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RESEARCH ARTICLE

In vitro evaluations for pharmacokinetic drug-drug interactions of a novel serotonin-dopamine activity modulator, brexpiprazole

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ABSTRACT

1. Brexpiprazole, a serotonin-dopamine activity modulator, is indicated for the treatment of schizophrenia and also adjunctive therapy to antidepressants for the treatment of Major Depressive Disorder. To determine the drug–drug interaction risk for cytochrome P450, and SLC and ABC transporters, brexpiprazole and its metabolite, DM-3411 were assessed in this *in vitro* investigation.
2. Brexpiprazole exhibited weak inhibitory effects (IC_{50} >13 μmol/L) on CYP2C9, CYP2C19, CYP2D6 and CYP3A4 activities, but had moderate inhibitor activity on CYP2B6 (IC_{50} 8.19 μmol/L). The ratio of systemic unbound concentration (3.8 nmol/L) to the K_i value was sufficiently low. DM-3411 had comparable inhibitory potentials with brexpiprazole only for CYP2D6 and CYP3A4. The mRNA expressions of CYP1A2, CYP2B6 and CYP3A4 were not changed by the exposure of brexpiprazole to human hepatocytes.
3. Brexpiprazole and DM-3411 exhibited weak or no inhibitory effects for hepatic and renal transporters (OATPs, OATs, OCTs, MATE1, and BSEP), except for MATE-2K (0.156 μmol/L of DM-3411), even for which the ratio to systemic unbound concentration (5.3 nmol/L) was sufficiently low.
4. Brexpiprazole effected the functions of P-gp and BCRP with IC_{50} values of 6.31 and 1.16 μmol/L, respectively, however, the pharmacokinetic alteration was not observed in the clinical concomitant study on P-gp and BCRP substrates.
5. These *in vitro* data suggest that brexpiprazole is unlikely to cause clinically relevant drug interactions resulting from the effects on CYPs or transporters mediating the absorption, metabolism, and/or disposition of co-administered drugs.

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Brexpiprazole; OPC-34712; REXULTI; CYPs; ABC transporter; SLC transporter; drug–drug interaction

Introduction

Brexpiprazole (REXULTI®; OPC-34712) is a serotonin–dopamine activity modulator that acts as a partial agonist at serotonin 5-HT_{1A} and dopamine D_2 receptors and a potent antagonist at serotonin 5-HT_{2A} receptors and noradrenaline α_{1B}/α_{2C} receptors (Maeda *et al.* 2014a, 2014b). It has some indications for the treatment of schizophrenia and major depressive disorder (MDD) as an adjunctive therapy to antidepressants in the United States, Canada, South America, and some of the Asian countries. It has also been approved for schizophrenia in Australia, as well as several European, and Asian countries including Japan.

In clinical setting, antipsychotic drugs are often administered concomitantly with other central nervous system (CNS) drugs to improve the overall treatment response. In patients with MDD who had experienced an inadequate response to antidepressant treatment, the add-on application of brexpiprazole has shown a beneficial therapeutic response. However, any adverse reactions rather than expected effects are occasionally induced when 2 or more medicines are administered together in patients. Paroxetine and fluoxetine are well-known antidepressant agents on the market which are CYP2D6 inhibitors (Richelson 1997). Since brexpiprazole is mainly metabolised by CYP2D6 and CYP3A4 (Sasabe *et al.* 2021), any combined application with antidepressant agents such as these and strong CYP3A4 inhibitors must be performed carefully. In general, most antidepressant and antipsychotic agents are metabolised by several cytochrome P450s (Richelson 1997), so this poses significant risk for drug interactions as the treatment of depression requires long-term management sometimes with different medication in order to effectively manage and optimise a patient's clinical response (Hicks *et al.* 2015, Hoffelt and Gross 2016). In such situation of managed treatment with medication, especially, the introduction of new agents to the area of psychotropic drug treatment can generate unexpected drug – drug interactions

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Supplemental data for this article can be accessed here.

(DDIs). As described above, DDIs can involve multiple mechanisms and the effects on the metabolic pathways (e.g. effects on CYP enzymes) are common sources of clinically significant adverse events. Thereby, in the present study, we investigated the potential metabolic effects of brexpiprazole as an inhibitor and inducer of various cytochrome P450 isozymes through a series of *in vitro* tests using human liver microsomes and hepatocytes, as well as human CYP-recombinant microsomes.

In addition to CYPs regulating the metabolism, multitude drug efflux transporters and uptake transporters regulate the absorption, distribution, and excretion of drugs in the intestinal epithelium, liver, kidney, vascular endothelium, and brain in which they are expressed (Giacomini *et al.* 2010). ABC efflux transporters, P-glycoprotein (MDR1/ABCB1, P-gp), and breast cancer resistance protein (BCRP/ABCG2) limit drug absorption through the intestinal epithelium and distribution through the blood–brain barrier into the brain. Moreover, a bile salt export pump (BSEP/ABCB11) can accelerate the biliary excretion of bile salts from the inside of hepatocytes. SLC transporters, organic anion transporting polypeptides (OATPs), organic anion transporters (OATs), and organic cation transporters (OCTs) function in the uptake of organic anionic and cationic compounds into hepatocytes and kidney cells on the plasma membrane. Multidrug and toxin extrusion proteins (MATEs) may accelerate urinary and biliary excretions of organic cationic compounds in the apical membrane side of hepatocytes and kidney cells, respectively. These transporters are major determinants not only of the dispositional fate of drugs, but also their organ/total clearances. Therefore, these transporter-mediated functions can result in DDIs and can be common sources of significant adverse events, similar to the effects of CYPs (König *et al.* 2013, Yu *et al.* 2018). For reference, these transporters are defined in the DDI guidelines of Food and Drug Administration (U.S. Department of Health and Human Services 2020), European Medicines Agency (EMA 2012), and Pharmaceuticals and Medical Devices Agency (PMDA 2019) as important drug transporters to evaluate whether an investigational drug is a potent substrate or inhibitor. Therefore, we conducted a series of *in vitro* tests evaluating the effect of brexpiprazole on the transport function of a number of substrates using the cell culture systems and membrane vesicles expressing transporters.

The pharmacokinetics of brexpiprazole were investigated in healthy volunteers and patients and were reported (Garnock-Jones 2016, Ishigooka *et al.* 2018), and and at the steady state during multiple once-daily oral dosing of brexpiprazole in Japanese patients with schizophrenia, its main metabolite, DM-3411 (Figure 1), represents 45%–70% of brexpiprazole exposure in plasma (Ishigooka *et al.* 2018). The potential inhibitory effects of the metabolite on the common drug metabolising enzymes and transporters should be considered. In addition, if the main metabolite is active and significantly contributes to pharmacological effects *in vivo*, altered exposure to this metabolite should be considered in terms of DDI risk. DM-3411 has substantially less potency in terms of pharmacologic activity compared with brexpiprazole, and does not appear to penetrate the brain (Sasabe *et al.* 2021). Consequently, it was concluded that DM-3411 does not to contribute to the therapeutic effects of brexpiprazole. Therefore, *in vitro* inhibition studies of DM-3411 were performed on the activities of CYPs and transporters alone, and substrate studies of DM-3411 were not considered necessary to evaluate DDI risk, taking into account the evaluation of the contribution of the metabolite to efficacy.

Taking these points into consideration, the results of this *in vitro* investigations will be in useful in fundamentally understanding the DDI potential of brexpiprazole particularly when it is concomitantly administered with several medicines for other diseases and other CNS medications like antidepressants.

Materials and methods

Chemicals and reagents

Brexpiprazole (7-{4-[4-(1-benzothiophen-4-yl)piperazin-1-yl] butyloxy}quinolin-2(1*H*)-one) (Figure 1) and its sulfoxide, DM-3411, were synthesised by Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan). [quinoline ring-4-^{14}C]Brexpiprazole (abbreviated as q-[^{14}C]brexpiprazole) and [benzothiophene-3-^{14}C]brexpiprazole (b-[^{14}C]brexpiprazole) were synthesised by Daiichi Pure Chemicals Co, Ltd (Naka-gun, Ibaraki, Japan) (Figure 1). Specific radioactivity was 5.07 MBq/mg and 4.92 MBq/mg, respectively. β-Nicotinamide adenine dinucleotide reduced form (β-NADPH) and β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADH) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Monosodium β-NADP$^+$ oxidised form, disodium glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan).

[^{3}H]Digoxin, [^{3}H]prazosin, [^{14}C]mannitol, [6,7-^{3}H(N)] oestrone 3-sulfate sodium salt, ([^{3}H]E3S), [6,7-^{3}H(N)]oestradiol 17β-D-glucuronide ([^{3}H]$E_2$17βG) and [^{3}H(G)]taurocholic acid ([^{3}H]TCA) were purchased from PerkinElmer Inc. (Waltham, MA). *p*-[glycyl-2-^{3}H]Aminohippuric acid ([^{3}H]PAH) and [biguanidine-^{14}C]metformin hydrochloride ([^{14}C]metformin) were purchased from American Radiolabeled Chemicals (St. Louis, MO) and Moravek Biochemicals (Brea, CA), respectively. Other chemicals were obtained from the following sources. Digoxin, PAH, E3S sodium salt, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. $E_2$17βG sodium salt and Ko143 were purchased from Santa Cruz Biotechnology (Dallas, TX) and Tocris Bioscience (Bristol, UK),

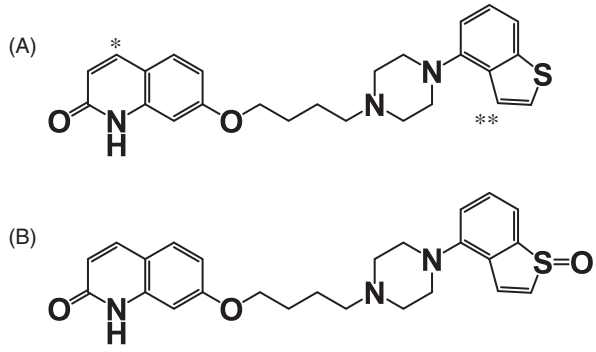

Figure 1. Chemical structures of brexpiprazole and its metabolite, DM-3411. (A) [^{14}C]Brexpiprazole; (B) DM-3411. *Labelled position of q-[^{14}C]brexpiprazole; **labelled position of b-[^{14}C]brexpiprazole. q-[^{14}C]Brexpiprazole and b-[^{14}C]brexpiprazole were separately synthesised, being labelled at each position.

respectively. TCA sodium salt was purchased from Wako Pure Chemical Industries Ltd (Tokyo, Japan). Paclitaxel and S-mephenytoin was purchased from Ultrafine Chemicals (Manchester, UK) and Toronto Research Chemicals Inc. (North York, ON, Canada), respectively. Other CYP's substrates, inhibitors, inducers and transporter's substrates and inhibitors were purchased from Sigma-Aldrich or Wako Pure Chemical Industries Ltd. All reagents and solvents were of analytical or HPLC grade, the highest grade or equivalent.

Human liver microsomes and human hepatocytes

Pooled human liver microsomes (20 mg/mL) for CYP inhibition experiments were obtained from BD Gentest (Franklin Lakes, NJ) or Xenotec LLC. (Lenexa, KS) and were stored at −70 °C or below. Fresh human hepatocytes for CYP induction were isolated from 3 samples of non-transplantable human liver tissue (3 males) and were cultured according to the methods by Quistorff (1990). Cryopreserved human hepatocytes (1 male and 2 females) for CYP2B6 induction experiment were purchased from Celsis *In Vitro* Technologies, Inc. (Chicago, IL)

LLC-PK1 cells expressing MDR1 or BCRP

Porcine kidney epithelial LLC-PK1 cells transfected with vectors containing human MDR1 cDNA and control LLC-PK1 cells transfected with vector only were used under a licence from BD Biosciences (Franklin Lakes, NJ). LLC-PK1 cells transfected with vectors containing human BCRP cDNA (Accession number: NM004827) were developed at the ADME-Tox. Research Institute, Sekisui Medical Co., Ltd. Prior to the experiments, the cells were cultured in 75 cm^2 bottom flasks and subjected to passage every 3–5 days. Cells expressing MDR1 (or empty vector) were seeded at 4×10^4 cells/insert, and cells expressing BCRP (or empty vector) were seeded at 2.5×10^4 cells/insert in 24-well transwell plates (0.3 cm^2 PET porous filter culture inserts of 3 μm pore size for MDR1-expressing cells or 0.4 μm pore size for BCRP-expressing cells, Cell Culture Insert 24-Well Companion Plate; BD Falcon, Bedford, MA). Seeded MDR1-expressing LLC-PK1 cells were incubated in a CO_2 incubator (37 °C, 5% CO_2) for 7–9 days and BCRP-expressing cells for 8 days to prepare cell monolayers for the determination of cellular transport activity. The medium in flasks and plates was changed every 2 or 3 days. The medium for passage in flasks was composed of Medium 199 containing 9% foetal bovine serum (FBS), 50 μg/mL gentamicin, and 100 μg/mL hygromycin B. The medium used during preparation of a monolayer for proliferation in 24-well plates was composed of Medium 199 containing FBS and gentamicin. The electrical resistances across the monolayer were measured prior to preincubation and were 486–591 Ωcm^2 for MDR1-expressing cells and 159–348 Ωcm^2 for BCRP-expressing and control cells, which were within the acceptable range (100–800 Ωcm^2).

HEK293 cells expressing OATP, OCT, and MATE

Human embryonic kidney (HEK293) cells transfected with a vector containing human OATP1B1/SLC21A6, OATP1B3/SLC21A8, OCT1/SLC22A1, OCT2/SLC22A2 or MATE1/SLC47A1 cDNA (Accession number: NM006446, NM019844, NM003057, NM003058, and NM018242, respectively), and control HEK293 cells were developed at Sekisui Medical Co., Ltd. Transient MATE2-K/SLC47A2-expressing cells and its control cells were prepared with vector alone and vector containing human MATE2-K cDNA (Accession number: NM001099646). Prior to the experiments, the cells were cultured in 75-cm^2 bottom flasks and subjected to passage every 3–4 days. The OATP1B1- and OATP1B3-expressing and control cells were seeded at 3.0×10^5 cells/well in 24-well plates coated with collagen I (BD Falcon) and then incubated in a CO_2 incubator for 1 day. The cells were further incubated for 1 day in 0.5 mL of the same medium plus 10 mmol/L butyric acid. The OCT1-, OCT2-, and MATE1-expressing and control cells were seeded at 2.5×10^5 cells/well in the same medium on 24-well plates coated with collagen I and incubated for 2 days.

For transient expression of MATE2-K and its control cells, HEK293 cells were seeded at a density of 3.5×10^5 cells/well in 12-well plates coated with collagen I and incubated for 1 day. The vector containing human MATE2-K cDNA, or vector alone, was introduced into the HEK293 cells, and the cells were further incubated for 2 days. The medium was composed of Dulbecco's Modified Eagle Medium containing 9% FBS, antibiotic-antimycotic and 2 mmol/L L-glutamine.

Renal proximal tubule (S_2) cells expressing OAT

Cells derived from the second segment of the proximal tubule of transgenic mice harbouring the temperature-sensitive simian virus 40 large T-antigen gene (here termed S_2 cells) and transfected with a vector, including human OAT1/SLC22A6 or OAT3/SLC22A8 cDNA (Accession number: NM004790 and NM004254, respectively), and control S_2 cells transfected with a vector alone, were established by Fuji Biomedix Co., Ltd (Tokyo, Japan), and the ownership was succeeded by Sekisui Medical Co., Ltd.

Prior to the experiments, OAT1- and OAT3-expressing S_2 cells and the corresponding control cells were cultured in 75-cm^2 bottom flasks and subjected to passage every 2 or 3 days. The cells were seeded at 2.5 or 4×10^5 cells/well in 24-well plates coated with collagen I (Corning, Tewksbury, MA) and incubated in a CO_2 incubator (33 °C, 5% CO_2) for 2 days. The passage and plating medium was composed of RITC80-7 containing 5% FBS, 10 μg/mL EGF, 0.08 unit/mL insulin and 10 μg/mL transferrin.

We used a number of different cell types in this study because of the development history in the facility where these experiments were performed. In the process of developing transporter-expressing cells, we first used HEK293 cells, which are widely used when creating recombinants, and as a result, we were able to construct an expression system for SLC transporters. We subsequently found that S_2 cells for OATs transporters were more resistant to toxicity in culture. On the other hand, since the ABC transporters of P-gp and

BCRP reflect bidirectional transports through the monolayer membrane, it is necessary to express the directional transporters on a monolayer membrane. The established LLC-PK cells having polarity and the established HEK293 cells and S_2 cells were suitable for the transport tests, depending on the compared results of the transports of the typical substrates to the corresponding control cells.

BSEP-expressing vesicles

BSEP-expressing and control vesicles (expression system of the insect cells infected with baculovirus) were obtained from GenoMembrane Co Ltd. (Yokohama, Kanagawa, Japan) and stored at −70 °C until use.

Microsomal metabolic experiments for CYP inhibition

The CYP enzymatic reactions and their substrates and inhibitors were described as below.

CYPs	Metabolic reactions	Substrate	Inhibitors
CYP1A2	Phenacetin *O*-deethylation	Phenacetin 50	Furafylline
CYP2A6	Coumarin 7-hydroxylation	Coumarin 5	8-Methoxypsoralen
CYP2B6	Bupropion hydroxylation	Bupropion 50	Orphenadrine
CYP2C8	Paclitaxel 6α-hydroxylation	Paclitaxel 10	Quercetin
CYP2C9	Diclofenac 4′-hydroxylation	Diclofenac 5	Sulfaphenazole
CYP2C19	S-Mephenytoin 4′-hydroxylation	S-Mephenytoin 100	Tranylcypromine
CYP2D6	Bufuralol 1′-hydroxylation	(±)-Bufuralol 20	Quinidine
CYP2E1	Chlorzoxazone 6-hydroxylation	Chlorzoxazone 50	Diethyldithiocarbamate
CYP3A4	Midazolam 1′-hydroxylation	Midazolam 100	Ketoconazole
	Testosterone 6β-hydroxylation	Testosterone 100	

The reaction mixture consisted of 100 mmol/L phosphate buffered saline (pH 7.4), 2.5 mmol/L β-NADPH, 2.5 mmol/L β-NADH, human liver microsome, and substrate and test compound as inhibitor at the designated concentration. The details of the experimental condition in the metabolic reaction are presented in Supplementary Table 1. The reaction mixture was preincubated without both β-NADPH and β-NADH at 37 °C for 5 min. After the addition of β-NADPH and β-NADH, the reactions were carried out at 37 °C at the designated time. In the case of mechanism-based inhibitors, furafylline (CYP1A2), 8-methoxypsoralen (CYP2A6), orphenadrine (CYP2B6), and diethyldithiocarbamate (CYP2E1), the reaction mixture was preincubated with β-NADPH and β-NADH and without the substrate at 37 °C for 15 min. After the addition of substrate, the reactions were carried out at 37 °C for the designated time. The reaction was stopped by the addition of 0.5 mL of IS solution (200 nmol/L of chlorpropamide in acetonitrile), and then the mixture was centrifuged at 15 000 rpm for 10 min at 4 °C (Himac CF15D, Hitachi Koki Co., Ltd. Tokyo, Japan). The resultant supernatant fraction was diluted with distilled water at a ratio of 1:4, and then analysed using the LC-ESI-MS/MS system. All reactions were carried out in duplicate. To evaluate the mechanism-based inactivation of brexpiprazole, after human liver microsomes were incubated with brexpiprazole at 100 μmol/L in the presence of β-NADPH at 37 °C for 30 min, the metabolic reactions were started by substrates for each CYP to the 10-fold diluted reaction mixtures and were subsequently incubated. The changing of the metabolic activity by brexpiprazole in the presence of β-NADPH through the preincubation and metabolic incubation was compared with that of the group for which β-NADPH was present only during the metabolic incubation (Venkatakrishnan *et al.* 2007).

mRNA assay and microsomal metabolic experiments for CYP induction

Three lots of fresh human cultured hepatocytes were used for the assessment of relative mRNA levels and metabolic activities of CYP1A2, CYP2C9, and CYP3A4. Hepatocytes were seeded at approximately 1.3×10^6 viable cells/mL on collagen coated 60 mm culture dishes and were placed in a humidified chamber (37 °C, 5% CO_2). After seeding, hepatocyte culture was performed according to the methods described in the previous report (Shimokawa *et al.* 2014). After the adaptation period, hepatocyte cultures were then treated daily for 3 consecutive days with 1 of the 3 concentrations of brexpiprazole (0.1, 1, or 10 μmol/L), omeprazole (100 μmol/L, CYP1A2 positive inducer), or rifampin (10 μmol/L, CYP2C9 and CYP3A4 positive inducer) dissolved in 0.1% (*v/v*) DMSO (vehicle). After the last treatment with test compounds, total RNA was extracted from the hepatocytes using TRIzol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit (Qiagen, Valencia CA). Subsequently, cDNA was prepared from mRNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosynterms, Foster City, CA) and amplification was conducted using TaqMan Gene Expression Master Mix (Applied Biosynterms) and the real-time polymerase chain reaction (PCR) sequence detection system (7300, Applied Biosynterms). For CYP2B6, the relative mRNA levels were determined using cultured human hepatocytes prepared from the cryopreserved hepatocytes, after 2-consecutive-day treatment days with brexpiprazole (0.05, 0.5, or 5 μmol/L) or phenobarbital (750 μmol/L, CYP2B6 positive inducer). The maximum incubation concentration of brexpiprazole was set to 5 or 10 μmol/L (for CYP2B6 and for other CYPs, respectively) which was sufficiently more than the required three orders of magnitude above the $C_{max,u}$ (0.0038 μmol/L) (Ishigooka *et al.* 2018). The details of PCR analysis were according to the methods described by Shimokawa *et al.* (2014). Microsomal samples were prepared from the human hepatocytes based on the method described by Madan *et al.* (1999). For each hepatocyte preparation, cell suspensions scraped from culture dishes were sonicated. Sonicated lysates were centrifuged at 7000 to 9000×*g* at 4 °C for 20 min, after which the supernatant fraction (S9) was removed and centrifuged at 80 000 to 90 000×*g* at 4 °C for 60 min. The resulting microsomal pellets were resuspended in 250 mmol/L sucrose aqueous solution.

Transwell transport experiments

In transwell plates, the apical-side volume of MDR1-expressing cell monolayers was maintained at 100 μL and the basal-

side volume at 600 μL. The apical-side volume of BCRP-expressing cell monolayers was maintained at 250 μL and the basal volume at 900 μL. Prior to flux assays, the medium surrounding both the apical and basal sides was removed and replaced with equal volumes of fresh 1% bovine serum albumin containing Hanks' balanced salt solution (pH = 7.4) (1% BSA-HBSS, for BCRP) or 4% BSA-HBSS (for BDR1) containing 0.2% DMSO (vehicle) or inhibitor solution, and the cells were preincubated at 37 °C for 1 h.

For the assessment of drug transport from the apical to basal sides, the apical-side medium was replaced with 1% or 4% BSA-HBSS containing the test substance as substrate/inhibitor or with [^{14}C]mannitol solution (indicator for the tight junction), and the basal side was replaced with 1% or 4% BSA-HBSS containing 0.2% DMSO or inhibitor solution as appropriate. Conversely, for the assessment of transport from the basal to apical sides, the apical-side medium was replaced with fresh 1% or 4% BSA-HBSS containing 0.2% DMSO or inhibitor solution as appropriate, and the basal side medium was replaced with the test substance as substrate/inhibitor or [^{14}C]mannitol solution. Then, the cells were incubated at 37 °C at the designated times (0.25, 0.5 and 1 h for [^{3}H]prazosin and 0.5, 1, and 2 h or 1, 2, and 4 h for [^{14}C]brexpiprazole and [^{3}H]digoxin). Incubation was conducted in triplicates. At the designated time, 70 μL (for MDR) or 100 μL (for BCRP) of the sample in the basal or apical sides was collected for the assessment of transport from apical to basal or basal to apical sides, respectively. The same volume of 1% or 4% BSA-HBSS containing 0.2% DMSO or inhibitor solution was quickly added to compensate for the sampled volume.

For experiments using radiolabelled substrate and reference compounds, scintillator cocktail (10 mL, Hionic-Fluor, PerkinElmer Inc.) was added to the collected samples. Permeability was calculated based on the transported amount and the concentration observed before incubation.

Cellular uptake experiments

The medium in each seeded well was replaced by fresh HBSS (1 mL), and the cells were preincubated at 37 °C for 15 min. Uptake was started by replacing of albumin-free HBSS (300 μL) containing 0.2% DMSO with the substrate solution or substrate solution containing inhibitor. The uptake reaction was performed in triplicate at 37 °C for each designated time (0.5, 1, 2, 5 and 10 min). The substrate solution was then removed, and the cells were washed once with 1 mL of ice-cold PBS containing 0.2% BSA and twice with 1 mL of ice-cold PBS. The intracellular fraction was isolated as described below. After PBS was removed, 0.1 mol/L NaOH (0.5 mL) was added to each well and mixed thoroughly by pipetting to dissolve the cells. An aliquot (0.3 mL) of the cell lysate was mixed with the scintillator cocktail (10 mL, Hionic-Fluor). Protein content was also determined in the aliquot. The uptake volume was calculated based on the amount of drug in the lysate and the concentration observed before incubation.

Vesicular uptake experiment

Prior to incubation, glass fibre filters (MF-membrane filters #HAWP02500, Merck Millipore, Burlington, MA) were washed once with an ice-cold stop solution of 10 mmol/L Tris/HEPES, 100 mmol/L KNO_3, and 50 mmol/L sucrose. Culture wells were preincubated at 37 °C for 5 min in the reaction buffer consisting of Tris/HEPES, KNO_3, 4 mmol/L Mg-ATP, and sucrose plus substrate or inhibitor (45 μL mixture). Then, a 5 μL aliquot of BSEP-expressing or control vesicle solution was added to start the uptake reaction, and the reaction mixture was allowed to proceed at 37 °C for 2 min. All incubations were conducted in triplicate. At the designed time, an ice-cold stop solution (200 μL) was added to terminate the reaction. An aliquot (200 μL) of each mixture was passed through the glass fibre filter by aspiration. Loaded filters were washed twice with 5 mL ice-cold stop solution, transferred to a vial and mixed with 10 mL scintillator cocktail (Insta-Gel Plus, PerkinElmer Inc.) for the measurement of the intravesicular radioactivity. An aliquot (20 μL) of the remaining reaction mixture was mixed with 10 mL of scintillator cocktail for the measurement of the remaining extravesicular radioactivity. The details of the experimental condition in the uptake and transcellular transport assay are presented in Supplementary Table 2.

Protein content assay

The protein content in the cell lysate was assayed using the BCA Protein Assay Kit (Thermo Fisher Scientific, Tokyo, Japan). Briefly, 20 μL of each cell lysate sample or BSA calibration solution (0, 0.05, 0.1, 0.25, 0.5 and 0.75 mg protein/mL) was allocated to 96-well plates, and 200 μL of reagent mixture from the reagent kit was added to each well. Mixtures were incubated at 37 °C for 30 min, and absorbance at 562 nm was measured on a microplate reader (SPECTRA$_{max}$ ^{TM}190, Molecular Devices Corp., San Jose, CA). Incubation was performed in duplicate.

LC-MS/MS analysis

LC-MS/MS analysis of the produced metabolites for microsomal CYP activity was conducted using an API4000 MS system with a Shimadzu LC20A HPLC system (Shimadzu Co., Ltd., Kyoto, Japan). Chromatographic separation was achieved on a Cadenza CD-C18 (3 μm, 2.0 mmID × 50 mm, Imataki Corp, Kyoto, Japan) using mixtures of 2 solvents, A [0.1% (*v/v*) acetic acid aqueous solution] and B [0.1% (*v/v*) acetic acid acetonitrile solution], at a constant flow rate of 0.2 mL/min. The elution steps were as follows: under the equilibration of 10% B flow, a linear gradient from 10% to 80% B was operated for 2.5 min after sample injection. Equilibration was achieved for 4 min after returning to the initial condition. The column effluent was delivered to the ESI ion source to detect in the positive multiple reaction monitoring mode except for chlorzoxazone (negative mode). The calibration of metabolites exhibited good linearity over the range 50–5000 nmol/L for acetaminophen (phenacetin'

metabolite) and 10–1000 nmol/L for others, using weighted (1/concentration^2) least-squares regression. The lower limit of qualification (LLOQ) defined as the lowest measurable concentration was 10 nmol/L except for acetaminophen, with an LLOQ of 50 nmol/L. The sensitivity of the LC-MS/MS analysis was sufficient to see microsomal CYP activities and related inhibition. Acceptance of analytical data was subject to the criterion that the analytical data of quality check samples must be within 85% to 115% of the nominal concentration.

Measurement of radioactivity

The radioactivity in each sample was measured for 2 min using liquid scintillation counting (LSC, 2500TR, 3100TR, or 1900CA, PerkinElmer Inc., LSC-6101 Aloka, Tokyo, Japan). The counting efficiency was corrected by the external standard source method. The detection limit of radioactivity was defined as the background radioactivity.

Calculations of parameters

In the monolayer experiments using MDR1-expressing and BCRP-expressing LLC-PK1 cells, the permeation volume and clearance were calculated from the determined permeated amounts across transporter-expressing or control cell monolayers and the initial concentration, according to the following equations:

$$\text{Permeation volume } (\mu\text{L/well}) = \frac{\text{Permeated amount } (\text{amount/well})}{\text{Initial concentration } (\text{amount}/\mu\text{L})}$$

and

$$\text{Permeation clearance ratio} = \frac{\text{Permeation clearance}_{\text{basal to apical}} \ (\mu\text{L/well/h})}{\text{Permeation clearance}_{\text{apical to basal}} \ (\mu\text{L/well/h})}$$

where permeation clearance was determined by a linear regression of permeation volume and incubation time. Further, a ratio of permeation clearance for transporter-expressing cells to the control cells was defined as a net transport ratio. In the uptake experiments using HEK293 or S_2 cells expressing OATs, OCTs, OATPs, and MATEs, the uptake volume was calculated from the amount of substrate in the cell lysate and the initial substrate concentration according to the following equation:

$$\text{Uptakevolume } (\mu\text{L/mg protein}) = \frac{\text{Uptake amount } (\text{amount/well})}{\text{Initial concentration } (\text{amount}/\mu\text{L}) \times \text{Protein content } (\text{mg protein/well})}$$

Further, the ratio of the uptake volume for transporter-expressing cells to the control cells was defined as the uptake ratio. In the vesicle experiment, the uptake amount and protein content were expressed per tube.

In the inhibition of monolayer permeation, the remaining activity (% of control) was determined as the ratio of the net transport in the presence and absence of an inhibitor as follows:

$$\%\text{ of control} = \frac{\text{Net transport ratio}_{\text{presence of inhibitor}}}{\text{Net transport ratio}_{\text{absence of inhibitor}}} \times 100$$

In the uptake inhibition experiments, the remaining activity was calculated according to the following equation:

$$\%\text{ of control} = \frac{\text{Uptake volume}_{\text{presence - absence of inhibitor}} \ (\text{expressing cells})}{\text{Uptake volume}_{\text{presence - absence of inhibitor}} \ (\text{control cells})} \times 100$$

The activity of each CYP enzymatic reaction and permeability were compared with those of the control, being expressed as the percentage of the remaining control activity. IC_{50} values were calculated using WinNonlin (Ver 5.2 or 6.1, Pharsight Co., Mountain View, CA). In the inhibition experiments, IC_{50} was calculated by the E_{max} equation:

$$\%\text{ of control} = \left(1 - \frac{I_{max} \times [I]^c}{[I]^c + IC_{50}{}^c}\right) \times 100$$

where I_{max} denotes the maximum inhibitory effect, [I] denotes the inhibitor concentration, and c denotes the Hill coefficient. When percentage of control at the maximum concentration tested was higher than 50.0%, IC_{50} was indicated as greater than the maximum concentration. For the K_i determination of brexpiprazole, the rates of metabolite formation following co-incubation of 3 concentrations (approximately 1/2 K_m, K_m, and 2 K_m) of the substrate with 0, 3, 10, and 30 μmol/L of brexpiprazole were determined. The calculation of the K_i value was performed using the Dixon plot.

Inactivation kinetic parameters were determined using the nonlinear regression of the data to the following expression:

$$k_{obs} = k_{obs[I]=0} + \frac{k_{inact} \times [I]}{K_I + [I]}$$

in which [I] denotes the concentrations of brexpiprazole in the inactivation preincubations, k_{obs} denotes the negative values of the slopes of the natural logarithm of the percentage of the remaining activity versus inactivation incubation time at various [I], $k_{obs[I]=0}$ denotes the apparent inactivation rate constant measured in the absence of brexpiprazole, k_{inact} denotes the limit maximum inactivation rate constant as [I]→∞, and K_I denotes the brexpiprazole concentration yielding k_{obs} at the sum of $k_{obs[I]=0}$ and 0.5 times k_{inact}.

Statistical analysis

The statistical significance of differences for the test compound and positive control compound for inhibition studies was determined by the Williams test (lower-tailed) and unpaired *t*-test (Student's or Welch), respectively, using Statistical Analysis System (SAS) software (Release 9.4, SAS Institute, Cary, NC). For induction study, The ANOVA was

followed by the Dunnett's *post hoc* test to identify the group means that were significantly different from the controls ($p < 0.05$). Analyses were performed using Sigma Stat Statistical Analysis System (version 2.03, Systat Software, Inc., Point Richmond, CA).

Results

Inhibition of CYP-mediated metabolic activity by brexpiprazole and DM-3411

Brexpiprazole exhibited several weak inhibitory effects on CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Table 1). The inhibition of brexpiprazole on CYP2B6 was the strongest among these CYPs, having an IC_{50} value of 8.19 μmol/L (K_i value = 5.01 μmol/L). Brexpiprazole exhibited no effects on the activities of CYP1A2, CYP2A6, CYP2C8, and CYP2E1. DM-3411 exhibited weak inhibitory effects on CYP2D6 and CYP3A4 (only midazolam 1′-hydroxylation), with similar IC_{50} values to those of brexpiprazole. For the effects of brexpiprazole, the preincubation of microsomes and brexpiprazole with β-NADPH make the 2 activities by CYP3A4 decreased over 20%. And when the preincubation time and the concentration of brexpiprazole varied for 0, 5, 10, 20, and 30 min, and at 0, 3, 10, 30, and 100 μmol/L, respectively in the inactivation experiment, K_I and K_{obs} for inactivation were obtained, both in the midazolam 1′-hydroxylation and testosterone 6β-hydroxylation (Table 1).

Induction of CYPs by brexpiprazole

At concentrations less 10 μmol/L, brexpiprazole did not affect the mRNA contents of CYP2B6 and CYP2C9 in human cultured hepatocytes. However, at 10 μmol/L, brexpiprazole produced a 9.12-fold increase in CYP1A2 mRNA levels, but this increase was less than 3% of that caused by omeprazole. The mRNA content increase (1.77-fold) in CYP3A4 at 10 μmol/L was statistically significant from the control, but, the difference was under 2-fold that is the DDI guideline's criterion for the clinical assessment of induction (Table 2). In addition, the metabolic activities mediated by CYP1A2, CYP2C9, and CYP3A4 were not significantly changed.

Transport of brexpiprazole across P-gp/MDR1- or BCRP-expressing cell monolayers

The permeation clearances of [^{14}C]brexpiprazole at 1 μmol/L across P-gp/MDR1-expressing LLC-PK1 cell monolayers were 2.87 ± 0.08 μL/well/h (mean ± SD, $n = 3$) from the apical to basal sides and 4.83 ± 0.16 μL/well/h from the basal to apical sides, and the permeation clearance ratio was 1.7 (Figure 2(A)). This was only slightly higher than that across control LLC-PK1 cell monolayers, in which the clearances were 2.85 ± 0.32 μL/well/h from the apical to basal sides and 2.68 ± 0.17 μL/well/h from the basal to apical sides, with a clearance ratio of 0.9. The ratio of the permeation clearance for transporter-expressing cells to the control cells (net transport ratio) was 1.9 under 2 that is criterion for assessment of transporter's substrate. The permeation clearances of [^{14}C]brexpiprazole across BCRP-expressing LLC-PK1 cell monolayers were 9.47 ± 1.94 μL/well/h from the apical to basal sides and 20.0 ± 0.9 μL/well/h from the basal to apical sides, and the permeation clearance ratio was 2.1 (Figure 2(C)). Meanwhile, the permeation clearances across the control cell monolayers were 13.4 ± 2.7 μL/well/h from the apical to basal sides and 17.4 ± 0.4 μL/well/h from the apical to basal sides, and a permeation clearance ratio was 1.3 (Figure 2(C)). These 2 permeation clearance values

Table 1. Inhibitory effects of brexpiprazole and its metabolite, DM-3411, on CYP-mediated metabolic activities in human liver microsomes.

	Brexpiprazole				DM-3411	Brexpiprazole		DM-3411
	w/o preincubation	with preincubation			w/o preincubation	w/o preincubation		
CYPs	IC_{50} [SE] (μmol/L)	Decrease from w/o (%)	K_I [SE] (μmol/L)	k_{inact} [SE] (min^{-1})	IC_{50} [SE] (μmol/L)	$C_{inlet,max,u}/IC_{50}$*	$C_{max,u}/IC_{50}$*	$C_{max,u}/IC_{50}$*
CYP1A2	>100**	1.2	–***	–	>100	–	–	–
CYP2A6	>100	4.0	–	–	>100	–		–
CYP2B6	8.19 [0.90] (5.01) ****	−2.6	–	–	>100	0.0012	0.00046	–
CYP2C8	>100	−4.5	–	–	>100	–	–	–
CYP2C9	22.2 [6.1]	5.1	–	–	>100	0.00044	0.00017	–
CYP2C19	39.8 [4.5]	−4.6	–	–	>100	0.00024	0.00010	–
CYP2D6	13.4 [2.4]	0.9	–	–	15.3 [1.0]	0.00072	0.00028	0.00034
CYP2E1	>100	–	–	–	>100			
CYP3A4#	29.9 [6.4]	30.5	32.1 [43.1]	0.0196 [0.0079]	25.8 [0.62]	0.00033	0.00013	0.00020
	40.8 [11.2]	41.4	4.7 [3.8]	0.0221 [0.0035]	>100	0.00024	0.00009	–

The test compound was incubated at 1, 3, 10, 30, and 100 μmol/L with human microsomes in the presence of β-NADPH/β-NADH. In the case of the w/o preincubation, the incubation for metabolic reaction was started by the addition of β-NADPH/β-NADH after preincubation of the test compound, substrate and human microsomes. In the case of the with preincubation, the incubation for metabolic reaction was started by the addition of substrate after preincubation of the test compound, human microsomes, and β-NADPH/β-NADH. The IC_{50} and the estimated standard error (SE) were obtained by the nonlinear regression of the mean remaining activity (% of control, triplicate determinations) from the control (0 μmol/L) to the E_{max} model equation, using WinNonlin (Ver. 5.2 or 6.1).

* The pharmacokinetic parameters when brexpiprazole was administered to Japanese patients at 4 mg for 14 days were used to calculate the ratios for drug–drug interaction (Ishigooka et al. 2018). The $C_{max,u}/IC_{50}$ value was calculated using free fraction (f_u: 0.01 for brexpiprazole, 0.035 for DM-3411) and plasma C_{max} (0.38 μmol/L for brexpiprazole and 0.15 μmol/L for DM-3411). $C_{inlet,max,u}$ was calculated using the equation $f_u \times [C_{max} + (k_a \times dose/Q_h)]$, where the absorption rate constant (k_a: 0.1 min^{-1}, regulatory maximum) was obtained from plasma concentration profiles, and hepatic blood flow (Q_h) of 23.4 mL/min/kg was used.

Upper: Midazolam 1′-hydroxylation, Lower: Testosterone 6β-hydroxylation.

>100: Inhibitory effects less than 50% at 100 μmol/L of the test compound. *–: not tested, ****The value in the parenthesis indicates the K_i value.

Table 2. Effects of brexpiprazole on CYP mRNA expression and CYP-mediated metabolic activities in human primary cultured hepatocytes.

	Fold increase						
	CYP1A2		CYP2C9		CYP3A4		
Concentration (μmol/L)	mRNA	Phenacetin O-dealkylation	mRNA	Diclofenac 4′-hydroxylation	mRNA	Testosterone 6β-hydroxyation	CYP2B6 mRNA
0 (Control)	1.00	1.00 ± 0.73	1.00	1.00 ± 0.49	1.00	1.00 ± 0.59	1.00
0.1 (0.05)*	1.34 ± 0.57	0.914 ± 0.103	1.09 ± 0.20	0.940 ± 0.052	1.06 ± 0.19	1.08 ± 0.16	1.02 ± 0.02
1 (0.5)*	1.61 ± 0.72	0.874 ± 0.160	1.23 ± 0.28	0.999 ± 0.070	1.15 ± 0.24	1.19 ± 0.04	1.18 ± 0.05
10 (5)*	9.12 ± 6.33	1.24 ± 0.28	1.03 ± 0.24	1.06 ± 0.08	1.77 ± 0.38¶	1.56 ± 0.61	1.14 ± 0.30
100 (Omeprazol)	372 ± 293#	18.8 ± 14.6#	1.86 ± 0.18#	1.72 ± 0.78#	2.90 ± 0.91	2.55 ± 1.31	– **
10 (Rifampin)	1.74 ± 0.94	1.68 ± 0.57	2.99 ± 0.20#	2.75 ± 1.01#	10.3 ± 2.8#	8.83 ± 5.67#	–
750 (Phenobarbital)	–	–	–	–	–	–	7.20 ± 0.48

The test compound was incubated with human hepatocytes (1.3×10^6 viable cells/well) for 48 h. The fold increase represents the ratio of mRNA expression or metabolic activity in the presence of inducer to that in the absence of inducer (0.1% (*v/v*) DMSO).
The value represents the mean ± standard deviation (SD) of 3 hepatocyte lots.
*The value in parenthesis indicates the concentration of brexpiprazole in the experiment involving mRNA expression of CYP2B6. **–: not tested.
¶Significantly different from the vehicle control as a result of one-way analysis of variance ($p < 0.05$) with the positive control groups (omeprazole and rifampin) excluded from the statistical analysis using Sigma Stat Statistical Analysis System (version 2.03).
#Significantly different from the vehicle control as a result of one-way analysis of variance ($p < 0.05$) with all treatment groups included in the statistical analysis.

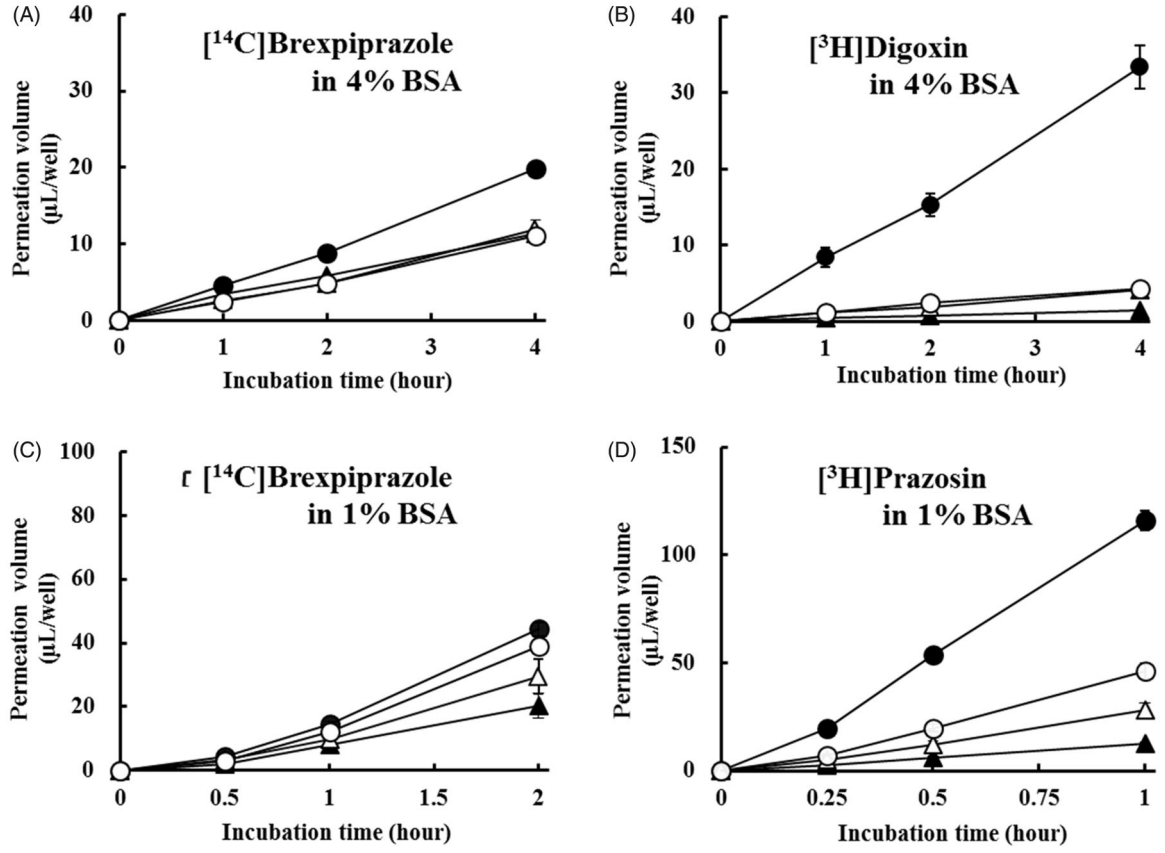

Figure 2. Time profiles for the transcellular transports of [^{14}C]brexpiprazole and [^{3}H]digoxin or [^{3}H]prazosin across MDR1-expressing or BCRP-expressing LLC-PK1 cells. The experiment was started by the addition of substrate to one compartment: (A) [^{14}C]brexpiprazole (1 μmol/L) and (B) [^{3}H]digoxin (1 μmol/L) across the MDR1-expressing LLC-PK1 cells in 4% BSA-HBSS; (C) brexpiprazole (1 μmol/L) and (D) [^{3}H]prazosin (1 μmol/L) across the BCRP-expressing LLC-PK1 cells in 1% BSA-HBSS. Circle symbols: basal to apical flux; triangle symbols: apical to basal flux; closed symbols: the transporter-expressing LLC-PK1 cells; open symbols: control LLC-PK1 cells. Data are expressed as mean ± standard deviation (SD) of triplicate determinations.

were close each other, and the net transport ratio was 1.6, which was lower than 2. The permeation clearance ratio of the known P-gp substrate, [^{3}H]digoxin, was significantly larger than that in the control cells (Figure 2(B)), and the net transport ratio was 20.0. The net transport ratio of BCRP substrate, [^{3}H]prazosin, was 5.6 (Figure 2(D)). Thus, compared with [^{3}H]digoxin and [^{3}H]prazosin in the monolayer flux experiments, brexpiprazole was not transported in significant amounts by P-gp/MDR1 and BCRP, indicating that brexpiprazole is not a substrate for the two efflux transporters; indeed if it was a substrate, it would be a weak and poor substrate.

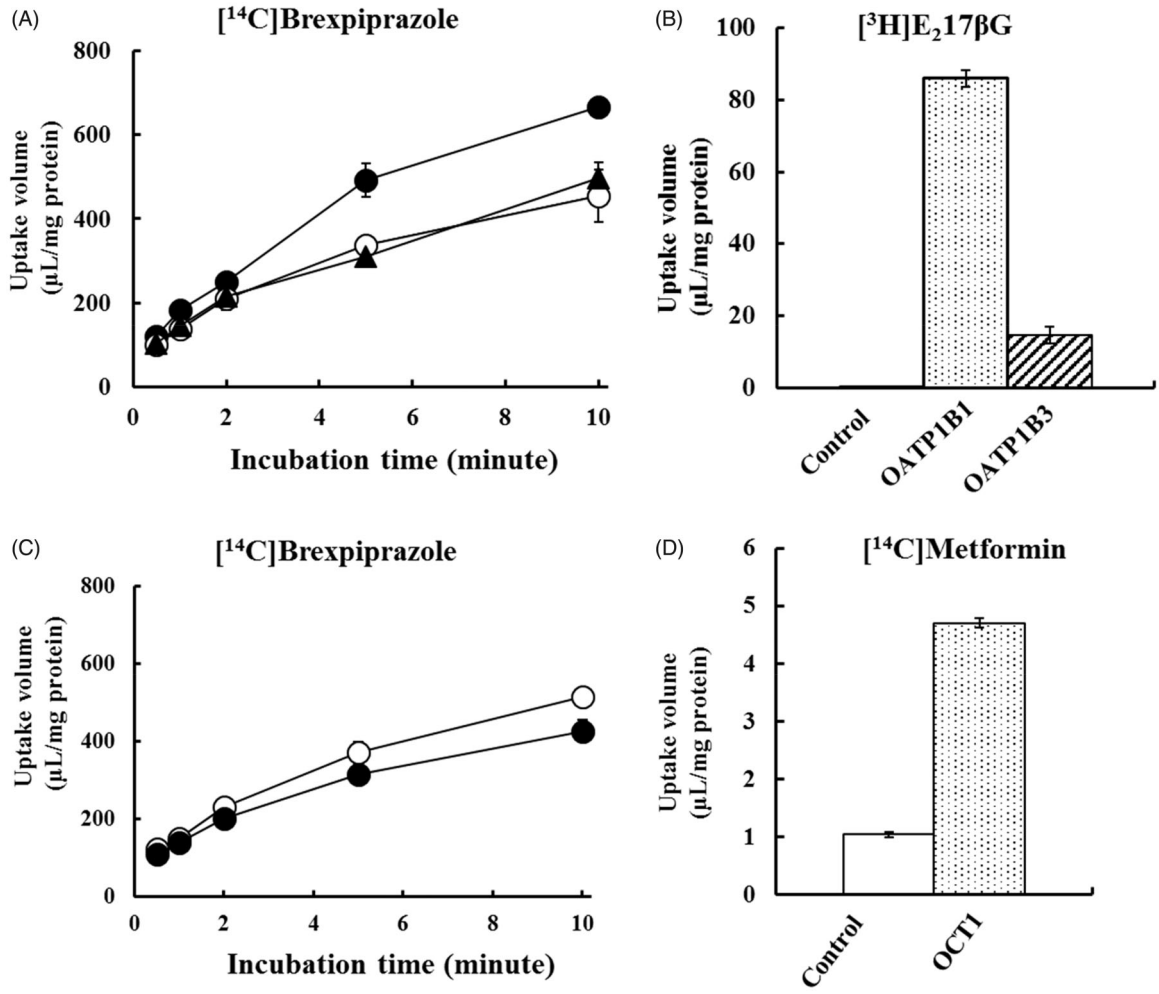

Figure 3. Time profiles for the uptake of [^{14}C]brexpiprazole and [^{3}H]$E_2$17βG into OATP1B1 or OATP1B3-expressing cells and for the uptake of [^{14}C]brexpiprazole and [^{14}C]metformin into OCT1-expressing HEK293 cells. The experiment was started by the addition of substrate: (A) (C) [^{14}C]brexpiprazole (1 μmol/L), (B) [^{3}H]$E_2$17βG (0.05 μmol/L), and (D) [^{14}C]metformin (10 μmol/L). Closed circle symbols: OATP1B1-expressing cells; closed triangle symbols: OATP1B3-expressing cells; open circle symbol: control HEK293 cells in (A). Open bar: control HEK293 cells; dotted bar: OATP1B1-expressing cells; hatched bar: OATP1B3-expressing cells in (B). Closed circle symbols: OCT1-expressing cells; open circle symbol: control HEK293 cells in (C). Open bar: Control HEK293 cells; dotted bar: OCT1-expressing cells in (D). The uptake volume of [^{3}H]$E_2$17βG and [^{14}C]metformin are at 2 and 5 min, respectively. Data are expressed as mean ± SD of triplicate determinations.

Uptake transport of brexpiprazole by OATP- or OCT1-expressing cells

The uptake volume of brexpiprazole was 665 ± 22 μL/mg protein at 10 min for OATP1B1-expressing HEK293 cells and 496 ± 38 μL/mg protein for OATP1B3-expressing cells (Figure 3(A)). The uptake volume of brexpiprazole into the common control cells was 455 ± 62 μL/mg protein (Figure 3(A)). The uptakes ratios of brexpiprazole for the OATP1B1- and OATP1B3-expressing cells were similar to the control cells throughout the uptake incubation time. Conversely, the known OATP substrate, [^{3}H]$E_2$17βG, showed substantial uptakes into the 2 expressing cells, 86.0 ± 2.3 and 14.7 ± 0.9 μL/mg protein for 2 min, respectively (0.355 ± 0.125 μL/mg protein into the control cells), and indicated functional uptake activity (Figure 3(B)).

The uptake volumes of brexpiprazole for OCT1-expressing HEK293 cells and the control were 427 ± 28 and 515 ± 12 μL/mg protein at 10 min, indicating slightly lower uptake than for control cells (Figure 3(C)). Conversely, the uptake volume of the known OCT1 substrate, [^{14}C]metformin, into the OCT1-expressing cells was 4.70 ± 0.08 μL/mg protein for 5 min (1.04 ± 0.05 μL/mg protein into the control cell), indicating substantially larger uptake than for control cells and functional uptake activity (Figure 3(D)). These results suggested that brexpiprazole is not a substrate of hepatic uptake transporters, OATP1B1, OATP1B3, and OCT1.

Inhibition of P-gp/MDR1 and BCRP-mediated transports by brexpiprazole and DM-3411

Brexpiprazole and DM-3411 had influences on the net efflux ratio of [^{3}H]digoxin across the P-gp/MDR1-expressing cell monolayers with similar IC_{50} values of 6.31 and 7.84 μmol/L, respectively (Tables 3 and 7). Compared with P-gp/MDR1, both compounds had more influence on the net efflux ratio of [^{3}H]prazosin across the BCRP expressing cells with IC_{50} values of 1.16 and 3.04 μmol/L, respectively (Tables 3 and 7).

Inhibition of OATP-, OAT-, OCT-, and MATE-mediated transports by brexpiprazole and DM-3411

Brexpiprazole and DM-3411 reduced the uptake of [^{3}H]$E_2$17βG by the OATP1B1 expressing cells with similar IC_{50}

Table 3. Inhibitory effects of brexpiprazole and its metabolite, DM-3411, on MDR1-mediated [^{3}H]digoxin transport and BCRP-mediated [^{3}H]prazosin transport.

Concentration (μmol/L)	MDR1-mediated [^{3}H]digoxin transport (Ratio of basal and apical transports)		BCRP-mediated [^{3}H]prazosin transport (Ratio of basal and apical transports)	
	% of control			
0 (Control)	15.4/1.0	33.0/1.0	9.0/1.7	
Inhibitor	Brexpiprazole	DM-3411	Brexpiprazole	DM-3411
0.1	96.1	90.0	86.8	111.3
0.3	119.5	58.5¶¶	98.1	103.8
1	135.7	77.9¶¶	62.3¶¶	81.1
3	78.6¶	67.3¶¶	39.6¶¶	62.3¶¶
10	24.7¶¶	50.3¶¶	30.2¶¶	47.2¶¶
30	15.6¶¶	33.9¶¶	20.8¶¶	28.3¶¶
30 (Verapamil)	11.7$^{\#\#}$	6.1$^{\#}$	–*	
1 (Ko123)	–	–	17.0$^{\#\#}$	

The values at 0 μmol/L represent the ratio of basal to apical transport against apical to basal transport (ratio for transporter-expressing LLC-PK1 cells/ratio for non-transporter-expressing LLC-PK1 cells). The % of control represents the net transport ratio in the presence of inhibitor to that in the absence of inhibitor (concentration 0 μmol/L).
The statistical significance of differences for the test compound and positive control compound for inhibition studies was determined by the Williams test (lower-tailed) and unpaired *t*-test (Student's or Welch), respectively, using SAS software (Release 9.4). $^{¶}p < 0.05$, $^{¶¶}P < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.
Each value represents the ratio of mean permeability rates obtained from triplicate determinations. *–: not tested.

Table 4. Inhibitory effects of brexpiprazole and its metabolites, DM-3411, on OATP-mediated [^{3}H]$E_2$17βG uptakes, OAT1-mediated [^{3}H]PAH uptake, and OAT3-mediated [^{3}H]E3S uptake.

Concentration (μmol/L)	Uptake volume into OATP1B1-expressing cells (μL/mg protein/2 min)		Uptake volume into OATP1B3-expressing cells (μL/mg protein/2 min)		Uptake volume into OAT1-expressing cells (μL/mg protein/2 min)		Uptake volume into OAT3-expressing cells (μL/mg protein/2 min)	
	81.4 ± 8.8 (0.27 ± 0.06)*	101 ± 6 (0.32 ± 0.07)*	17.3 ± 1.6 (0.27 ± 0.06)*	15.4 ± 2.0 (0.32 ± 0.07)*	43.9 ± 2.5 (0.65 ± 0.18)*	41.7 ± 2.2 (1.10 ± 0.70)*	34.2 ± 2.5 (2.59 ± 0.26)*	41.1 ± 3.8 (2.49 ± 0.37)*
	% of control							
0 (Control) Inhibitor	Brexpiprazole	DM-3411	Brexpiprazole	DM-3411	Brexpiprazole	DM-3411	Brexpiprazole	DM-3411
0.1	108.0	90.9	90.5	109.2	95.5	92.6	103.3	77.7¶
0.3	115.1	112.8	119.4	122.7	99.4	99.1	105.3	80.2¶
1	115.7	104.9	103.6	121.2	91.9	88.9	129.7	78.0¶
3	84.7¶	93.7	120.0	126.9	95.2	98.6	130.2	87.9¶
10	62.7¶¶	58.5¶¶	111.0	122.5	83.6	85.9¶¶	116.9	66.5¶¶
30	37.8¶¶	29.0¶¶	98.4	89.7	95.2	63.7¶¶	102.7	48.7¶¶
10 (Rifampin)	5.9$^{\#\#}$	5.1$^{\#\#}$	1.6$^{\#\#}$	4.8$^{\#\#}$	–**	–	–	–
100 (Probenecid)	–	–	–	–	7.8$^{\#\#}$	5.5$^{\#\#}$	15.4$^{\#\#}$	12.7$^{\#\#}$

OATP1B1- and OATP1B3-mediated uptake volumes of [^{3}H]$E_2$17βG are the values in transporter-expressing HEK293 cells minus the corresponding values in non-expressing control HEK293 cells. OAT1- and OAT2-mediated uptake volumes of [^{3}H]PAH and [^{3}H]E3S are the values in transporter-expressing S_2 cells minus the corresponding values in non-expressing control S_2 cells. The % of control represents the ratio of the uptake volume in the presence of inhibitor to that in the absence of inhibitor (concentration 0 μmol/L).
The statistical significance of differences for the test compound and positive control compound for inhibition studies was determined by the Williams test (lower-tailed) and unpaired *t*-test (Student's or Welch), respectively, using SAS software (Release 9.4). $^{¶}p < 0.05$, $^{¶¶}P < 0.01$, $^{\#\#}p < 0.01$.
The uptake volume and the % of control are expressed as mean ± SD or mean of triplicate determinations.
*The value in the parenthesis represents the uptake volume into non-expressing cells, indicating large transporter-mediated uptake by a comparison of transporters-expressing and non-expressing cells. **–: not tested.

values of 8.39 and 9.13 μmol/L, respectively, whereas brexpiprazole and DM-3411 at 30 μmol/L had no effect on the [^{3}H]$E_2$17βG uptakes by OATP1B3 (Tables 4 and 7). Brexpiprazole did not affect the [^{3}H]PAH and [^{3}H]E3S uptakes by OAT1- and OAT3-expressing S_2 cells, respectively, whereas DM-3411 diminished their uptake slightly (Table 4).

Brexpiprazole and DM-3411 inhibited the uptakes of [^{14}C]metformin by OCT1-, OCT2-, MATE1-, or MATE2-K-expressing HEK293 cells (Table 5). The IC_{50} values for brexpiprazole and DM-3411 were similar with the exception of MATE2-K, in which the IC_{50} value of DM-3411 was 10-fold lower than that of brexpiprazole (0.154 μmol/L for DM-3411 and 1.57 μmol/L for brexpiprazole, Table 7).

Inhibition of BSEP vesicular transports by brexpiprazole and DM-3411

Brexpiprazole and DM-3411 at 30 μmol/L slightly inhibited the BSEP-mediated uptake of [^{3}H]TCA into vesicles which was measured by subtracting the uptake into control vesicles from that into BSEP-expressing vesicles (Table 6 and 7).

Discussion

Brexpiprazole is mainly eliminated through metabolism by CYP3A4 and CYP2D6 (Sasabe *et al.* 2021). When brexpiprazole was concomitantly administered with strong inhibitors,

Table 5. Inhibitory effects of brexpiprazole and its metabolites, DM-3411, on OCT-mediated [^{14}C]metformin uptakes, MATE-mediated [^{14}C]metformin uptakes.

Concentration (μmol/L)	Uptake volume into OCT1-expressing cells (μL/mg protein/5 min)		Uptake volume into OCT2-expressing cells (μL/mg protein/2 min)		Uptake volume into MATE1-expressing cells (μL/mg protein/5 min)		Uptake volume into MATE2-K-expressing cells (μL/mg protein/5 min)	
	4.91 ± 0.64 (1.18 ± 0.03)*	7.45 ± 0.50 (1.83 ± 0.13)*	76.2 ± 1.0 (0.64 ± 0.06)*	68.4 ± 2.0 (0.67 ± 0.12)*	18.3 ± 0.9 (1.88 ± 0.22)*	20.8 ± 2.1 (1.57 ± 0.24)*	10.9 ± 0.9 (1.06 ± 0.04)*	
	% of control							
0 (Control)								
Inhibitor	Brexpiprazole	DM-3411	Brexpiprazole	DM-3411	Brexpiprazole	DM-3411	Brexpiprazole	DM-3411
0.1	87.4	101.6	79.7	96.5	99.6	77.9¶¶	83.8¶¶	59.0¶¶
0.3	73.7¶	102.1	105.6	103.7	78.4¶¶	80.4¶¶	75.4¶¶	39.3¶¶
1	74.8¶	82.2	99.7	102.8	71.1¶¶	55.0¶¶	47.4¶¶	16.6¶¶
3	84.4¶	110.7	82.6¶¶	96.0	45.9¶¶	35.3¶¶	39.6¶¶	12.4¶¶
10	71.1¶¶	82.5	40.3¶¶	50.0¶¶	32.3¶¶	17.0¶¶	11.6¶¶	4.9¶¶
30	18.4¶¶	27.9¶¶	35.0¶¶	39.1¶¶	13.2¶¶	7.3¶¶	5.1¶¶	2.2¶¶
100 (Quinidine)	−6.0$^{##}$	0.8$^{##}$	1.2$^{##}$	1.1$^{##}$	–**	–	–	–
10					15.6$^{##}$	14.1$^{##}$		
100 (Cimetidine)							10.7$^{##}$	

OCT1-, OCT2-, MATE1-, and MATE2-K-mediated uptake volumes of [^{14}C]metformin are the value in transporter-expressing HEK293 cells minus the corresponding values in non-expressing control HEK293 cells. The % of control represents the ratio of the uptake volume in the presence of inhibitor to that in the absence of inhibitor (concentration, 0 μmol/L).
The statistical significance of differences for the test compound and positive control compound for inhibition studies was determined by the Williams test (lower-tailed) and unpaired *t*-test (Student's or Welch), respectively, using SAS software (Release 9.4).$^{¶}p < 0.05$,$^{¶¶}P < 0.01$,$^{##}p < 0.01$.
The uptake volume and the % of control are expressed as mean ± SD or mean of triplicate determinations.
*The value in the parenthesis represents the uptake volume into non-expressing cells, indicating large transporter-mediated uptake by a comparison of transporter-expressing and non-expressing cells. **–: not tested.

Table 6. Inhibitory effects of brexpiprazole and its metabolite, DM-3411, on BSEP-mediated vesicular uptake of [^{3}H]TCA.

Concentration (μmol/L)	Uptake volume into BSEP-expressing vesicles (μL/mg protein/2 min)	
0 (Control)	341.5 ± 65.5	340.2 ± 102.0
	% of control	
Inhibitor	brexpiprazole	DM-3411
0.3	99.1	83.7
1	134.4	108.8
3	119.7	102.5
10	103.9	81.8
30	66.4¶	54.0¶¶
30 (Cyclosporin A)	0.0$^{#}$	9.1$^{#}$

The BSEP-mediated uptake volume of [^{3}H]TCA is the value in the BSEP-expressing vesicles minus that in the non-expressing control vesicles.
The % of control represents the ratio of the uptake volume in the presence of inhibitor to that in the absence of inhibitor (concentration 0 μmol/L).
The statistical significance of differences for the test compound and positive control compound for inhibition studies was determined by the Williams test (lower-tailed) and unpaired *t*-test (Student's or Welch), respectively, using Statistical SAS software (Release 9.4). $^{¶}p < 0.05$, $^{¶¶}P < 0.01$, $^{#}p < 0.05$.
The uptake volume and the % of control are expressed as mean ± SD or mean of triplicate determinations.

itraconazole (CYP3A4) or quinidine (CYP2D6), both of AUC values increased to approximately two fold (Garnock-Jones 2016), respectively. Additionally, when brexpiprazole was also administered to the poor metaboliser of CYP2D6, the AUC value was approximately twice of that in the extensive metaboliser (Ishigooka *et al.* 2018). Therefore, CYP3A4 and CYP2D6 are thought to contribute equally to the metabolic elimination profile of brexpiprazole. In the labelling sheet of brexpiprazole, the results were summarised as 'the dosage adjustments of brexpiprazole are recommended when brexpiprazole is administered with strong CYP2D6 inhibitors (e.g. paroxetine, fluoxetine, and quinidine) or to CYP2D6 poor metaboliser'. Conversely as demonstrated in this study, brexpiprazole and DM-3411 exhibited weak inhibitory effects on CYP2B6, CYP2D6, CYP2C9, CYP2C19, and CYP3A4 with IC_{50} values sufficiently higher than the unbound C_{max} in human (Table 1). For each these $C_{max,u}/IC_{50}$ ratios were less than 0.02 which represents the regulatory cut-off value for potential DDI risk (EMA 2012, PMDA 2019, U.S. Department of Health and Human Services 2020). It is important to note that the f_u value is 0.01, as in humans brexpiprazole is highly protein bound (99.8%) (Sasabe *et al.* 2021). Moreover, brexpiprazole has a weak potency of mechanism-based inactivation (MBI) against CYP3A4. The DDI which resulted from MBI is known to occur after the dosing period of the inhibitory agent. When bupropion (CYP2B6 substrate), dextromethorphan (CYP2D6), or lovastatin (CYP3A4) were administered with multiple doses of brexpiprazole in a clinical DDI study (Otsuka America Pharmaceutical, Inc 2015), the AUC values of these three agents were not changed, when compared to each alone.

In the *in vitro* CYP induction studies using human hepatocytes, brexpiprazole did not exhibit strong induction potency for CYP1A2, CYP2B6, CYP2C9, or CYP3A4 (Table 2). For *in vitro* evaluation of drug–drug interaction for an NME, CYP enzymes CYP3A4, CYP2B6 and CYP1A2 should always be included as markers of induction in the basic methodology of CYP induction as described by the EMA (2012) guideline. This approach is based on the molecular mechanisms of CYP induction *via* nuclear receptors of pregnane X receptor (PXR, for CYP3A4 and CYP2C9), constitutive androstane receptor (CAR, for CYP2B6) and the aryl hydrocarbon receptor (AhR, for CYP1A2). Recently, CYP2D6 has been reported to be inducible in one paper (Farooq *et al.* 2016). However, this induction is currently limited to endogenous and exogenous corticosteroids and rifampin, and its exact inductive molecular mechanisms and direct implications for CYP2D6 induction in a clinical setting are still not clear. Though brexpiprazole

Table 7. Summary of the inhibitory effects of brexpiprazole and its metabolite, DM-3411, on ABC transporters and SLC transporters.

	Brexpiprazole	DM-3411	Brexpiprazole			DM-3411
Transporters	IC_{50} [SE] (μmol/L)		Dose/250 mL/IC_{50}*	$C_{inlet,max,u}/IC_{50}$*	$C_{max,u}/IC_{50}$*	$C_{max,u}/IC_{50}$*
MDR1	6.31 [2.38]	7.84 [5.04]	5.8	0.0015	0.00060	0.00066
BCRP	1.16 [0.32]	3.04 [1.56]	32	0.0084	0.0033	0.0017
OATP1B1	8.39 [5.57]	9.13 [2.49]	–**	0.0012	0.00045	0.00057
OATP1B3	>30***	>30	–	–	–	
OAT1	>30	>30	–	–	–	
OAT3	>30	>30	–	–	–	
OCT1	13.0 [9.3]	11.4 [2900] ¶	–	0.00075	0.00029	0.00046
OCT2	4.27 [1.82]	6.50 [0.75]	–	–	0.00089	0.00080
MATE1	2.79 [1.65]	1.83 [1.07]	–	0.0035	0.0014	0.0028
MATE2-K	1.57 [0.77]	0.154 [0.014]	–	–	0.0024	0.034
BSEP	>30	>30	–	–	–	–

The value and inequality sign indicate the maximum concentration of the test compound in the inhibition study when 50% inhibition was not observed even at the maximum concentration. The IC_{50} and the estimated SE were obtained by the nonlinear regression of the mean remaining activity (% of control, triplicate determinations) from the control (0 μmol/L) to the E_{max} model equation, using WinNonlin (Ver. 6.1).

* The parameters when brexpiprazole was administered to Japanese patients at 4 mg for 14 days were used to calculate the ratios for drug–drug interaction (Ishigooka et al. 2018). The $C_{max,u}/IC_{50}$ value was calculated using free fraction (f_u: 0.01 for brexpiprazole, 0.035 for DM-3411) and plasma C_{max} (0.38 μmol/L for brexpiprazole and 0.15 μmol/L for DM-3411) . $C_{inlet,max,u}$ was calculated using the equation $f_u \times [C_{max} + (k_a \times \text{dose}/Q_h)]$, where the absorption rate constant (k_a: 0.1 min^{-1}, regulatory maximum) was obtained from plasma concentration profiles and hepatic blood flow (Q_h: 23.4 mL/min/kg).

–: not applicable. *>30: Inhibitory effects less than 50% at 30 μmol/L of the test compound.

¶The estimated SE is large compared to the IC_{50} value. The two point values at the lower concentration of the test compound are poorly fitted because they show the maximum variation that could be tolerated, resulting in a large computer estimate of the SE value. As there was sufficient inhibition at 30 μmol/L which is a higher concentration than the estimated IC_{50}, we concluded that was confirmed that the estimated IC_{50} value could be adopted.

was mainly metabolised by CYP2D6 and CYP3A4, given this background, we performed the CYP induction study with respect to CYP1A2, CYP2B6 and CYP3A4, based on the molecular mechanisms of CYP induction, not self-induction. For CYP1A2, CYP2C9 and CYP3A4, both mRNA level and activity were measured and compared to levels in untreated control hepatocytes. The results indicated very similar conclusions that brexpiprazole has no induction potency. For CYP2B6, changes in the mRNA level were hardly observed on treatment with brexpiprazol. Considering that mRNA is a much more sensitive method that provides greater dynamic range, while showing a good relationship with enzyme activity (Fahmi and Ripp 2010), the enzyme activity of CYP2B6 is also expected not to be changed by drug treatment.

The more hepatocyte sample are used in the testing, the better the system will be. For *in vitro* evaluation of drug–-drug interactions for a New Molecular Entity (NME), the FDA, EMA, and PMDA specify use of hepatocytes derived from three or more donors, since cultured human hepatocytes have great inter-individual variability or lot-to-lot variation (EMA 2012, PMDA 2019, U.S. Department of Health and Human Services 2020). With this background, we used hepatocytes from three donors under established and validated experimental procedures (Supplementary Tables 3 and 4), with which the increase seen with control inducers could be observed at a level that is comparable to reported results, in this study and also in previous in-house studies. Therefore, we believe that the results presented here could verify the inductive potency of test compounds. In addition, many results using hepatocytes from three individuals have been reported from pharmaceutical company researchers and academic researchers, just as in our experiment, and the results of *in vitro* studies are conclusive. From the results obtained in these studies, one can conclude that brexpiprazole and its primary metabolie, DM-3411, will have low to negligible DDI risk for CYP enzymes in the clinical setting.

Brexpiprazole was transported neither by P-gp nor by BCRP (Figure 2), which limit the absorption of oral hydrophobic compounds in the apical membrane of the intestine. These *in vitro* results are consistent with the report that the bioavailability of brexpiprazole in human is nearly 100%, and the absorptive fraction is thought to complete when brexpiprazole is orally administered (Garnock-Jones 2016). Contrarily, the inhibition of brexpiprazole for the transport functions of P-gp and BCRP was observed in *in vitro* transport studies across the expressing cell monolayers. When their IC_{50} values were compared with the expected maximum concentration in the gut lumen after oral dosing of 4 mg (4 mg ÷ 250 mL = 37 μmol/L), the ratios were greater than 10, which is the cut-off position for three regulatory agencies for conduction of clinical study to evaluate of DDI risk (Table 7). It may follow that brexpiprazole has a potential for DDI risk by P-gp and BCRP. However, the AUC values of fexofenadine and rosuvastatin, which are substrates for P-gp and BCRP, respectively, were not changed when both were co-administered with brexpiprazole in a clinical DDI study (Otsuka America Pharmaceutical, Inc 2015).

When inhibition or transport studies of intestinal transporters such as P-gp and BCRP, are conducted *in vitro*, these evaluations are required at a broad range of inhibitor and substrate's concentrations, considering the physiologically relevant concentrations described above. However, the inhibition of brexpiprazole was evaluated in the experiment of the inhibitor concentration until 30 μmol/L, which is limited due to the solubility in the transport buffer. But, the IC_{50} values of brexpiprazole could be obtained accurately. Meanwhile, the transport experiment of brexpiprazole was performed in the cell monolayers only at low donor

concentrations of 1 μmol/L. In the cell monolayers, the membrane permeability generally constitutes passive diffusion and transporter-mediated efflux. Thereby, the function of the transporter at high concentration may be diminished by saturation. For example, the apical to basal permeability of the substrates for P-gp (quinidine, verapamil, and vinblastine) increased when their apical side concentration was increased and reached a maximum value at the higher concentration range, whereas the basal to apical permeability was decreased (Shirasaka *et al.* 2008). These results demonstrated that saturation of the efflux transporters would decrease the permeability ratio, suggesting that the assay for a substrate of efflux transporters would be less sensitive at higher concentrations. It was confirmed that brexpiprazole is not a substrate of P-gp and BCRP at a sufficiently low concentration (1 μmol/L); thereby, it is considered that brexpiprazole would not be a substrate of these transporters even when testing at assumed higher concentrations in the gut lumen.

Next, when the interaction risks of DM-3411 for P-gp and BCRP are considered by comparison of the compound concentration with IC_{50}, the systemic unbound concentration should be used rather than the concentration in the gut lumen, because, the metabolite is produced in the systemic compartment after absorption. The ratios of systemic $C_{max,u}$ to the IC_{50} values are lower than 0.02, which is 3 regulator's criteria (Table 7), and are sufficiently low, thus not affecting the transport function of P-gp and BCRP. The P-gp- and BCRP-mediated interactions of brexpiprazole and the main metabolite, DM-3411, are considered to be not induced in the clinical setting.

Brexpiprazole is mainly eliminated through metabolism, as the parent compound was not detected in human urine probably due to its high lipophilic property and high protein binding (Garnock-Jones 2016). Brexpiprazole has undergone several oxidative reactions, for example, hydroxylation, dehydrogenation, and N-dealkylation. These reactions usually occurred in the liver after transferring to the inside of hepatocytes. SLC transporters, OATPs and OCT1 recognise anionic organic compounds and cationic organic compounds, respectively, on the plasma membrane of hepatocytes. These transporters take in such substrates into the inside of hepatocytes from circulating blood. As a result, they accelerate the subsequent hepatic metabolism (Izumi *et al.* 2017). Brexpiprazole is not a substrate for any uptake transporters (Figures 2 and 3). Thus, it is considered that brexpiprazole transfers through the plasma membrane only by passive diffusion. This evidence suggests that brexpiprazole has hardly undergone the uptake transporter-mediated pharmacokinetic changing by any concomitant agents. On the contrary, brexpiprazole and DM-3411 exhibit inhibitory effects on the transport activities of OATP1B1, OCT1, OCT2, MATE1, and MATE2-K in the *in vitro* studies on transporter-expressing cells (Table 7). Except MATE2-K, the ratio values of systemic concentration and IC_{50} values were much lower than 0.1 (FDA, PMDA guideline's cut-off) and 0.02, (EMA's cut-off) (Table 7). Thereby, DDI through these uptake transporters is not considered to be induced in the clinical concomitant dosing with brexpiprazole. For the MATE2-K inhibition of DM-3411, the ratio (0.034) of the maximum plasma unbound concentration and IC_{50} values are above the regulators' cut-off (0.02). The accurate prediction of interaction for MATEs is challenging, because MATEs and OCT2 are thought to often share the same substrate, and the inhibition of MATEs may cause only an increase in intracellular concentration of substrates, not reflecting the systemic exposure (Yoshida *et al.* 2017). Although the systemic unbound concentration (5.3 nmol/L) is sufficiently much lower than the IC_{50} value, a close observation on the renal function may be needed when a MATE substrate, like the antidiabetic agent, metformin is concomitantly administered in the clinic.

BSEP is expressed on the bile canalicular membrane of hepatocytes and functions as an exporter of bile acids to bile. Its drug-mediated functional dysfunction leads to the intrahepatic accumulation of bile acids, which results in severe liver damage, a form of drug-induced liver injury (DILI) (Funk *et al.* 2001, Yoshikado *et al.* 2013). Brexpiprazole did not inhibit BSEP and therefore, may have a low clinical risk for DILI due to the function of BSEP.

In summary, in the *in vitro* assessments using the human hepatic microsomes, brexpiprazole and its major metabolite, DM-3411 exhibited weak inhibitory effects on the activities of several CYPs (CYP2B6, CYP2D6, CYP2C9, CYP2C19, and CYP3A4). In the comparisons with the IC_{50} (or K_i value) and the systemic concentration ($C_{max,u}$ or $C_{inlet,max,u}$), the ratio was low and therefore, not believed to induce any drug interaction when a potential CYP substrate is concomitantly administered with brexpiprazole. In studies using human transporter-expressing-cells, brexpiprazole exhibited weak or negligible inhibitory effects at the hepatic and renal SLC transporters (OATPs, OCTs, OATs, and MATEs) and efflux transporters (MDR1, BCRP, and BSEP) investigated. When a comparison of IC_{50} to systemic $C_{max,u}$ or $C_{inlet,max,u}$ is performed, the risk for DDI is considered to be minimal, this is also true for CYPs, as well. Overall, brexpiprazole did not exhibit relevant changes in the clinical DDI studies performed together with their CYP or transporter substrates. These *in vitro* data suggest that brexpiprazole is unlikely to cause clinically relevant drug interactions resulting from the effects on CYPs or transporters mediating the absorption, metabolism, and/or disposition of co-administered drugs. These findings are particularly significant for patients with mental disorders who are treated with brexpiprazole in addition to other medications to manage their psychiatric symptoms.

Disclosure statement

The authors report no declarations of interest.

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