (g)	Administered doses	
					Radioactivity (MBq)	Mass (nmol/kg)
^{[11]C} Loperamide	WT	Control	4	28.5 ± 2.7	20.8 ± 2.5	23.9 ± 4.7
		Ketoconazole	4	25.8 ± 7.3	18.5 ± 3.0	29.3 ± 15.4
	P-gp KO	Control	3	26.3 ± 0.7	18.2 ± 2.3	27.9 ± 4.1
		Ketoconazole	4	30.3 ± 3.2	19.2 ± 5.5	24.3 ± 12.8
^{[11]C} dLop	WT	Control	3	26.2 ± 2.1	25.1 ± 2.1	6.4 ± 4.3
		Ketoconazole	2	24.0; 24.2	27.2; 26.8	2.91; 13.6
	P-gp KO	Control	3	29.4 ± 5.6	28.3 ± 1.9	6.0 ± 2.9
		Ketoconazole	3	29.3 ± 6.3	25.7 ± 3.2	7.2 ± 4.3