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Influence of breast cancer resistance protein and P-glycoprotein on tissue distribution and excretion of Ko143 assessed with PET imaging in mice

Severin Mairinger^a, Viktoria Zoufal^a, Thomas Wanek^a, Alexander Traxl^a, Thomas Filip^a, Michael Sauberer^a, Johann Stanek^{a,b}, Claudia Kuntner^a, Jens Pahnke^{c,d,e}, Markus Müller^b, and Oliver Langer^{a,b,f,*}

^aBiomedical Systems, Center for Health & Bioresources, AIT Austrian Institute of Technology GmbH, Seibersdorf, Austria

^bDepartment of Clinical Pharmacology, Medical University of Vienna, Vienna, Austria

^cDepartment of Neuro-/Pathology, University of Oslo (UiO) and Oslo University Hospital (OUS), Oslo, Norway

^dLIED, University of Lübeck, Germany

^eLeibniz-Institute of Plant Biochemistry, Halle, Germany

^fDivision of Nuclear Medicine, Department of Biomedical Imaging and Image-guided Therapy, Medical University of Vienna, Vienna, Austria

Abstract

Ko143 is a reference inhibitor of the adenosine triphosphate-binding cassette (ABC) transporter breast cancer resistance protein (humans: ABCG2, rodents: Abcg2) for *in vitro* and *in vivo* use. Previous *in vitro* data indicate that Ko143 binds specifically to ABCG2/Abcg2, suggesting a potential utility of Ko143 as a positron emission tomography (PET) tracer to assess the density (abundance) of ABCG2 in different tissues. In this work we radiolabeled Ko143 with carbon-11 (¹¹C) and performed small-animal PET experiments with [¹¹C]Ko143 in wild-type, Abcg2^(-/-), Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice to assess the influence of Abcg2 and Abcb1a/b on tissue distribution and excretion of [¹¹C]Ko143.

[¹¹C]Ko143 was extensively metabolized *in vivo* and unidentified radiolabeled metabolites were found in all investigated tissues. We detected no significant differences between wild-type and Abcg2^(-/-) mice in the distribution of [¹¹C]Ko143-derived radioactivity to Abcg2-expressing organs (brain, liver and kidney). [¹¹C]Ko143 and possibly its radiolabeled metabolites were transported by Abcb1a and not by Abcg2 at the mouse blood-brain barrier. [¹¹C]Ko143-derived radioactivity underwent both hepatobiliary and urinary excretion, with Abcg2 playing a possible role in mediating the transport of radiolabeled metabolites of [¹¹C]Ko143 from the kidney into urine. Experiments in which a pharmacologic dose of unlabeled Ko143 (10 mg/kg) was co-

*Corresponding author at: Department of Clinical Pharmacology, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria oliver.langer@meduniwien.ac.at (O. Langer).

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administered with [^{11}C]Ko143 revealed pronounced effects of the vehicle used for Ko143 formulation (containing polyethylene glycol 300 and polysorbate 80) on radioactivity distribution to the brain and the liver, as well as on hepatobiliary and urinary excretion of radioactivity.

Our results highlight the challenges associated with the development of PET tracers for ABC transporters and emphasize that inhibitory effects of pharmaceutical excipients on membrane transporters need to be considered when performing *in vivo* drug-drug interaction studies. Finally, our study illustrates the power of small-animal PET to assess the interaction of drug molecules with membrane transporters on a whole body level.

Keywords

PET; [^{11}C]Ko143; breast cancer resistance protein; P-glycoprotein; blood-brain barrier; pharmacokinetic disposition; pharmaceutical excipients

1 Introduction

Breast cancer resistance protein (humans: ABCG2, rodents: Abcg2) is an adenosine triphosphate-binding cassette (ABC) transporter which can recognize many different, structurally unrelated drugs and drug metabolites as its substrates and thereby exert a major influence on drug disposition (Mao and Unadkat, 2015). In the canalicular (bile-facing) membrane of hepatocytes and in the brush-border membrane of kidney proximal tubule cells, ABCG2 promotes the excretion of drugs and drug metabolites into bile and urine, respectively. In the epithelium of the small intestine, ABCG2 restricts absorption of orally administered drugs from the intestinal lumen, and at the blood-brain barrier (BBB), ABCG2 - mostly in cooperation with P-glycoprotein (humans: ABCB1, rodents: Abcb1a/b) - limits the brain distribution of many drugs. Drug-drug interactions, single nucleotide polymorphisms in the gene encoding ABCG2 and many diseases may change ABCG2 expression and function, which may cause variability in the pharmacokinetics and tissue distribution of ABCG2 substrates, potentially affecting drug safety and drug efficacy (Giacomini et al., 2010).

An emerging tool to study ABC transporters *in vivo* is positron emission tomography (PET) with carbon-11- (^{11}C , half-life: 20.4 min) or fluorine-18- (^{18}F , half-life: 109.8 min) labeled transporter substrates (Wanek et al., 2013; Raaphorst et al., 2015; Langer, 2016). Whereas effective PET tracers for measuring ABCB1 function both at the rodent and human BBB have been developed ((*R*)-[^{11}C]verapamil and [^{11}C]N-desmethyl-loperamide), the development of ABCG2-selective PET tracers is hindered by the overlapping substrate specificities between ABCG2 and ABCB1. Although some dual ABCB1/ABCG2 substrates have been proposed for PET imaging (e.g. [^{11}C]tariquidar, [^{11}C]elacridar, [^{11}C]erlotinib, [^{11}C]gefitinib and [^{18}F]gefitinib), only few attempts have been made so far to develop ABCG2-selective PET tracers (Mairinger et al., 2010; Takada et al., 2010; Hosten et al., 2013; Takashima et al., 2013). One approach to develop PET tracers for ABC transporters is the radiolabeling of prototypical transporter inhibitors, as illustrated by [^{11}C]elacridar, [^{11}C]tariquidar and [^{11}C]laniquidar (Wanek et al., 2013; Raaphorst et al., 2015). For

ABCG2, however, there is a scarcity of selective inhibitors available for *in vivo* use (Lee et al., 2015).

The fumitremorgin C derivative Ko143 [(3*S*,6*S*,12*aS*)-1,2,3,4,6,7,12,12*a*-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxypyrazino[1',2':1,6]pyrido[3,4-*b*]indole-3-propanoic acid 1,1-dimethylethyl ester] (Allen et al., 2002) is a potent and relatively selective reference ABCG2 inhibitor which is commonly used for *in vitro* experiments and which has also been used *in vivo* in preclinical species (Giri et al., 2008; Chen et al., 2009; Wanek et al., 2012; Bakhsheshian et al., 2013). In addition to being a potent ABCG2 inhibitor with nanomolar half-maximum inhibitory concentrations, Ko143 at higher, micromolar concentrations also inhibits ABCB1 and ABCC1 (Allen et al., 2002; Matsson et al., 2009; Weidner et al., 2015). Moreover, there is evidence that Ko143 inhibits solute carrier (SLC) transporters of the SLCO family (Karlgren et al., 2012). Ko143 is rapidly metabolized *in vivo* through enzymatic hydrolysis of the *tert*-butyl ester group into its free acid, which was found to lack an inhibitory effect on ABCG2 and ABCB1 (Weidner et al., 2015; Liu et al., 2017). Although some preliminary data are available on Ko143 tissue distribution in mice (Liu et al., 2012; Liu et al., 2017), the influence of ABCG2 and ABCB1 on disposition of Ko143 has not yet been systematically assessed. Weidner et al. performed *in vitro* uptake experiments with low concentrations of [³H]Ko143 in cell lines overexpressing different ABC transporters (ABCG2, Abcg2, ABCB1, Abcb1a and multidrug resistance-associated protein 1, ABCC1) (Weidner et al., 2015). Whereas there was no difference in cellular accumulation of [³H]Ko143 between ABCB1-, Abcb1a- and ABCC1-overexpressing and control cells, respectively, they found an approximately 2-fold higher accumulation of [³H]Ko143 in ABCG2- and Abcg2-overexpressing cells, which was displaceable by an excess of unlabeled Ko143 or fumitremorgin C. This led them to conclude that Ko143 binds specifically to ABCG2/Abcg2, suggesting a potential utility of Ko143 as a PET tracer for measuring the density of ABCG2 in different tissues (Weidner et al., 2015). In this work, we performed PET imaging with ¹¹C-labeled Ko143 in mice to assess the influence of Abcg2 and Abcb1a/b on tissue distribution and excretion of Ko143 and to assess the suitability of [¹¹C]Ko143 as a PET tracer for ABCG2.

2 Material and methods

2.1 Chemicals

Unless otherwise stated, all chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Schnelldorf, Germany). Ko143 was obtained from Axon Medchem BV (Groningen, The Netherlands). Before each administration, Ko143 was dissolved in dimethyl sulfoxide (DMSO) and diluted with a mixture of sterile water / polyethylene glycol 300 (PEG300) / polysorbate 80 (65/25/10, v/v/v) to a final DMSO concentration of 5% (v/v). Formulated Ko143 solution was mixed with radiotracer dissolved in the same vehicle and the mixture was injected intravenously (i.v.) into mice at a volume of 4 mL/kg body weight. The ABCB1 inhibitor tariquidar dimesylate was freshly dissolved in 2.5% (w/v) aqueous (aq.) dextrose solution before each administration and injected i.v. into mice at a volume of 4 mL/kg. [¹¹C]Methane was produced in a PETtrace cyclotron equipped with a methane target system (GE Healthcare, Uppsala, Sweden). [¹¹C]Methyl iodide was prepared

in a TRACERlab FX C Pro synthesis module (GE Healthcare) and converted into [^{11}C]methyl triflate as described before (Jewett, 1992). The radiolabeling precursor 9-*O*-desmethyl-Ko143 was synthesized at the Department of Medicinal Chemistry (University of Vienna) as described elsewhere (Mairinger, 2016).

2.2 Radiotracer synthesis

Using a TracerLab FX C Pro synthesis module, [^{11}C]methyl triflate was led into a solution of 9-*O*-desmethyl-Ko143 (0.15 mg, 0.33 μmol) in acetonitrile (0.3 mL) containing aq. sodium hydroxide solution (2M, 3 μL , 6 μmol , 18 equivalents). After heating for 5 min at 75°C, the reaction mixture was cooled (40°C), diluted with 10 mM aq. hydrochloric acid (0.7 mL) and injected into a built-in high-performance liquid chromatography (HPLC) system. A Chromolith Performance RP 18-e (100 x 10 mm, 5 μm) HPLC column equipped with a Chromolith RP 18-e guard column (10 x 10 mm) (Merck KGaA, Darmstadt, Germany) was eluted with acetonitrile / water (45/55, v/v) at a flow rate of 2.5 mL/min. The HPLC eluate was monitored in series for radioactivity and ultraviolet (UV) absorption (wavelength: 220 nm). Radiolabeling precursor 9-*O*-desmethyl-Ko143 and product [^{11}C]Ko143 eluted with retention times of 7.3 min and 15.9 min, respectively. The product fraction was diluted with water (100 mL) and passed over a pre-conditioned (5 mL of ethanol and subsequently 10 mL of water) C18 Sep-Pak Plus cartridge (Waters, Milford, MA, USA). The cartridge was then washed with water (10 mL) and [^{11}C]Ko143 was eluted with ethanol (3 mL). The ethanol was then removed on a rotary evaporator. For i.v. injection into animals, the product was formulated at an approximate concentration of 370 MBq/mL in either 0.9% aq. saline solution / polysorbate 80 (100/0.1, v/v) for baseline scans, or in a mixture of sterile water / PEG300 / polysorbate 80 / DMSO (60/25/10/5, v/v/v/v) for scans in which unlabeled Ko143 was co-injected. Radiochemical purity and molar radioactivity of [^{11}C]Ko143 were determined by analytical radio-HPLC using an Agilent Eclipse XDB C18 (150 x 4.6 mm) column eluted with acetonitrile containing 0.1% aq. ammonium hydroxide solution / 0.1% aq. ammonium hydroxide solution (45/55, v/v) at a flow rate of 1 mL/min. UV detection was performed at a wavelength of 228 nm and fluorescent detection with excitation and emission wavelengths set at 295 nm and 350 nm, respectively. The retention time of [^{11}C]Ko143 was 9.6 min on this HPLC system. (*R*)-[^{11}C]verapamil was synthesized as described elsewhere (Brunner et al., 2005) and formulated at a concentration of 370 MBq/mL in the same vehicle as [^{11}C]Ko143 for co-injection with unlabeled Ko143.

2.3 Animals

Female wild-type, *Abcg2*^(-/-), *Abcb1a/b*^(-/-) and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice with a FVB genetic background were obtained from Taconic Biosciences Inc. At the time of experiment, animals were 7-8 weeks old and weighed 25 ± 2 g. An acclimatization period of at least 1 week was allowed before the animals were used in the experiments. The study was approved by the national authorities (Amt der Niederösterreichischen Landesregierung) and study procedures were in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU).

2.4 Experimental design

An overview of animal groups examined with [^{11}C]Ko143 is given in Table 1. Each animal received a maximum of two radiotracer injections within an interval of at least 7 days between the two injections. Groups of wild-type, *Abcg2*^(-/-), *Abcb1a/b*^(-/-) and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice each received a baseline PET scan with [^{11}C]Ko143 followed by a second PET scan in which [^{11}C]Ko143 was co-injected with unlabeled Ko143 (10 mg/kg). Additional groups of wild-type mice underwent [^{11}C]Ko143 PET scans with co-injection of Ko143 vehicle or after pre-treatment with the ABCB1 inhibitor tariquidar at a dose which was shown to lead to complete inhibition of *Abcb1a* at the mouse BBB (15 mg/kg administered i.v. at 2 h before the PET scan) (Wanek et al., 2015). In addition, wild-type and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice were used for determination of radiolabeled metabolites of [^{11}C]Ko143. Furthermore, one group of wild-type mice ($n = 4$) underwent PET scans with the ABCB1 substrate radiotracer (*R*)-[^{11}C]verapamil, which was co-injected with unlabeled Ko143 (10 mg/kg).

2.5 PET imaging

Imaging experiments were performed under isoflurane anesthesia. Animals were warmed throughout the experiment and body temperature and respiratory rate were constantly monitored. Mice were placed in a custom-made imaging chamber and the lateral tail vein was cannulated for i.v. administration. A microPET Focus220 scanner (Siemens Medical Solutions, Knoxville, TN, USA) was used for PET imaging. Dynamic emission scans (60 min) were started with the i.v. injection of [^{11}C]Ko143 alone (26.6 ± 10.5 MBq in a volume of 0.1 mL, corresponding to 0.59 ± 0.52 nmol of unlabeled Ko143) or [^{11}C]Ko143 (26.5 ± 6.4 MBq in a volume of 0.15 mL) mixed with a pharmacologic dose of unlabeled Ko143 (10 mg/kg). For injection of [^{11}C]Ko143 alone, the employed vehicle resulted in an administered dose of polysorbate 80 of approximately 4 mg/kg. For co-injection of unlabeled Ko143, the employed vehicle resulted in administered doses of approximately 330 mg/kg for DMSO, 640 mg/kg for polysorbate 80 and 1690 mg/kg for PEG300. In addition, dynamic 60-min emission scans were performed with the i.v. injection of (*R*)-[^{11}C]verapamil (47.7 ± 12.5 MBq in a volume of 0.15 mL, corresponding to 0.76 ± 0.06 nmol of unlabeled verapamil) mixed with a pharmacologic dose of unlabeled Ko143 (10 mg/kg). List-mode data were acquired with a timing window of 6 ns and an energy window of 250-750 keV. At the end of the PET scan, a blood sample (20 - 30 μL) was collected from the retro-orbital sinus and animals were sacrificed by cervical dislocation under deep anesthesia.

2.6 PET data analysis

The dynamic PET data were sorted into 23 frames, which incrementally increased in time length. PET images were reconstructed using Fourier re-binning of the 3-dimensional sinograms followed by a 2-dimensional filtered back-projection with a ramp filter giving a voxel size of $0.4 \times 0.4 \times 0.796$ mm³. Using AMIDE software (Loening and Gambhir, 2003), left kidney, duodenum, intestine, liver, brain, gall bladder and urinary bladder were manually outlined on the PET images to derive concentration-time curves expressed in percent of the injected dose per gram or milliliter (%ID/g or %ID/mL). In addition, the left ventricle of the

heart was outlined to obtain an image-derived blood curve. It was assumed, that the sum of radioactivity in the gallbladder, the duodenum and the intestine represented biliary radioactivity excreted from the liver. A graphical analysis method (integration plot) was used to estimate the rate constants for transfer of radioactivity from blood into brain ($k_{\text{uptake,brain}}$, mL/min/g brain), liver ($k_{\text{uptake,liver}}$, mL/min/g liver) and kidney ($k_{\text{uptake,kidney}}$, mL/min/g kidney), using data measured from 0.3 min - 3.5 min after radiotracer injection, as described in detail elsewhere (Traxl et al., 2017). Moreover, the rate constants for excretion of radioactivity from kidney into urine (k_{urine} , min⁻¹) and from liver into bile (k_{bile} , min⁻¹) were determined from 4.5 min - 45 min after radiotracer injection (Traxl et al., 2017). Total distribution volume (V_T) of [¹¹C]Ko143 in brain, liver and kidney was estimated by using Logan graphical analysis over the interval of 6.25 min - 45 min after radiotracer injection (Logan et al., 1990). V_T corresponds to the organ-to-blood ratio of radioactivity at steady state.

2.7 Analysis of radiolabeled metabolites

Radiolabeled metabolites of [¹¹C]Ko143 were assessed in two dedicated groups of wild-type ($n = 3$) and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) ($n = 4$) mice. [¹¹C]Ko143 (28.4 ± 4.1 MBq in a volume of 0.1 mL, corresponding to 1.24 ± 0.53 nmol of unlabeled Ko143) was administered i.v. under isoflurane anesthesia. After a period of 20 min, blood was collected from the retro-orbital sinus and animals were sacrificed by cervical dislocation under deep anesthesia. Liver, kidneys, brain, bile and urine were collected. Blood was centrifuged to obtain plasma. Plasma proteins were precipitated by the addition of 1 μ L acetonitrile per μ L plasma. Liver, kidneys and brain were homogenized and proteins were precipitated with acetonitrile (1 mL per liver, 0.2 mL per kidneys/brain). Bile and urine were precipitated with acetonitrile (15 μ L per 5 μ L bile, 20 μ L per 5 μ L urine). All solutions were vortexed and then centrifuged (12,000 g, 5 min, 4°C). Each supernatant (3 μ L) and diluted radiotracer solution were spotted on thin-layer chromatography (TLC) plates (silica gel 60F 254 nm, 10 x 20 cm; Merck, Darmstadt, Germany) and plates were developed in ethyl acetate / hexane (7/3, v/v). For detection, TLC plates were placed on multisensitive phosphor screens (Perkin-Elmer Life Sciences, Waltham, MA). Using a PerkinElmer Cyclone® Plus Phosphor Imager (Perkin-Elmer Life Sciences), the screens were scanned at a resolution of 300 dpi. Images were analyzed using Optiquant v5.0 software. The retardation factor (R_f) for [¹¹C]Ko143 was approximately 0.3.

2.8 In vitro autoradiography

One female FVB wild-type, *Abcg2*^(-/-) and *Abcb1a/b*^(-/-)/*Abcg2*^(-/-) mouse was euthanized by cervical dislocation. The brain was immediately dissected, embedded in Tissue Freezing Medium (Tissue-Tek O.C.T Compound, Sakura Finetec), snap frozen in liquid nitrogen and stored at -80°C. After defrosting from -80°C to -20°C the brain was cut in coronal 10 μ m slices with a cryostat (Microm HM 550, Walldorf, Germany). The slices were mounted on coated slides (VWR Superfrost Plus) and stored at -80°C. The thawed brain slices were washed in phosphate-buffered saline solution containing 0.05% (v/v) polysorbate 20 (PBST) for 10 min. Subsequently, brain slices ($n = 4$) were incubated with either [¹¹C]Ko143 alone (2.7 nM) or with [¹¹C]Ko143 and unlabeled Ko143 (10 μ M) at room temperature for 30 min. To remove non-specifically bound radiotracer, slices were washed twice for 5 min with a

mixture of PBST and ethanol (80/20, v/v) and once for 2 min with distilled water. Washing steps were performed at 4°C. The slides were air-dried for 10 min at 37°C and exposed overnight to a multisensitive phosphor screen (MS, Perkin-Elmer Life Sciences, Waltham, MA, USA). The screens were then removed from the film cassettes under dim light. Using a Cyclone storage phosphor system (Perkin-Elmer Life Sciences, Waltham, MA, USA), the screens were scanned at a resolution of 600 dpi. Images were analyzed with OptiQuant v5.0 software.

2.9 Statistical analysis

Differences between groups were analyzed by one-way ANOVA followed by a Tukey's multiple comparison test using Prism 7 software (GraphPad Software, La Jolla, CA, USA). The level of statistical significance was set to a p value of less than 0.05. All values are given as mean $\pm$ standard deviation (S.D.).

3 Results

3.1 Radiosynthesis of [^{11}C]Ko143

[^{11}C]Ko143 was synthesized by *O*-[^{11}C]methylation of 9-*O*-desmethyl-Ko143 with [^{11}C]methyl triflate (Fig. 1). [^{11}C]Ko143 ready for i.v. injection was obtained in a decay-corrected radiochemical yield of $4.0 \pm 1.8\%$ ($n = 22$, based on [^{11}C]methane) in a total synthesis time of 35 min with a molar radioactivity at end of synthesis of $266 \pm 157\text{ GBq}/\mu\text{mol}$. The identity of [^{11}C]Ko143 was confirmed by HPLC co-injection with unlabeled Ko143.

3.2 Influence of Abcg2 and Abcb1a/b on brain distribution of [^{11}C]Ko143

In Fig. 2, representative PET images depicting distribution of radioactivity to the brain of one wild-type and one Abcb1a/b^(-/-)Abcg2^(-/-) mouse are shown. Radioactivity concentration in the brain was lower than in the surrounding head tissue in the wild-type mouse and comparable to surrounding head tissue in the Abcb1a/b^(-/-)Abcg2^(-/-) mouse. To determine radioactivity concentrations in the blood, we generated an image-derived blood curve by placing a region of interest into the left ventricle of the heart. Radioactivity concentrations measured with PET in the left ventricle of the heart showed a good correlation (Pearson correlation coefficient $r = 0.837$, $p < 0.0001$, slope = 2.06 ± 0.21) with radioactivity concentrations measured with a gamma counter in a venous blood sample collected at the end of the PET scan. Radioactivity concentrations in the blood of wild-type, Abcg2^(-/-), Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice were higher than radioactivity concentrations in the brain (Fig. 3). Brain concentration-time curves in wild-type and Abcg2^(-/-) mice were almost superimposable and approximately 2-fold lower than in Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice (Fig. 3C). To quantitatively evaluate brain distribution of [^{11}C]Ko143, we used graphical analysis approaches (integration plot and Logan plot) to estimate the rate constant for transfer of radioactivity from blood into brain ($k_{\text{uptake,brain}}$) and $V_{\text{T brain}}$ (Fig. S1 in the supplemental file). $K_{\text{uptake,brain}}$ and $V_{\text{T brain}}$ values were comparable in Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice and significantly (1.7 - 2.5-fold) higher than in wild-type and Abcg2^(-/-) mice (Fig. 4). Pre-treatment of wild-type mice with the Abcb1a/b inhibitor tariquidar increased $k_{\text{uptake,brain}}$ and $V_{\text{T brain}}$ values to comparable levels as in Abcb1a/b^(-/-)

and Abcb1a/b^(-/-)Abcg2^(-/-) mice. To study dose linearity in the distribution of [¹¹C]Ko143 to the brain and to assess possible displacement effects of Abcg2-specific binding, we co-injected one group of wild-type mice with a pharmacologic dose of unlabeled Ko143 with the radiotracer. Co-injection of unlabeled Ko143 was well tolerated and significantly increased $k_{\text{uptake,brain}}$ and $V_{\text{T brain}}$ values in wild-type mice to similar values as in Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice (Fig. 4). As the vehicle for formulation of unlabeled Ko143 was different from the vehicle used for radiotracer formulation, we examined one further group of wild-type mice, which was co-injected with Ko143 vehicle alone. In vehicle-co-injected wild-type mice, too, $k_{\text{uptake,brain}}$ and $V_{\text{T brain}}$ values were significantly higher than in wild-type mice which received [¹¹C]Ko143 without Ko143 vehicle. We further examined Abcg2^(-/-), Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice co-injected with a pharmacologic dose of unlabeled Ko143 (Fig. 4). Co-injection of unlabeled Ko143 abolished the differences seen in brain distribution between the four mouse genotypes when they were injected with a microdose of [¹¹C]Ko143 only. Radioactivity in wild-type and Abcg2^(-/-) mice increased markedly, to comparable levels as in Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice (Fig. 4). To further assess the Abcb1a/b inhibitory effect of Ko143 and its vehicle, we co-injected one group of wild-type mice with unlabeled Ko143 and the ABCB1 substrate radiotracer (*R*)-[¹¹C]verapamil. Co-injection of Ko143 increased brain concentration-time curves of (*R*)-[¹¹C]verapamil to similar levels as in tariquidar pre-treated wild-type mice and in Abcb1a/b^(-/-) mice (see Fig. S2 in the supplemental file).

3.3 Influence of Abcg2 and Abcb1a/b on excretion of [¹¹C]Ko143

Over the time course of the PET scan, both hepatobiliary and urinary excretion of radioactivity occurred. Concentration-time curves of radioactivity in the liver and the kidney were similar for all four mouse genotypes (Fig. 5). In wild-type mice, mean k_{uptake} values were 0.689 mL/min/g tissue for the liver and 0.262 mL/min/g tissue for the kidney, which corresponded to extraction ratios of 0.69 for the liver and 0.06 for the kidney (assuming hepatic and renal blood flow rates in mice of 1.0 and 4.1 mL/min/g tissue, respectively) (Davies and Morris, 1993). K_{uptake} and V_{T} values in the liver and kidney were similar for the four mouse genotypes (Figs. 7A,B and 8A,B). We also estimated the rate constants for excretion of radioactivity from liver *via* bile into the intestine (k_{bile}) and from kidney into urine (k_{urine}) (Fig. S1 in the supplemental file). K_{bile} values were not significantly different among the four mouse genotypes (Fig. 7C), whereas k_{urine} values were significantly lower in Abcg2^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice as compared with wild-type mice (Fig. 8C). Co-injection of unlabeled Ko143 or Ko143 vehicle in wild-type mice caused a marked reduction in initial radioactivity uptake in the liver (Fig. 6A) and significant decreases in both $k_{\text{uptake,liver}}$ and $V_{\text{T liver}}$ values (Fig. 7A,B). In addition, the amount of radioactivity excreted into the intestine was significantly decreased, with 1.9- and 2.4-fold lower k_{bile} values in wild-type mice co-injected with unlabeled Ko143 or Ko143 vehicle, respectively, as compared with wild-type mice injected with [¹¹C]Ko143 alone (Fig. 7C). In the kidneys, co-injection of unlabeled Ko143 significantly decreased $k_{\text{uptake,kidney}}$ values (Fig. 8A). Moreover, k_{urine} values were almost decreased to zero for co-injection of both unlabeled Ko143 and Ko143 vehicle (Fig. 8C).

3.4 Assessment of radiolabeled metabolites of [^{11}C]Ko143

In separate groups of wild-type and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice, radiolabeled metabolites of [^{11}C]Ko143 were assessed at 20 min after radiotracer injection with radio-TLC in plasma, brain, liver, kidney, bile and urine (Table 2). In Fig. S3 of the supplemental file a representative radio-TLC is shown for an *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mouse. In plasma, brain, liver and kidney both unchanged [^{11}C]Ko143 and unidentified [^{11}C]metabolites were detected. In the brain, the percentage of unchanged [^{11}C]Ko143 was significantly higher in *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice as compared with wild-type mice, however in the other organs no significant differences were found. In bile, only a very low percentage of unchanged [^{11}C]Ko143 was detected, and only radiolabeled metabolites were found in the urine (Table 2).

3.5 In vitro autoradiography with [^{11}C]Ko143

In vitro autoradiography was performed on brain slices of wild-type, *Abcg2*^(-/-) and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice incubated with [^{11}C]Ko143 alone or with [^{11}C]Ko143 and unlabeled Ko143 (10 μM) (Fig. S4 of the supplemental file). Brain distribution of radioactivity was uniform, and there were no significant differences in [^{11}C]Ko143 binding between the three studied mouse genotypes. Co-incubation with unlabeled Ko143 had no effect on binding of [^{11}C]Ko143 to brain slices of wild-type and *Abcg2*^(-/-) mice, whereas in *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice a significant increase in binding (+41%) was observed.

4 Discussion

4.1 PET imaging of ABC transporters

Two approaches have been proposed for the development of PET tracers for ABC transporters (Wanek et al., 2013; Raaphorst et al., 2015). The first approach, which uses radiolabeled substrates to visualize the function of transporters, has produced very useful radiotracers such as (*R*)-[^{11}C]verapamil and [^{11}C]N-desmethyl-loperamide. The second approach uses compounds which bind to the transporter without being transported and which should theoretically allow for mapping the density (abundance) of the transporter in different tissues. This second approach may potentially be very useful, in particular for tissues which are protected by ABC transporters, such as the brain, for which radiolabeled transporter substrates afford very low imaging signals. However, this approach has remained unsuccessful so far, presumably due to the low density of ABC transporters, in particular in the brain (~1 nM), combined with insufficiently high binding affinities of the tested compounds (Bauer et al., 2013; Kannan et al., 2013; Müllauer et al., 2013). Based on available *in vitro* data with [^3H]Ko143 (Weidner et al., 2015), we were interested in evaluating if Ko143 can serve as a PET tracer to map the density of ABCG2. To this end we synthesized an *O*-desmethyl precursor of Ko143 (Mairinger, 2016), which was radiolabeled with ^{11}C (Fig. 1). We used small-animal PET imaging in wild-type as well as in *Abcg2* and/or *Abcb1a/b* knockout mice to assess the influence of *Abcg2* and *Abcb1a/b* on the distribution of [^{11}C]Ko143 to different tissues which express *Abcg2* (kidney, liver and brain). Moreover, we assessed if these transporters play a role in the excretion of [^{11}C]Ko143. PET is a powerful tool to assess the interaction of drugs with ABC and SLC transporters (Kusuhara, 2013; Testa et al., 2015; Langer, 2016). A great advantage of small-

animal PET studies in mice is that the entire animal is within the field of view of the PET scanner, so that kinetic information on radiotracer distribution can be obtained from all organs and tissues in a single PET scan. We generated an image-derived blood curve and used graphical analysis approaches to obtain pharmacokinetic parameters, which were shown in previous studies to be sensitive to membrane transporter function in different organs (V_T , k_{uptake} , k_{bile} and k_{urine}) (Bankstahl et al., 2008; Takashima et al., 2013; Shingaki et al., 2015; Traxl et al., 2015; Traxl et al., 2017). Image-derived blood radioactivity concentrations overestimated the true blood values measured in a gamma counter, most likely due to spillover/partial volume effects. However, as the magnitude of these spillover/partial volume effects is unlikely to differ among different mouse groups, our analytical approach appears valid to assess relative differences in outcome parameters between the studied mouse groups. An advantage of k_{uptake} is that this parameter is derived from early PET data (< 5 min after radiotracer injection) and should therefore be minimally affected by tissue uptake of radiolabeled metabolites. [^{11}C]Ko143 was extensively metabolized, and radiolabeled metabolites were detected along with unchanged [^{11}C]Ko143 in all investigated tissues (brain, liver and kidney) (Table 2). Although we were not able to verify the identity of the radiolabeled metabolites, it is reasonable to assume - based on previous data (Weidner et al., 2015; Liu et al., 2017) - that they mainly consisted of ^{11}C -labeled Ko143 free acid. These radiolabeled metabolites may either be formed by plasma carboxylesterases and then taken up into different tissues, or they may also be directly formed by tissue carboxylesterases (Liu et al., 2017).

4.2 Influence of Abcg2 and Abcb1a/b on brain distribution of [^{11}C]Ko143

At the BBB, ABCB1 and ABCG2 cooperate with each other in limiting brain distribution of dual ABCB1/ABCG2 substrates, such as tyrosine kinase inhibitors (Kodaira et al., 2010; Agarwal et al., 2011). Only when both ABCB1 and ABCG2 are knocked out or inhibited are substantial increases in brain distribution of dual ABCB1/ABCG2 substrates achieved. We detected no differences in brain distribution of [^{11}C]Ko143-derived radioactivity between wild-type and *Abcg2*^(-/-) mice, indicating a lack of *Abcg2*-specific binding of [^{11}C]Ko143 at the murine BBB. Unexpectedly, we found that [^{11}C]Ko143 is transported by *Abcb1a* at the murine BBB. $K_{\text{uptake,brain}}$ and V_T brain values were similar in *Abcb1a/b*^(-/-) and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice and about 2-fold higher as compared with wild-type and *Abcg2*^(-/-) mice, which indicates that [^{11}C]Ko143 and possibly its radiolabeled metabolites are only transported by *Abcb1a* and not by *Abcg2* at the murine BBB (Fig. 4). *Abcb1a* transport of [^{11}C]Ko143-derived radioactivity was further confirmed by *Abcb1a* inhibition experiments with tariquidar. In addition, our observation that the percentage of unchanged [^{11}C]Ko143 was higher in brains of *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice as compared with wild-type mice further supported transport of [^{11}C]Ko143 by *Abcb1a* (Table 2). Our data are in good agreement with data by Liu et al., who assessed the brain distribution of Ko143 in wild-type, *Abcb1a/b*^(-/-), *Abcg2*^(-/-) and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice and found comparable brain concentrations of Ko143 in *Abcb1a/b*^(-/-) and *Abcb1a/b*^(-/-)*Abcg2*^(-/-) mice. The latter were about 3-fold higher than in *Abcg2*^(-/-) mice (Liu et al., 2012). Weidner et al. found that Ko143 stimulated human ABCB1 ATPase activity, suggesting substrate affinity for ABCB1 (Weidner et al., 2015). On the other hand, the same authors found no *in vitro* transport of [^3H]Ko143 in human ABCB1- and mouse *Abcb1a*-overexpressing cells, which contradicts

our *in vivo* PET results (Weidner et al., 2015). This makes the selection of candidate compounds for the development of PET tracers for ABC transporters very challenging, as *in vitro* data, even when acquired with low concentrations of ^3H -labeled compounds (Weidner et al., 2015; Raaphorst et al., 2017), do not necessarily predict the *in vivo* behavior of PET tracers.

We were interested in finding out if [^{11}C]Ko143 also showed Abcb1a transport at the murine BBB when administered at pharmacologically relevant doses. To this end, we studied brain distribution of [^{11}C]Ko143-derived radioactivity in wild-type and transporter knockout mice which were co-injected with unlabeled Ko143 at a dose which was previously shown to inhibit Abcg2 at the murine BBB (Wanek et al., 2012; Bakhsheshian et al., 2013). Co-injection of unlabeled Ko143 (10 mg/kg) abolished the differences in brain distribution of radioactivity between the four mouse genotypes which were observed when they were injected with a microdose of [^{11}C]Ko143 (Fig. 4). Similarly, co-injection of Ko143 (10 mg/kg) with the validated ABCB1 substrate radiotracer (*R*)-[^{11}C]verapamil (Luurtsema et al., 2003; Römermann et al., 2013) increased brain distribution of (*R*)-[^{11}C]verapamil to comparable levels as in tariquidar pre-treated wild type mice and in Abcb1a/b^(-/-) mice (Fig. S2 of the supplemental file). We found that the observed Abcb1a inhibitory effect of Ko143 could be attributed to the vehicle used for formulation of Ko143 rather than to Ko143 itself (Fig. 4). Ko143 is a highly lipophilic compound with very poor aqueous solubility, requiring co-solvents and surfactants for solubilization. The vehicle which was used in our study (sterile water / PEG300 / polysorbate 80 / DMSO, 60/25/10/5) was adopted from previously published work (Shimanovich et al., 2010). It ensured that Ko143 remained in solution for the required amount of time while containing safe levels of excipients. It is well known that pharmaceutical excipients can exert inhibitory effects on membrane transporters such as ABCB1 (Sosnik, 2013). For instance, Hugger et al. reported that PEG300 (20%, v/v) caused almost complete *in vitro* inhibition of ABCB1 activity in both Caco-2 and MDR1-MDCK cell monolayers, whereas polysorbate 80 (0.05%, w/v) only partially inhibited ABCB1 activity in Caco-2 cells (Hugger et al., 2002). Our results emphasize that possible excipient effects need to be considered when assessing drug-drug interactions *in vivo*, in particular for poorly soluble compounds. The Ko143 vehicle employed in our work precluded an assessment of whether Ko143 transport by Abcb1a at the mouse BBB is saturated at pharmacologic Ko143 doses. Allen et al. reported a half-maximum effect concentration of 1.0 μM for *in vitro* reversal of ABCB1-mediated paclitaxel resistance by Ko143 (Allen et al., 2002). The reported plasma concentration of Ko143 in mice at 1 h after i.v. administration of a dose of 10 mg/kg is 420 nM (Zhang et al., 2011). The unbound fraction of Ko143 in rat plasma is 0.0293 (Liu et al., 2014). Assuming that plasma protein binding does not differ between rats and mice, the unbound plasma concentration of Ko143 in mice at 1 h after an i.v. 10 mg/kg dose can be estimated as 12 nM, which is far too low to achieve ABCB1 inhibition. This is also supported by our own previous results, in which no increase in brain uptake of the dual Abcb1a/Abcg2 substrate [^{11}C]tariquidar was observed in Abcg2^(-/-) mice pre-treated at 1 h before PET with 10 mg/kg Ko143 (Wanek et al., 2012). To assess if the discrepancy between the *in vitro* results reported by Weidner indicating ABCG2-specific binding of Ko143 and our *in vivo* PET results may be due to rapid *in vivo* metabolism of [^{11}C]Ko143, we performed *in vitro* autoradiography experiments with [^{11}C]Ko143 on brain

slices of wild-type and Abcg2 knockout mice (Fig. S4 of the supplemental file). These experiments, too, failed to reveal Abcg2-specific binding of [^{11}C]Ko143. Based on these data, it cannot be entirely excluded that Ko143 binds to Abcg2; the affinity of Ko143 to Abcg2 may be too low to achieve an Abcg2-specific binding signal in brain tissue. The ABCG2/Abcg2-overexpressing cell lines used in the previously published *in vitro* experiments may express higher levels of ABCG2/Abcg2, so that ABCG2/Abcg2-specific binding was measurable *in vitro* (Weidner et al., 2015).

4.3 Influence of Abcg2 and Abcb1a/b on excretion of [^{11}C]Ko143

We also assessed distribution of [^{11}C]Ko143-derived radioactivity to the kidney and liver, which have an 168- and 7-fold higher density of Abcg2, respectively, as compared with brain microvessels (Dallas et al., 2016). In these two organs, too, there was no difference in k_{uptake} and V_T values between wild-type and Abcg2^(-/-) mice, suggesting a lack of Abcg2-specific binding. Remarkably, co-injection of both Ko143 and Ko143 vehicle significantly reduced $k_{\text{uptake,liver}}$ values in wild-type mice, pointing to an inhibition of basolateral uptake transporters in hepatocytes (Figs. 6A and 7A). As Ko143 has been found to inhibit human SLCO transporters (SLCO1B1 and SLCO2B1) (Karlgrén et al., 2012), it is tempting to speculate that [^{11}C]Ko143 and possibly its radiolabeled metabolites are taken up into the mouse liver by the murine orthologues of these transporters. Since Abcg2 mediates excretion of compounds from the liver *via* bile into the intestine and from the kidneys into urine, we also estimated k_{bile} and k_{urine} values of radioactivity following injection of [^{11}C]Ko143 (Figs. 7C and 8C). We observed that [^{11}C]Ko143-derived radioactivity undergoes both hepatobiliary and urinary excretion. Our data indicate that urinary excretion of radioactivity is dependent on Abcg2, as k_{urine} values were significantly reduced in mice which lacked Abcg2 (Abcg2^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice). However, as radio-TLC analysis only found [^{11}C]metabolites and no unchanged parent in mouse urine (Table 2), Abcg2 most likely mediates urinary excretion of one or more of these [^{11}C]metabolites. Interestingly, Ko143 vehicle strongly inhibited urinary as well as intestinal excretion of radioactivity, further emphasizing that effects of pharmaceutical excipients can be observed *in vivo* in multiple organs. As PEG300 and polysorbate 80 are commonly used excipients, their inhibitory effects on ABC and SLC transporter need to be taken into account for *in vivo* drug-drug interaction studies to avoid a misinterpretation of transporter effects.

5 Conclusion

We assessed the suitability of [^{11}C]Ko143 to measure the density of Abcg2 in different mouse tissues and failed to detect any Abcg2-specific binding of the radiotracer, which highlights the challenges associated with the development of PET tracers to measure ABC transporter densities in tissues. We discovered that [^{11}C]Ko143 and possibly its radiolabeled metabolites are transported by Abcb1a and not by Abcg2 at the murine BBB, and that Abcg2 contributes to urinary excretion of the radiolabeled metabolites of [^{11}C]Ko143. Moreover, we observed pronounced effects of the vehicle used for formulation of Ko143 on tissue distribution and excretion of [^{11}C]Ko143-derived radioactivity, which emphasizes the importance of considering the effects of pharmaceutical excipients on ABC and SLC transporters for *in vivo* studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Agarwal S, Hartz AM, Elmquist WF, Bauer B. Breast cancer resistance protein and P-glycoprotein in brain cancer: two gatekeepers team up. *Curr Pharm Des.* 2011; 17:2793–2802. [PubMed: 21827403]
- Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Koomen GJ, Schinkel AH. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther.* 2002; 1:417–425. [PubMed: 12477054]
- Bakhsheshian J, Wei BR, Chang KE, Shukla S, Ambudkar SV, Simpson RM, Gottesman MM, Hall MD. Bioluminescent imaging of drug efflux at the blood-brain barrier mediated by the transporter ABCG2. *Proc Natl Acad Sci U S A.* 2013; 110:20801–20806. [PubMed: 24297888]
- Bankstahl JP, Kuntner C, Abraham A, Karch R, Stanek J, Wanek T, Wadsak W, Kletter K, Müller M, Löscher W, Langer O. Tariquidar-induced P-glycoprotein inhibition at the rat blood-brain barrier studied with (R)-¹¹C-verapamil and PET. *J Nucl Med.* 2008; 49:1328–1335. [PubMed: 18632828]
- Bauer M, Karch R, Zeitlinger M, Stanek J, Philippe C, Wadsak W, Mitterhauser M, Jager W, Haslacher H, Müller M, Langer O. Interaction of ¹¹C-tariquidar and ¹¹C-elacridar with P-glycoprotein and breast cancer resistance protein at the human blood-brain barrier. *J Nucl Med.* 2013; 54:1181–1187. [PubMed: 23833270]
- Brunner M, Langer O, Sunder-Plassmann R, Dobrozemsky G, Müller U, Wadsak W, Krcal A, Karch R, Mannhalter C, Dudczak R, Kletter K, et al. Influence of functional haplotypes in the drug transporter gene ABCB1 on central nervous system drug distribution in humans. *Clin Pharmacol Ther.* 2005; 78:182–190. [PubMed: 16084852]
- Chen Y, Agarwal S, Shaik NM, Chen C, Yang Z, Elmquist WF. P-glycoprotein and breast cancer resistance protein influence brain distribution of dasatinib. *J Pharmacol Exp Ther.* 2009; 330:956–963. [PubMed: 19491323]
- Dallas S, Salphati L, Gomez-Zepeda D, Wanek T, Chen L, Chu X, Kunta J, Mezler M, Menet MC, Chasseigneaux S, Decleves X, et al. Generation and characterization of a breast cancer resistance protein humanized mouse model. *Mol Pharmacol.* 2016; 89:492–504. [PubMed: 26893303]
- Davies B, Morris T. Physiological parameters in laboratory animals and humans. *Pharm Res.* 1993; 10:1093–1095. [PubMed: 8378254]
- Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, et al. Membrane transporters in drug development. *Nat Rev Drug Discov.* 2010; 9:215–236. [PubMed: 20190787]
- Giri N, Shaik N, Pan G, Terasaki T, Mukai C, Kitagaki S, Miyakoshi N, Elmquist WF. Investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) on pharmacokinetics and central nervous system penetration of abacavir and zidovudine in the mouse. *Drug Metabol Dispos.* 2008; 36:1476–1484.
- Hosten B, Boisgard R, Jacob A, Goutal S, Saubamea B, Dolle F, Scherrmann JM, Cisternino S, Tournier N. [¹¹C]befloxatone brain kinetics is not influenced by Bcrp function at the blood-brain barrier: a PET study using Bcrp TGEM knockout rats. *Eur J Pharm Sci.* 2013; 50:520–525. [PubMed: 23981334]

- Hugger ED, Novak BL, Burton PS, Audus KL, Borchardt RT. A comparison of commonly used polyethoxylated pharmaceutical excipients on their ability to inhibit P-glycoprotein activity in vitro. *J Pharm Sci.* 2002; 91:1991–2002. [PubMed: 12210046]
- Jewett DM. A simple synthesis of [^{11}C]methyl triflate. *Appl Radiat Isot.* 1992; 43:1383–1385.
- Kannan P, Pike VW, Halldin C, Langer O, Gottesman MM, Innis RB, Hall MD. Factors that limit positron emission tomography imaging of p-glycoprotein density at the blood-brain barrier. *Mol Pharm.* 2013; 10:2222–2229. [PubMed: 23597242]
- Karlgrén M, Vildhede A, Norinder U, Wisniewski JR, Kimoto E, Lai Y, Haglund U, Artursson P. Classification of inhibitors of hepatic organic anion transporting polypeptides (OATPs): influence of protein expression on drug-drug interactions. *J Med Chem.* 2012; 55:4740–4763. [PubMed: 22541068]
- Kodaira H, Kusuhashi H, Ushiki J, Fuse E, Sugiyama Y. Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. *J Pharmacol Exp Ther.* 2010; 333:788–796. [PubMed: 20304939]
- Kusuhashi H. Imaging in the study of membrane transporters. *Clin Pharmacol Ther.* 2013; 94:33–36. [PubMed: 23615038]
- Langer O. Use of PET imaging to evaluate transporter-mediated drug-drug interactions. *J Clin Pharmacol.* 2016; 56(Suppl 7):S143–156. [PubMed: 27385172]
- Lee CA, O'Connor MA, Ritchie TK, Galetin A, Cook JA, Ragueneau-Majlessi I, Ellens H, Feng B, Taub ME, Paine MF, Polli JW, et al. Breast cancer resistance protein (ABCG2) in clinical pharmacokinetics and drug interactions: practical recommendations for clinical victim and perpetrator drug-drug interaction study design. *Drug Metabol Dispos.* 2015; 43:490–509.
- Liu K, Zhu J, Huang Y, Li C, Lu J, Sachar M, Li S, Ma X. Metabolism of KO143, an ABCG2 inhibitor. *Drug Metab Pharmacokinet.* 2017; 32:193–200. [PubMed: 28619281]
- Liu X, Cheong J, Ding X, Deshmukh G. Use of cassette dosing approach to examine the effects of P-glycoprotein on the brain and cerebrospinal fluid concentrations in wild-type and P-glycoprotein knockout rats. *Drug Metab Dispos.* 2014; 42:482–491. [PubMed: 24398459]
- Liu X, Ding X, Deshmukh G, Liederer BM, Hop CE. Use of the cassette-dosing approach to assess brain penetration in drug discovery. *Drug Metab Dispos.* 2012; 40:963–969. [PubMed: 22328585]
- Loening AM, Gambhir SS. AMIDE: a free software tool for multimodality medical image analysis. *Mol Imaging.* 2003; 2:131–137. [PubMed: 14649056]
- Logan J, Fowler JS, Volkow ND, Wolf AP, Dewey SL, Schlyer DJ, MacGregor RR, Hitzemann R, Bendriem B, Gatley SJ, et al. Graphical analysis of reversible radioligand binding from time-activity measurements applied to [^{11}C -methyl]-(-)-cocaine PET studies in human subjects. *J Cereb Blood Flow Metab.* 1990; 10:740–747. [PubMed: 2384545]
- Luurtsma G, Molthoff CF, Windhorst AD, Smit JW, Keizer H, Boellaard R, Lammertsma AA, Franssen EJ. (*R*)- and (*S*)-[^{11}C]verapamil as PET-tracers for measuring P-glycoprotein function: in vitro and in vivo evaluation. *Nucl Med Biol.* 2003; 30:747–751. [PubMed: 14499333]
- Mairinger S. Radiosynthesis and bioevaluation of ABC efflux transporter substrates and inhibitors. PhD thesis at the University of Vienna, Faculty of Life Sciences; 2016. <http://othes.univie.ac.at/43376/>
- Mairinger S, Langer O, Kuntner C, Wanek T, Bankstahl JP, Bankstahl M, Stanek J, Dörner B, Bauer F, Baumgartner C, Löscher W, et al. Synthesis and in vivo evaluation of the putative breast cancer resistance protein inhibitor [^{11}C]methyl 4-((4-(2-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl)phenyl)amino-carbonyl)-2-(quinoline-2-carboxylamino)benzoate. *Nucl Med Biol.* 2010; 37:637–644. [PubMed: 20610168]
- Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport—an update. *AAPS J.* 2015; 17:65–82. [PubMed: 25236865]
- Matsson P, Pedersen JM, Norinder U, Bergström CA, Artursson P. Identification of novel specific and general inhibitors of the three major human ATP-binding cassette transporters P-gp, BCRP and MRP2 among registered drugs. *Pharm Res.* 2009; 26:1816–1831. [PubMed: 19421845]
- Müllauer J, Karch R, Bankstahl JP, Bankstahl M, Stanek J, Wanek T, Mairinger S, Müller M, Löscher W, Langer O, Kuntner C. Assessment of cerebral P-glycoprotein expression and function with PET

- by combined [^{11}C]inhibitor and [^{11}C]substrate scans in rats. *Nucl Med Biol.* 2013; 40:755–763. [PubMed: 23774004]
- Raaphorst RM, Savolainen H, Cantore M, van de Steeg E, van Waarde A, Colabufo NA, Elsinga PH, Lammertsma AA, Windhorst AD, Luurtsema G. Comparison of in vitro assays in selecting radiotracers for in vivo P-glycoprotein PET imaging. *Pharmaceuticals.* 2017; 10(3) pii:E76.
- Raaphorst RM, Windhorst AD, Elsinga PH, Colabufo NA, Lammertsma AA, Luurtsema G. Radiopharmaceuticals for assessing ABC transporters at the blood-brain barrier. *Clin Pharmacol Ther.* 2015; 97:362–371. [PubMed: 25669763]
- Rörmann K, Wanek T, Bankstahl M, Bankstahl JP, Fedrowitz M, Müller M, Löscher W, Kuntner C, Langer O. (R)-[^{11}C]verapamil is selectively transported by murine and human P-glycoprotein at the blood-brain barrier, and not by MRP1 and BCRP. *Nucl Med Biol.* 2013; 40:873–878. [PubMed: 23845421]
- Shimanovich, R., Berry, V., Morgan, C., Waldon, DJ. Infusion formulation of a specific BCRP inhibitor enables mechanistic investigation of clearance mechanism in vivo [symposium abstract]. *Pharmaceutical Sciences World Congress 2010 T3432*; 2010.
- Shingaki T, Hume WE, Takashima T, Katayama Y, Okauchi T, Hayashinaka E, Wada Y, Cui Y, Kusuha H, Sugiyama Y, Watanabe Y. Quantitative evaluation of mMate1 function based on minimally invasive measurement of tissue concentration using PET with [^{11}C]metformin in mouse. *Pharm Res.* 2015; 32:2538–2547. [PubMed: 25715695]
- Sosnik A. Reversal of multidrug resistance by the inhibition of ATP-binding cassette pumps employing "Generally Recognized As Safe" (GRAS) nanopharmaceuticals: A review. *Adv Drug Deliv Rev.* 2013; 65:1828–1851. [PubMed: 24055628]
- Takada Y, Ogawa M, Suzuki H, Fukumura T. Radiosynthesis of [2- ^{11}C -carbonyl]dantrolene using [^{11}C]phosgene for PET. *Appl Radiat Isot.* 2010; 68:1715–1720. [PubMed: 20395154]
- Takashima T, Wu C, Takashima-Hirano M, Katayama Y, Wada Y, Suzuki M, Kusuha H, Sugiyama Y, Watanabe Y. Evaluation of breast cancer resistance protein function in hepatobiliary and renal excretion using PET with ^{11}C -SC-62807. *J Nucl Med.* 2013; 54:267–276. [PubMed: 23287578]
- Testa A, Zanda M, Elmore CS, Sharma P. PET tracers to study clinically relevant hepatic transporters. *Mol Pharm.* 2015; 12:2203–2216. [PubMed: 26034841]
- Traxl A, Komposch K, Glitzner E, Wanek T, Mairinger S, Langer O, Sibilica M. Hepatocyte-specific deletion of EGFR in mice reduces hepatic Abcg2 transport activity measured by [^{11}C]erlotinib and positron emission tomography. *Drug Metab Dispos.* 2017; 45:1093–1100. [PubMed: 28790147]
- Traxl A, Wanek T, Mairinger S, Stanek J, Filip T, Sauberer M, Müller M, Kuntner C, Langer O. Breast cancer resistance protein and P-glycoprotein influence in vivo disposition of ^{11}C -erlotinib. *J Nucl Med.* 2015; 56:1930–1936. [PubMed: 26359257]
- Wanek T, Kuntner C, Bankstahl JP, Mairinger S, Bankstahl M, Stanek J, Sauberer M, Filip T, Erker T, Müller M, Löscher W, et al. A novel PET protocol for visualization of breast cancer resistance protein function at the blood-brain barrier. *J Cereb Blood Flow Metab.* 2012; 32:2002–2011. [PubMed: 22828996]
- Wanek T, Mairinger S, Langer O. Radioligands targeting P-glycoprotein and other drug efflux proteins at the blood-brain barrier. *J Labelled Comp Radiopharm.* 2013; 56:68–77. [PubMed: 24285312]
- Wanek T, Rörmann K, Mairinger S, Stanek J, Sauberer M, Filip T, Traxl A, Kuntner C, Pahnke J, Bauer F, Erker T, et al. Factors governing p-glycoprotein-mediated drug-drug interactions at the blood-brain barrier measured with positron emission tomography. *Mol Pharm.* 2015; 12:3214–3225. [PubMed: 26202880]
- Weidner LD, Zoghbi SS, Lu S, Shukla S, Ambudkar SV, Pike VW, Mulder J, Gottesman MM, Innis RB, Hall MD. The inhibitor Ko143 is not specific for ABCG2. *J Pharmacol Exp Ther.* 2015; 354:384–393. [PubMed: 26148857]
- Zhang, J., Chen, Y., Fu, T., Yang, Y., Li, Y., Liu, H., Cheng, Z. Use or misuse of Ko143 for Bcrp chemical knockout mice [symposium abstract]. *CPSA Shanghai*; Shanghai: 2011. 2011

Fig. 1.
Radiosynthesis of $[^{11}\text{C}]\text{Ko143}$.

Fig. 2. [¹¹C]Ko143 PET images. Shown are representative coronal (left) and sagittal (right) PET summation images (0-60 min) of the head region obtained after i.v. injection of a microdose of [¹¹C]Ko143 into one wild-type and one Abcb1a/b^(-/-)Abcg2^(-/-) mouse. Brain is highlighted with a white broken line.

Fig. 3.

Pharmacokinetics of $[^{11}\text{C}]\text{Ko143}$ in blood and brain. Shown are concentration-time curves (mean $\pm$ S.D.) in blood (derived from the left ventricle of the heart) (A) and in whole brain (C) measured after i.v. injection of a microdose of $[^{11}\text{C}]\text{Ko143}$ into wild-type, $\text{Abcg2}^{-/-}$, $\text{Abcb1a/b}^{-/-}$ and $\text{Abcb1a/b}^{-/-}\text{Abcg2}^{-/-}$ mice. In B and D, concentration-time curves (mean $\pm$ S.D.) in blood and whole brain are shown for wild-type mice injected with a microdose of $[^{11}\text{C}]\text{Ko143}$ (wild-type), for wild-type mice injected with a microdose of $[^{11}\text{C}]\text{Ko143}$ at 2 h after i.v. pre-treatment with tariquidar (15 mg/kg) (wild-type + tariquidar), for wild-type mice injected with a mixture of $[^{11}\text{C}]\text{Ko143}$ and a pharmacologic dose of unlabeled Ko143 (10 mg/kg) (wild-type + Ko143) and for wild-type mice injected with a mixture of $[^{11}\text{C}]\text{Ko143}$ and Ko143 vehicle (wild-type + Ko143 vehicle).

Fig. 4. Pharmacokinetic parameters for the brain. Shown are $k_{\text{uptake,brain}}$ (A) and $V_T \text{ brain}$ (B) values (mean $\pm$ S.D.) in groups of wild-type, Abcg2^(-/-), Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice injected with a microdose of [¹¹C]Ko143 or with a mixture of [¹¹C]Ko143 and a pharmacologic dose of unlabeled Ko143 (10 mg/kg), wild-type mice injected with a mixture of [¹¹C]Ko143 and Ko143 vehicle (wild-type + Ko143 vehicle) and wild-type mice injected with a microdose of [¹¹C]Ko143 at 2 h after i.v. pre-treatment with tariquidar (15 mg/kg)

(wild-type + TQD). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$, for comparison with wild-type using one-way ANOVA with Tukey's multiple comparison test.

Fig. 5. Pharmacokinetics of $[^{11}\text{C}]\text{Ko143}$ in excretory organs. Shown are concentration-time curves (mean $\pm$ S.D.) in liver (A), left kidney (B), intestine (C) and urinary bladder (D) measured after i.v. injection of a microdose of $[^{11}\text{C}]\text{Ko143}$ into wild-type, $\text{Abcg2}^{-/-}$, $\text{Abcb1a/b}^{-/-}$ and $\text{Abcb1a/b}^{-/-}\text{Abcg2}^{-/-}$ mice.

Fig. 6.

Pharmacokinetics of $[^{11}\text{C}]\text{Ko143}$ in excretory organs. Shown are concentration-time curves (mean $\pm$ S.D.) in liver (A), left kidney (B), intestine (C) and urinary bladder (D) for wild-type mice injected with a microdose of $[^{11}\text{C}]\text{Ko143}$ (wild-type), wild-type mice injected with a mixture of $[^{11}\text{C}]\text{Ko143}$ and a pharmacologic dose of unlabeled Ko143 (10 mg/kg) (wild-type + Ko143) and wild-type mice injected with a mixture of $[^{11}\text{C}]\text{Ko143}$ and Ko143 vehicle (wild-type + Ko143 vehicle).

Fig. 7.

Pharmacokinetic parameters for hepatobiliary excretion of [¹¹C]Ko143. Shown are $k_{\text{uptake,liver}}$ (A), $V_{T,liver}$ (B) and k_{bile} (C) values (mean $\pm$ S.D.) in groups of wild-type, Abcg2^(-/-), Abcb1a/b^(-/-) and Abcb1a/b^(-/-)Abcg2^(-/-) mice injected with a microdose of [¹¹C]Ko143, wild-type mice injected with a mixture of [¹¹C]Ko143 and a pharmacologic dose of unlabeled Ko143 (10 mg/kg) (wild-type + Ko143) and wild-type mice injected with a mixture of [¹¹C]Ko143 and Ko143 vehicle (wild-type + Ko143 vehicle). * $p < 0.05$, ** $p < 0.01$.

0.01 and *** $p < 0.001$, for comparison with wild-type using one-way ANOVA with Tukey's multiple comparison test.

Fig. 8. Pharmacokinetic parameters for renal excretion of $[^{11}\text{C}]\text{Ko143}$. Shown are $k_{\text{uptake,kidney}}$ (A), V_T kidney (B) and k_{urate} (C) values (mean $\pm$ S.D.) in groups of wild-type, $Abcg2^{-/-}$, $Abcb1a/b^{-/-}$ and $Abcb1a/b^{-/-}Abcg2^{-/-}$ mice injected with a microdose of $[^{11}\text{C}]\text{Ko143}$, wild-type mice injected with a mixture of $[^{11}\text{C}]\text{Ko143}$ and a pharmacologic dose of unlabeled Ko143 (10 mg/kg) (wild-type + Ko143) and wild-type mice injected with a mixture of $[^{11}\text{C}]\text{Ko143}$ and Ko143 vehicle (wild-type + Ko143 vehicle). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

0.01 and *** $p < 0.001$, for comparison with wild-type using one-way ANOVA with Tukey's multiple comparison test.

Table 1Overview of animal groups and numbers examined with [^{11}C]Ko143.

	Baseline	Ko143 (10 mg/kg) ^a	Ko143 vehicle ^b	Tariquidar (15 mg/kg) ^c	Metabolism ^d
Wild-type	5	4	4	4	4
Abcg2 ^(-/-)	5	4	-	-	-
Abcb1a/b ^(-/-)	5	4	-	-	-
Abcb1a/b ^(-/-) Abcg2 ^(-/-)	4	4	-	-	3

^a Ko143 was co-injected with radiotracer, same animals used as for baseline scan^b Ko143 vehicle was co-injected with radiotracer^c PET imaging performed at 2 h after i.v. administration of tariquidar^d no PET imaging performed

Table 2

Percentage of unchanged radiotracer in different mouse tissues and fluids collected at 20 min after i.v. injection of [^{11}C]Ko143.

	Wild-type	Abcb1a/b ^(-/-) Abcg2 ^(-/-)
<i>n</i>	4	3
Plasma	36.0 ± 1.5	34.7 ± 0.4
Brain	51.5 ± 13.9	73.2 ± 2.4 ^a
Liver	17.4 ± 2.7	16.0 ± 1.8
Kidney	35.6 ± 9.6	21.6 ± 2.0
Bile	15.2 ± 21.0	1.5 ± 1.0
Urine	n.d.	n.d.

^a significantly different from wild-type (p < 0.05)

n.d. not detected