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# Brain penetration of WEB 2086 (Apafant) and Dantrolene in Mdr1a (P-gp) and Bcrp knockout rats

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## Running Title

BRAIN PENETRATION OF WEB 2086 AND DANTROLENE IN KNOCKOUT RATS

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**List of nonstandard abbreviations**: AtoB, apical to basal; BBB, blood-brain barrier; BCRP breast cancer transport protein; BPR, brain-to-plasma concentration ratio; BtoA, basal to apical; HPLC-MS/MS, high performance liquid chromatography coupled to tandem mass spectrometry; MPR, muscle-to-plasma concentration ratio; MRT, mean residence time; Papp, apparent permeability coefficient; P-gp, P-glycoprotein.

## Abstract

Transporter gene knockout rat models are attracting increasing interest for mechanistic studies of new drugs as transporter substrates or inhibitors in vivo. However, limited data are available on the functional validity of such models at the blood-brain barrier. Therefore, the present study evaluated Mdr1a (P-gp), Bcrp and combined Mdr1a/Bcrp knockout rat strains for the influence of P-gp and BCRP transport proteins on brain penetration of the selective test substrates [$^{14}$C]WEB 2086 (apafant) for P-gp, and dantrolene for BCRP. Brain-to-plasma ratios (BPR) were measured after intravenous co-infusions of 5.5 µmol/kg/h [$^{14}$C]WEB 2086 and 2 µmol/kg/h dantrolene for 2h in groups of knockout or wildtype rats. Compared to wildtype controls, mean BPR of [$^{14}$C]WEB 2086 increased 8-fold in Mdr1a knockouts, 9.5-fold in double Mdr1a/Bcrp knockouts and 7.3fold in zosuquidar-treated wildtype rats, but was unchanged in Bcrp knockout rats. Mean BPR of dantrolene increased 3.3fold in Bcrp-knockouts and and 3.9-fold in double Mdr1a/Bcrp knockouts compared to wildtype, but was unchanged in the Mdr1a knockouts. The human intestinal CaCo-2 cell bidirectional transport system in vitro confirmed the in vivo finding that [$^{14}$C]WEB 2086 is a substrate of P-gp but not of BCRP. Therefore, Mdr1a, Bcrp and combined Mdr1a/Bcrp knockout rats provide functional absence of these efflux transporters at the blood-brain barrier and are a suitable model for mechanistic studies on the brain penetration of drug candidates.

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## Introduction

Transporter gene knockout rats are attracting increasing attention for the characterization of drug substances regarding their properties as transporter substrates or inhibitors, which can potentially affect pharmacokinetics (Zamek-Gliszczynski et al., 2012, Zamek-Gliszczynski et al., 2013). Knockout rats have advantages compared to previously established mouse knockout models (Kodaira et al., 2010, Bundgaard et al., 2012), due to the high relevance of rats for toxicological testing during preclinical drug development and their better suitability for pharmacokinetic studies using repeated blood sampling.

Transport of drug substances across the blood-brain barrier (BBB) is of particular therapeutic interest, and merits detailed investigation in these knockout models. The important transporters for restricting the brain penetration of drugs across the BBB in rodents and humans are Mdr1a (P-glycoprotein, P-gp) and breast cancer resistance protein (BCRP) (Uchida et al., 2011; Hoshi et al., 2013, Nicolazzo and Katneni, 2009). Both are efflux transporters of the ATP-binding cassette type and limit access of xenobiotics into the brain. In addition, a considerable overlap in substrate properties exists and many P-gp substrates are also BCRP substrates and vice versa (Agarwal et al., 2011, Ni et al., 2010). Data are gradually accumulating that demonstrate the validity of transporter gene knockout rat models in vivo on a functional level at the BBB using reference compounds. Zamek-Gliszczynski et al. (2012) reported that the brain distribution of paclitaxel was markedly enhanced in Mdr1a knockout rats. In addition, Bundgaard et al. (2012) found that the brain distribution of several CNS drugs and active metabolites showed the same changes in brain penetration in rats as in mice when Mdr1a knockout animals were compared with wild-type animals. Liu et al (2014) have recently used a cassette dosing approach to study BCRP and P-gp substrates and inhibitors in Mdr1a knockout rats.

The aim of the present work was to functionally validate the brain penetration of selective P-gp and BCRP substrates in the 3 transporter gene knockout rat strains that lack mdr1a, Bcrp and Mdr1a/Bcrp respectively. For brevity, P-gp is used synonymously here to mdr1a, acknowledging that in rodents the isoform mdr1b is also expressed in various tissues outside the brain. Normal Sprague-Dawley rats, which are the background for the knockout strains, were used as wild-type controls. As a P-gp substrate, the compound WEB 2086 (apafant) was used (Leusch et al., 2002). Dantrolene was selected as a specific BCRP substrate based on in vitro and in vivo literature data (Kodaira et al., 2010, Xiao et al., 2012, Enokizono et al., 2008). Both [$^{14}$C]WEB 2086 and dantrolene are known to exhibit a short mean residence time (MRT) in rats: MRT of WEB 2086 is 0.44 h (unpublished data), and MRT of dantrolene was reported to be 0.52 h by Meyler et. al. (1979) and 3.1 h by Karan et al. (1996). Thus, it was expected that close to steady-state conditions would be achieved after a combined intravenous infusion of [$^{14}$C]WEB 2086 and dantrolene for 2 h, enabling the function of both brain efflux transporters to be assessed in the same set of animals. In addition, [$^{14}$C]WEB 2086 brain and muscle penetration were compared in Mdr1a knockout rats and wild-type rats given intravenous infusions of the chemical P-gp inhibitor zosuquidar. [$^{14}$C]WEB 2086 was further characterized in vitro in a human system using bidirectional transcellular transport across a confluent monolayer of the colon carcinoma-derived cell line (CaCo-2).

## Materials and Methods

### Chemicals

[$^{14}$C]-radiolabeled WEB 2086 free base (apafant) with specific radioactivity 2.149 MBq/µmol and non-labelled WEB 2086 were obtained from the Isotope Chemistry Laboratory, Boehringer Ingelheim, Biberach, Germany. Dantrolene sodium salt was obtained from Sigma-Aldrich, Germany. Zosuquidar·HCl was synthesized by Boehringer Ingelheim, Biberach, Germany. [$^{13}C_3$]-labelled dantrolene was purchased from Toronto Research Chemicals Inc., Canada. All other chemicals were of the highest reagent grade available from commercial sources.

### Animals and cells

Male homozygous rats were obtained from SAGE-Labs, Boyertown, USA. The strains (strain code, product number) were: wildtype Sprague Dawley (CRL:SD, TGRSW); Mdr1a knockout (SD- Abcb1a$^{tm1sage}$ -TGRS3570); Bcrp knockout (SD- Abcg2$^{tm1sage}$, TGRS4200); Mdr1a/Bcrp Knockout (SD-cb1a$^{tm1sage}$/ Abcg2$^{tm1sage}$, TGRS7490). CaCo-2 cells were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany. All animal experiments were conducted in accordance with the German Animal Welfare Act (Tierschutzgesetz) and approved by the local authorities.

### Brain penetration of [$^{14}$C]WEB 2086 and dantrolene in transporter gene knockout rats

The experiments were conducted in five periods. In each period, four rats were used, one from each of the strains wild type, Mdr1a knockout, Bcrp knockout and Mdr1a/Bcrp knockout. [$^{14}$C]WEB 2086 was dissolved together with non-labeled WEB 2086 in deionized water to a concentration of 1 mg/mL and specific radioactivity of 0.8 MBq/mL. Dantrolene was dissolved in 2-hydroxylpropyl-beta-cyclodextrin (10%). The infusion solution contained both substance formulations in a volume ratio 1:1, with a final concentration of 0.5 mg/mL [$^{14}$C]WEB 2086 and 0.137 mg/mL dantrolene. The intravenous infusion rate was 5 mL/kg/h

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for two hours (5.5 µmol/kg/h [$^{14}$C]WEB 2086 and 2 µmol/kg/h dantrolene). Rats were anaesthetized with Inactin®/Ketamin® (100 mg/kg i.p. / 25 mg/kg i.m.) and the intravenous catheter was inserted into the left jugular vein. An intratracheal tube was inserted. At 1 and 1.5 h after start of the infusion, blood was collected in EDTA-coated tubes by retrobulbar punction. After 2 h, terminal blood sampling was performed in the still anaesthetized animals by cardiac puncture, plasma was obtained by centrifugation, and the brain was removed.

**Brain penetration of [$^{14}$C]WEB 2086 in zosuquidar treated rats and Mdr1a knockout rats**

Three groups of 4-5 male rats were used, 2 with wildtype animals and a third comprising Mdr1a knockouts. The wildtype groups received intravenous infusions of either vehicle (saline) or zosuquidar 10 mg/kg/h for 4 hours, and a 1 mg/kg intravenous bolus injection of [$^{14}$C]WEB 2086 (formulation see above) after 3 h. The Mdr1a knockouts also received intravenous infusions of saline for 4 hours and a 1 mg/kg intravenous bolus of [$^{14}$C]WEB 2086 after 3 h. Zosuquidar free base was dissolved in physiological saline. Anaesthesia and intravenous dosing were as described above. After 4h, terminal blood (EDTA, cardiac puncture), whole brain and samples of skeletal muscle tissue were collected. Water was added to brain and muscle tissue in a ratio of about 1 to 2 and the tissues were homogenized. Aliquots of 300 mg muscle tissue homogenate were used. From each homogenate, 2 aliquots were transferred into scintillation vials pre-filled with 1.5 mL soluene/isopropanol (1:1, v/v). After 24 h when tissue structures had dissolved, [$^{14}$C]WEB 2086 radioactivity was measured by liquid scintillation counting.

**Radioactivity measurements of [$^{14}$C]WEB 2086 in rat plasma and brain**

[$^{14}$C]WEB 2086 radioactivity in rat plasma was analyzed by liquid scintillation counting (LSC). Counting time was 10 min in vials containing 4 ml scintillation fluid, using a Perkin Elmer TriCarb® 2900 TR counter. The radioactivity was quantified using the transferred

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Spectral Index of External Standard method for quench correction. For the rat brain sample workup with combined extraction of [$^{14}$C]WEB 2086 and dantrolene, the pineal gland was removed, 2 volumes of water were added per volume of brain and the samples homogenized. The extraction procedure was performed in 4 steps, using at each step methanol/acetonitrile (1:1) with phosphate buffered saline (up to 2:1) added to 1 volume brain homogenate, followed by centrifugation. Two aliquots of 500 µL supernatant were measured by LSC for [$^{14}$C]WEB 2086 radioactivity and 1 mL was used for HPLC-MSMS determination of dantrolene.

**HPLC-MS/MS analysis of dantrolene in rat plasma and brain**

Dantrolene was analyzed in rat plasma and in extracts from rat brain homogenates by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Isotope-labeled [$^{13}C_3$]dantrolene was used as an internal standard. Calibration standards and quality controls were prepared either in rat EDTA blank plasma for the analysis of rat plasma samples, or in phosphate buffered saline for the analysis of brain extract samples. For both matrices, the assay comprised sample clean-up by liquid-liquid extraction in the 96-well plate format. To 100 µL of phosphate buffered saline, 100 µL of internal standard solution (100 nmol/L [$^{13}C_3$]dantrolene in 0.6 M HCl) and 100 µL of sample (rat plasma or brain extract) was added and extracted 3 times with 300 µL tert.-butyl-methyl ether. Organic supernatants were combined and evaporated to dryness in a gentle stream of nitrogen at 40°C. Finally, the dried residue was reconstituted with 100 µL acetonitrile/$H_2O$ (1+4, v/v). Chromatography was performed on a Phenomenex Luna C18(2), 3 µm, 30 mm x 2.0 mm analytical HPLC column. Mobile phases were 0.1 % aqueous formic acid (A) and 0.1 % formic acid in acetonitrile (B) using a programmed linear gradient [0.0 – 1.0 minutes: 30 % (B); 1.0 – 3.0 minutes: from 30 % to 80 % (B); 3.0 – 3.6 minutes: 80 % (B); 3.6 – 3.7 minutes: from 80 % to 30 % (B); 3.7 – 5.0 minutes: 30 % (B); at a flow rate of 0.3 mL/min].

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Analytes were quantified on an AB Sciex QTrap® 6500 mass spectrometer (AB Sciex, Darmstadt, Germany) operated in positive electrospray MS/MS mode. Transitions from m/z = 315.2 to m/z = 244.1 and from m/z = 318.2 to m/z = 244.9 were recorded for dantrolene and the internal standard. The calibration curves were linear over the concentration range from 1.00 to 1000 nmol/L. Over the whole study, mean inaccuracy and imprecision of plasma calibration standards and QCs was within ±11.4 % and 10.5 %, respectively. For buffer calibration standards and QCs they were within ±8.4 % and 10.5 %.

**Bidirectional transcellular transport assay**

CaCo-2 cells were seeded on Transwell filter inserts (3396, Corning, USA) and cultured at 37°C, 8% $CO_2$ and 95% relative humidity to confluency (14-16 days) with Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, L-glutamate, non-essential amino acids, and antibiotic-antimycotic mixture. For drug permeation and inhibition experiments, [$^{14}$C]WEB 2086, [$^{3}$H]digoxin or [$^{3}$H]estrone 3-sulfate was added to the donor compartment and nonlabelled inhibitors such as zosuquidar, fumitremorgin C or WEB 2086 were added at the same concentration to both donor and receiver compartments. As the medium for transport assay, Hank's balanced salts solution supplemented with 15 mM HEPES after adjusting the pH to 7.4 was used. CaCo-2 cells were incubated at 37°C for 90 minutes. Samples were taken at 0 minutes from the donor compartment, representing the actual start concentrations in the donor compartment, and samples were also taken at 0, 30, 60 and 90 minutes from the receiver compartment, for measurement of the amounts of compound that had passed through the monolayer. Sample aliquots withdrawn at each time point from the receiver compartment were immediately replaced with an equal volume of fresh receiver solution. Each transport experiment consisted of triplicate incubations using three different filter inserts for both the apical-to-basal (AtoB) and basal-to-apical (BtoA) directions. The net

permeability coefficient (Papp) (cm/s) and efflux ratio was calculated by equations previously reported (Ishiguro et al., 2014).

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## Results and Discussion

**Brain penetration of apafant and dantrolene in knockout and zosuquidar-treated rats.**

The effects of the transporter gene knockout rat strains on brain-to-plasma ratios (BPRs) for [$^{14}$C]WEB 2086 and dantrolene compared to wildtype after simultaneous 2 h infusions are shown in Fig. 1A. The mean BPR of [$^{14}$C]WEB 2086 in the wildtype rats was 0.023±0.005 (mean±SD, n=4-5). This increased 7.9fold in Mdr1a-knockout rats (0.181±0.037) and 9.3fold in the double Mdr1a/Bcrp-knockouts (0.215±0.026), but was unchanged in the Bcrp-knockout rats (0.027±0.007). The mean BPR of dantrolene in wildtype rats was 0.042±0.016, increased 3.7 fold in Bcrp-knockouts (0.156±0.010) and 3.9fold in double Mdr1a/Bcrp-knockouts (0.163±0.024), but was unchanged in the Mdr1a-knockout rat group (0.047±0.009). Plasma and brain concentration data are given in Supplemental Table 1.

WEB 2086 (apafant) was selected as a P-gp substrate because its P-gp dependent brain penetration has already been demonstrated in vivo using Mdr1a knockout mice (Leusch et al., 2002). Dantrolene was chosen as a BCRP substrate because in contrast to the majority of BCRP substrates it is not or only a weak substrate for P-gp in vitro and in vivo (Kodaira et al. 2010, Enokizono et al., 2008, Xiao et al., 2012). The BPRs of WEB 2086 were calculated on the basis of total [$^{14}$C]WEB 2086-derived radioactivity. This was considered an indicator for the brain penetration of parent compound WEB 2086, as in the previous study in knockout mice (Leusch et al, 2002). However, it cannot be excluded that putative metabolites of WEB 2086 contributed to the results.

The very low BPR of [$^{14}$C]WEB 2086 in wildtype rats is similar to the vascular space fraction of about 0.02 (Ohno et al., 1978), confirming that WEB 2086 and its putative metabolites are virtually completely excluded from the brain. Fig. 1A shows that WEB 2086 is a selective substrate for P-gp at the BBB of rats in vivo, and no relevant contribution of BCRP could be detected. Thus, lack of P-gp at the BBB on a functional level was demonstrated in these

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knockout rats. Only minor and most probably functionally irrelevant compensatory changes of the respective knockout strains were reported (Zamek-Gliszcynski et al., 2013). Up to now, only limited literature on the brain penetration of P-gp substrates in Mdr1a knockout rats exists. Zamek-Gliszczynski et al. (2013) showed a 4-fold increase of paclitaxel brain partitioning in Mdr1a knockout rats. Similarly to the results here for WEB 2086, no increase of the brain distribution of paclitaxel was observed in Bcrp knockout rats. The BPR of dantrolene determined in wildtype rats was slightly higher than the vascular space fraction, which may indicate very limited brain penetration. The data in Fig 1A clearly indicate that dantrolene is a selective substrate for the BCRP transporter at the rat BBB, and that P-gp has no relevance for the brain distribution of dantrolene in vivo in rats. This is in agreement with previous data obtained in mice (Kodaira et al., 2010, Enokizono et al., 2008).

Figure 1B shows the effect of i.v. infusion with zosuquidar (10 mg/kg/h) on BPR and muscle-to-plasma concentration ratios (MPRs) of [$^{14}$C]WEB 2086 1 h after intravenous bolus injection of 1 mg/kg, in comparison to wildtype vehicle controls and Mdr1a knockouts. In preceding dose-finding experiments with varying infusion rates of zosuquidar, it had been found that 10 mg/kg/h for 3 h was required to attain essentially complete inhibition of P-gp mediated efflux of WEB 2086 from the rat brain. In the wildtype vehicle control rats, the [$^{14}$C]WEB 2086 BPR was low (0.0742 ± 0.00785, mean ± SD, n=4-5) compared to a MPR of near unity (1.37 ± 0.266). In the zosuquidar treated group, the BPR increased 7.3fold to 0.543 ± 0.094 but the MPR differed only slightly (0.973 ± 0.123, a 0.71fold decrease). In the Mdr1a knockout rats, the [$^{14}$C]WEB 2086 BPR increased 6.8fold to 0.505 ± 0.106, whereas the MPR again changed only slightly (1.05 ± 0.0451, a 0.76fold decrease).

The BPR of [$^{14}$C]WEB 2086 observed in wildtype rats (0.0742) was higher than in the single/double knockout experiment (0.023; Figs 1B and 1A), but still reflects a low brain penetration of WEB 2086 that is only slightly higher than the vascular space fraction. The

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marked BPR increases in the zosuquidar and Mdrla groups (Fig. 1B) were in turn similar to the BPR increases seen in Fig. 1A. Thus, the increased brain permeability of WEB 2086 in Mdr1a knockout rats compared to wildtype rats is similar to that attained after maximum chemical inhibition of P-gp by the specific inhibitor zosuquidar. The MPR in wildtype rats was hardly affected by zosuquidar or Mdr1a knockout, which is consistent with the known lack of expression of P-gp in this tissue.

**CaCo-2 cell studies.** The selectivity of zosuquidar and fumitremorgin C, which are reported to be selective inhibitors of P-gp and BCRP respectively (Mease et al., 2012) was investigated in the CaCo-2 cells used in the study. Zosuquidar at 1 μM inhibited transcellular transport of digoxin which is an in vitro P-gp probe substrate (efflux ratio: 23 and 1.3 in the absence and presence of zosuquidar), but it did not inhibit transcellular transport of estrone-3-sulfate which is an in vitro BCRP probe substrate (efflux ratio: 13 and 12 in the absence and presence of zosuquidar, respectively) (Fig. 2A and 2B). Fumitremorgin C showed opposite results to those of zosuquidar (Fig. 2A and 2B). Involvement of P-gp and BCRP on WEB 2086 transport was investigated by evaluating in vitro bidirectional transport of [$^{14}$C]WEB 2086 across CaCo-2 cell monolayers in the absence and presence of zosuquidar, fumitremorgin C and combination of both inhibitors (Fig. 2C). In the substrate assay in the absence of test inhibitors, transport of [$^{14}$C]WEB 2086 was asymmetric at 1 μM and the resulting efflux ratio was 8.4. Marked inhibition was observed by the addition of zosuquidar, resulting in an efflux ratio of 0.68 by zosuquidar alone and 0.74 by combination of zosuquidar and fumitremorgin C. Addition of fumitremorgin C alone showed no effect (efflux ratio: 7.6). These findings indicate the involvement of P-gp but not BCRP in the transcellular transport of [$^{14}$C]WEB 2086 across CaCo-2 cell monolayers. Vectorial transport of WEB 2086 was reported to be saturable with a high estimated $K_m$ value of 100-500 μM for P-gp (Leusch et al., 2002). The results indicate that P-gp plays a role in the efflux of WEB 2086, but its P-gp-mediated

transport will only be saturated if WEB 2086 concentrations surrounding P-gp exceed the $K_m$ value.

The potential for WEB 2086 to inhibit P-gp and BCRP was also assessed in the CaCo-2 system. WEB 2086 demonstrated weak concentration-dependent inhibition on the efflux of P-gp probe substrate digoxin, but no concentration-dependent inhibition on the efflux of BCRP probe substrate estrone 3-sulfate. The $IC_{50}$ of WEB 2086 was estimated to be in the range from 100 to 500 μM for P-gp and >500 μM for BCRP (details are given in Supplemental Fig.1). WEB 2086 did not relevantly inhibit BCRP transport, and dantrolene was reported not to inhibit P-gp activity represented by digoxin transcellular transport across CaCo-2 monolayer (Xiao et al., 2011), suggesting no mutual inhibition after co-infusion of both WEB 2086 and dantrolene.

**Conclusions.** Taken together, the results obtained in the Mdr1a, Bcrp and combined Mdr1a/ Bcrp knockout rats provide functional absence of these efflux transporters at the BBB. Thus, these animal models can be used for pharmacokinetic and mechanistic studies investigating the complete absence of one or both of these transporters at the BBB. They have the advantage over chemical transporter knockout procedures that such studies can be carried out for an extended observation period in rats without anaesthesia. Rats are better suited than mice for pharmacokinetic studies regarding blood sampling, are usually the rodent species for toxicology studies and are therefore an important and relevant species for nonclinical PK studies. Although the species differences of P-gp between human and rat need to be elucidated, it was suggested that WEB 2086 is a selective substrate for P-gp and conversely that dantrolene is a selective Bcrp substrate in rats. In conclusion, the investigated knockout rats are a valid model to investigate the influence of Mdr1a and Bcrp on the brain penetration of test compounds in rats.

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## Authorship contributions

*Participated in research design*

Fuchs, Ishiguro

*Conducted experiments*

Fuchs, Kishimoto, Gansser, Ishiguro

*Performed data analysis*

Fuchs, Kishimoto, Gansser, Ishiguro

*Wrote or contributed to the writing of the manuscript*

Fuchs, Kishimoto, Gansser, Ishiguro, Tanswell

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## Footnotes

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## Figure Legends

Figure 1

A. Brain-to-plasma ratios of [$^{14}$C]WEB 2086 derived radioactivity and dantrolene after intravenous co-infusions in wildtype and knockout rats. B. Brain-to-plasma and muscle-to-plasma ratios of [$^{14}$C]WEB 2086 after i.v. bolus of 1 mg/kg in wildtype rats, rats given 10 mg/kg/h zosuquidar infusion, and Mdr1a knockout rats.

Mean SD, n=4-5 per group.

Figure 2

Papp values and efflux ratios of [$^{3}$H]Digoxin (1 µM) (A), [$^{3}$H]E-sul (10 µM) (B), and WEB 2086 (1 µM) (C), in the absence and presence of the P-gp inhibitor zosuquidar (1 µM), the BCRP inhibitor fumitremorgin C (1 µM), and both inhibitors.

Figure 1

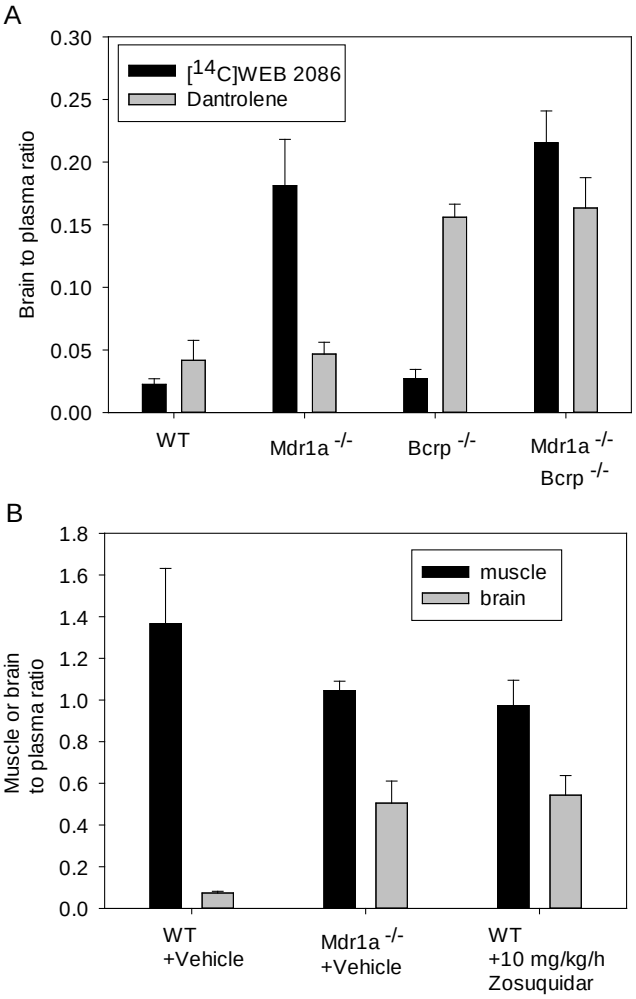

Figure 2