N-desmethyl-Loperamide is selective for P-glycoprotein among three ATP-binding cassette transporters at the blood-brain barrier

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ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; Ca-AM, calcein-AM; CsA, cyclosporin A; FTC, fumitremorgin C; dLop, *N-desmethyl*-loperamide; IC_{50}, inhibitory concentration at 50%; Lop, loperamide; Mrp1, multidrug resistance protein 1; MTX, mitoxantrone; PET, positron emission tomography; P-gp, P-glycoprotein; Rh123, rhodamine 123; RR, resistance ratio; SPECT, single photon emission computed tomography; SUV, standard uptake value.

ABSTRACT

[^{11}C]N-*desmethyl*-loperamide ([^{11}C]dLop) is used in positron emission tomography (PET) to measure the *in vivo* activity of efflux transporters that block the passage of drugs across the blood-brain barrier. The three most prevalent ATP-binding cassette (ABC) efflux transporters at the blood-brain barrier are P-glycoprotein (P-gp), multidrug resistance protein 1 (Mrp1), and breast cancer resistance protein (BCRP). We sought to measure the selectivity of dLop among these three transporters. The selectivity of dLop at low concentrations (≤ 1 nM) was measured both as the accumulation of [^{3}H]dLop in human cells that over-express each transporter and as the uptake of [^{11}C]dLop in brains of mice that lack genes encoding P-gp, Mrp1, or BCRP. The selectivity of dLop at high concentrations (≥ 20 µM) was measured as the inhibition of uptake of a fluorescent substrate and the change in cytotoxicity of drugs effluxed at each transporter. Accumulation of [^{3}H]dLop was lowest in cells over-expressing P-gp, and the uptake of [^{11}C]dLop was highest in brains of mice lacking P-gp. At high concentrations, dLop selectively inhibited P-gp function and also decreased the resistance of only the P-gp-expressing cells to cytotoxic agents. dLop is selective for P-gp among these three transporters, but its activity is dependent on concentration. At low concentrations (≤ 1 nM), dLop acts only as a substrate; at high concentrations (≥ 20 µM), it acts as both a substrate and an inhibitor (*i.e.*, a competitive substrate). Since low concentrations of radiotracer are used for PET imaging, [^{11}C]dLop acts selectively and only as a substrate for P-gp.

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INTRODUCTION

Efflux transporters of the ATP binding cassette (ABC) family block the entry into cells of many diverse of foreign compounds and thereby protect the body from potential toxins (Gottesman, 2002). At the blood-brain barrier, the three most prevalent ABC transporters are P-glycoprotein (P-gp; encoded by *ABCB1*), multidrug resistance protein 1 (Mrp1; encoded by *ABCC1*), and breast cancer resistance protein (BCRP; encoded by *ABCG2*) (Loscher and Potschka, 2005a). They are expressed in the walls of the blood vessels and not only protect the brain from toxins but also impede delivery of therapeutic drugs (Gottesman, 2002; Graff and Pollack, 2004). Among these three transporters, P-gp has been most studied, in part because it likely causes pathology in both brain and periphery. At the blood-brain barrier, over-expression of P-gp may contribute to drug-resistant epilepsy (Loscher and Potschka, 2005b), while its under-expression may contribute to Alzheimer's disease (Vogelgesang et al., 2004; Cirrito et al., 2005). In the periphery as well as in the brain, over-expression of P-gp in cancer cells is one cause of multidrug resistance to chemotherapy (Gottesman et al., 2002; Szakacs et al., 2006).

The *in vivo* function of P-gp has been quantified using substrates radiolabeled for single photon emission computed tomography (SPECT) or positron emission tomography (PET) (Kannan et al., 2009). The SPECT radiotracer [^{99m}Tc]sestamibi was the first substrate to image the function of P-gp at the blood-brain barrier and in multidrug resistant cancers (Piwnica-Worms et al., 1993). However, this radiotracer was later found to be a substrate also for Mrp1, thereby compromising its ability to quantify the function of only P-gp (Hendrikse et al., 1998).

In our search for an improved substrate radiotracer to selectively image P-gp, we evaluated *N-desmethyl*-loperamide (dLop), the major metabolite of loperamide, a potent opiate agonist used to treat diarrhea. Loperamide (Lop) has no opiate effects on the central nervous

system because ABC transporters avidly block its entry into brain (Kurnik et al., 2008). Our PET

imaging studies with [^{11}C]dLop in animals suggest, but do not prove, that dLop is selective for P-

gp. We found that brain uptake of [^{11}C]dLop is negligible in wild-type mice and in monkeys, but

markedly increased in *abcb1a/b*-knockout mice and in monkeys treated with an inhibitor of P-gp

(Lazarova et al., 2008; Zoghbi et al., 2008; Liow et al., 2009). These studies do not definitively

prove selectivity for P-gp because we did not test mice with knockout of the other two

transporters at the blood-brain barrier (*abcc1a* and *abcg2*) and because the inhibitor (tariquidar)

used in monkeys is likely not selective at high concentrations for P-gp (Robey et al., 2004).

We used a two-system approach to investigate the selectivity of dLop for P-gp. Since

substrate recognition by P-gp is known to differ between species (Syvanen et al., 2009), the first

goal of this study was to determine selectivity in human tissues using three pairs of human cell

lines that over-express P-gp, MRP1, or BCRP. However, given that *in vitro* conditions also differ

from those *in vivo*, the second goal was to determine the selectivity of dLop in three strains of

mice that were selectively knocked out for the genes that encode P-gp, Mrp1, and BCRP.

Nomenclature.

For these three efflux transporters, we use upper case italic for the human gene, lower

case italic for the mouse gene, and plain font for the expressed protein: 1) *ABCB1*, *abcb1a/b*, P-

gp; 2) *ABCC1, abcc1a,* Mrp1; and 3) *ABCG2, abcg2,* BCRP. The names of the expressed

proteins are the same for both species.

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MATERIALS AND METHODS

Chemicals

[*N*-methyl-^{3}H]dLop (American Radiolabeled Chemicals, Inc., St. Louis, MO) was synthesized by [^{3}H$_3$]methylation of di-desmethyl-loperamide (Lazarova et al., 2008), and had a radiochemical purity of 97.7% by high performance liquid chromatography analysis, a specific activity of 3.0 GBq/µmol, and a concentration of 37 MBq/mL. [^{11}C]dLop was prepared on four separate occasions as a solution in sterile saline (0.9% w/v) containing ascorbic acid (1 mg), as described previously (Lazarova et al., 2008). [^{11}C]dLop was obtained with a radiochemical purity > 99% and had a specific radioactivity of 78.3 ± 16.8 GBq/µmol at the time of injection. Unless otherwise specified, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines

Three pairs of cell lines were cultured, each pair consisting of a parental (control) and a resistant (ABC transporter expressing) line. The three pairs were: the human adenocarcinoma cell line KB-3-1 and its *ABCB1*-expressing variant KB-8-5-11; the human breast cancer cell line MCF-7 and its *ABCC1*-expressing variant MCF-7/VP16; and the human adenocarcinoma cell line H460 and its *ABCG2*-expressing variant H460/MX20. The KB and MCF-7 lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM), while the H460 lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. Resistant cell lines were additionally cultured in the following cytotoxic drugs to maintain ABC transporter expression: colchicine (250 nM; 100 ng/mL) for KB-8-5-11(Shen et al., 1986); etoposide (4 µM) for MCF-7/VP16 (Schneider et al., 1994); and mitoxantrone (20 nM) for H460/MX20 (Henrich et al., 2007). All

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culture media were supplemented as previously reported (Lee et al., 1998), and cell lines were

grown at 37 ºC in 5% CO_2.

Animals

Four strains of mice (four animals per strain) were used for imaging studies. Three of the

strains were knocked out for the genes encoding either P-gp (26.0 ± 3.4 g; *abcb1a/b -/-*; model

001487), Mrp1 (23.0 ± 1.1 g; *abcc1a -/-*; model 001486), or BCRP (25.5 ± 2.6 g; *abcg2 -/-*;

model 002767); the fourth strain was wild-type (23.6 ± 0.9 g; *abcb1a/b +/+*; model FVB). Mice

were purchased from Taconic Farm (Germantown, NY). Animal experiments were performed in

accordance with the Guide for Care and Use of Laboratory Animals (Clark et al., 1996) and were

approved by the National Institute of Mental Health Animal Care and Use Committee.

Western Blot Analysis.

The expression of ABC transporter in each cell line was visualized by Western blot

analysis. Protein samples were prepared and run on a gel as reported by Brimacombe et al.

(2009). In brief, lysed cells were incubated in sodium dodecyl sulfate (5x) buffer, loaded onto a

3-8% NuPAGE® Novex ® Tris-Acetate gel (Invitrogen Corp., Carlsbad, CA, USA), and

transferred to nitrocellulose membranes. Dry blots were blocked in 20% milk for 30 min at 21

ºC. Blots were then probed for expression of three ABC transporters using three primary

antibodies (Supplemental Table 1) for 60 min at 21 ºC, washed 3 x 10 min, immunoprobed with

a secondary antibody (Supplemental Table 1) for 60 min at 21 ºC, and washed again. To ensure

that the same amount of protein was loaded per lane, each blot was immunoprobed for

glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a protein uniformly expressed in

cells.

Cellular Accumulation of [^{3}H]dLop

The selectivity of dLop (at nanomolar concentrations) for the three ABC transporters was measured *in vitro* as the accumulation of [^{3}H]dLop in all cell lines. Cells (2.5 x 10^5 cells/well) were seeded in 24-well plates and incubated for 24 h at 37 ℃ to allow attachment. To ensure stable uptake of [^{3}H]dLop over time, accumulation of [^{3}H]dLop was measured in KB-3-1 cells over the course of 60 min. To initiate the time course assay, [^{3}H]dLop (1 nM) was added to each well. Following incubation at 37 ℃ for the appropriate time, the growth media were aspirated, and the wells were washed with ice-cold 1x phosphate buffered saline (0.5 mL/well; PBS). Cells were then lysed with trypsin (0.1 mL/well) by incubation for 90 min at 37 ℃. Radioactivity in the lysates was measured with liquid-scintillation counting. Adsorption of [^{3}H]dLop to the plates was determined by adding [^{3}H]dLop (1 nM) to three wells containing no cells. For the purposes of standardizing accumulation, the number of attached cells was counted in three wells per plate using a Cellometer Automatic Cell Counter (Nexcelcom, Lawrence, MA). Radioactivity was corrected for adsorption, cell counts, and expressed as fmol per 10^6 cells.

To achieve virtually complete inhibition of P-pg, cells from the *ABCB1* pair were pre-incubated with cyclosporin A (10 µM) for 30 min at 37 ℃ before adding [^{3}H]dLop (1 nM). Cells were then incubated for 45 min at 37 ℃, washed, lysed, and counted as described above.

Uptake of [^{11}C]dLop in Mice Brains

The selectivity of dLop was also measured *in vivo* as the uptake of [^{11}C]dLop in the brains of four strains of mice. Four mice from each strain (16 total) were simultaneously

anesthetized with 1.5% isoflurane and injected via the tail vein with [^{11}C]dLop (16.0 ± 4.6 MBq; 0.230 ± 0.084 nmol; 0.1 mL). Mice brains were then scanned using a Focus 220 microPET camera (Siemens Medical Solutions, Knoxville, TN), which has a transaxial field of view of 19 cm and an axial one of 7.6 cm. Serial dynamic scans were acquired for 60 minutes as previously described (Lazarova et al., 2008) and were reconstructed using a Fourier Rebinning + 2-D Ordered Subset Expectation Maximization algorithm, resulting in an image resolution of 1.6 mm at full-width half-maximum. No attenuation or scatter correction was applied.

Images were analyzed with PMOD (pixelwise modeling computer softward; PMOD group, Zurich, Switzerland). Regions of interest were drawn on coronal slices of the brain. Brain concentration of radioactivity (decay-corrected till injection time) was expressed as the percentage of the standardized uptake value (%SUV), which normalizes for injected activity and body weight: %SUV = (%injected activity/cm^3 of brain) × (g of body weight).

Inhibition of Transporter Function

Since substrates (at high concentrations) can inhibit ABC transporter function as competitive substrates (Ambudkar et al., 1999), we determined whether dLop could selectively inhibit the function of human P-gp. In addition, we determined the selectivity of loperamide (Lop) because it is administered at pharmacological doses to treat diarrhea. We measured their inhibitory activity by the uptake of a fluorescent substrate (one preferentially effluxed by P-gp, Mrp1, or BCRP) in the presence of high concentrations (micromolar) of dLop or Lop. Four conditions for each transporter were tested: untreated (negative control), inhibitor-treated (positive control), dLop-treated, and Lop-treated. For each condition, 2 x10^5 cells were suspended in 1 mL of Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% fetal bovine serum.

The cells were first pre-treated with inhibitor, dLop, or Lop for 10 min in a 37 ℃ water bath. Pre-treatment conditions were as follows. Untreated cells were incubated in drug-free medium. Inhibitor-treated cells were incubated with one of the following inhibitors of ABC transporters: 10 µM cyclosporin A for P-gp (Kimchi-Sarfaty et al., 2007), 50 µM MK-571 for Mrp1 (Mitra et al., 2006), or 5 µM fumitremorgin C for BCRP (Wu et al., 2007). dLop- and Lop-treated cells were incubated in media containing 10, 20, or 50 µM.

Following pre-treatment, cells were isolated by centrifugation and resuspended in 1 mL IMDM containing the same concentration of inhibitor, dLop, or Lop used during pre-treatment. All cells were also incubated with a fluorescent substrate that could detect the activity of each transporter: 4 µM rhodamine 123 for P-gp (Kimchi-Sarfaty et al., 2007); 0.25 µM calcein-AM for Mrp1 (Wu et al., 2007); or 5 µM mitoxantrone for BCRP (Wu et al., 2007). Although calcein-AM is not itself a fluorescent substrate for Mrp1, it is cleaved by cellular esterases to yield calcein, which is a fluorescent substrate effluxed Mrp1. After addition of the fluorescent substrate, cells were incubated in the dark for 45 min in a 37 ℃ water bath. They were then centrifuged, resuspended in 300 µL of 0.1% bovine serum albumin in 1x PBS, and kept on ice until analysis. For each cell treatment, fluorescence intensity (cellular uptake of fluorescent substrate) was recorded for a total of 10,000 cells using a FACS Calibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA) under the FL-2 (rhodamine 123), FL-1 (calcein-AM), and FL-3 (mitoxantrone) channels. FACS data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Cytotoxicity Assay

The ability of dLop and Lop to selectively inhibit the function of each ABC transporter

was additionally measured by their effects on the resistance of cells to cytotoxic substrates for each transporter. Cytoxicity was measured with a colorimetric viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Molecular Probes, Eugene, OR). Cytotoxic drugs known to be substrates for each ABC transporter were selected: doxorubicin for P-gp, etoposide for Mrp1, and mitoxantrone for BCRP. Stock solutions of cytotoxic drugs were prepared in DMEM or RPMI medium containing either 0 or 20 µM dLop or Lop. Cultured cells were seeded and incubated in serial dilutions of the stock solutions as described by Brimacombe et al (2009). Cytotoxicity (IC_{50}) was defined as the drug concentration that reduced cell viability to 50% of the untreated control and was calculated from three independent experiments. Resistance ratios (RR) are also reported for each resistant cell line, determined by dividing the IC_{50} of the resistant cell line by that of the parental cell line.

Statistical Analysis

Data are expressed as mean ± SD from three observations for radioactivity accumulation assays, from four observations for mouse imaging studies, from three observations for inhibition assays, and from at least nine observations for cytotoxicity assays. After the data were tested for homogeneity of variance, statistical significance was evaluated for radioactivity accumulation and cytotoxicity assays by the Student's *t*-test (unpaired, two-tailed, α = 0.05), and for imaging studies by a two-way ANOVA followed by the Bonferroni post *t*-test (α = 0.05).

RESULTS

Each Resistant Cell Line Exclusively Expressed One Functional ABC Transporter

We confirmed that each ABC transporter was exclusively expressed by one of the resistant cell lines using Western analysis: KB-8-5-11 expressed only P-gp; MCF-7/VP16 expressed only Mrp1; and H460/MX20 expressed only BCRP (Figure 1). None of the parental cell lines had detectable expression of any of these three ABC transporters. Based on band width and intensity, immunoprobing for GAPDH expression showed roughly equal loading of protein in all lanes.

We also confirmed that each ABC transporter was functional: for each cell pair, the resistant cell line had a significantly higher cytotoxicity (IC_{50}) value than its parental line. The *ABCB1*-expressing cell line was 71-fold more resistant to doxorubicin than its parental line; the *ABCC1*-expressing cell line was 79-fold more resistant to etoposide than its parental line; and the *ABCG2*-expressing cell line was 37-fold more resistant to mitoxantrone than its parental line (Table 1).

At Low Concentrations (≤ 1 nM), dLop is a Substrate Selective for Human ABCB1

[^{3}H]dLop accumulation was stable after 30 minutes (Figure 2) and was significantly different between the parental and resistant cells of only the *ABCB1* pair (Figure 3). Parental cells of the *ABCB1* pair (210 ± 15 fmol/10^6 cells) accumulated 4 times more [^{3}H]dLop than *ABCB1*-expressing cells (42 ± 5 fmol/10^6 cells; $P < 0.001$; Figure 3). After treatment with cyclosporin A, *ABCB1*-expressing cells (124 ± 25 fmol/10^6 cells) accumulated 3 times more [^{3}H]dLop than untreated ones (42 ± 5 fmol/10^6 cells; $P < 0.001$; data not shown). Furthermore, after treatment with cyclosporin A, accumulation of [^{3}H]dLop in *ABCB1*-expressing cells was equivalent to that in parental cells ($P = 0.10$). In contrast to these findings of the *ABCB1* pair, the

parental and resistant cells of the *ABCC1* and *ABCG2* pairs accumulated the same amount of

[^{3}H]dLop ($P = 0.86$ for *ABCC1*, $P = 0.18$ for *ABCG2*; Figure 3).

dLop is Selectively Taken up into Brains of abcb1a/b-Knockout Mice

After injection of [^{11}C]dLop into fours strains of mice, brain concentration of

radioactivity in *abcb1a/b*-knockout mice ($25.5 \pm 4.1\%$ SUV; $P <0.0001$) was at least 2.5-fold

higher than in *abcc1a*-knockout ($7.9 \pm 2.1\%$ SUV), *abcg2*-knockout ($9.6 \pm 1.6\%$ SUV), or wild-

type ($6.0 \pm 2.1\%$ SUV) mice (Figure 4).

At High Concentrations (≥ 20 µM), dLop and Lop Selectively Inhibit P-gp Function

Using inhibition and cytotoxicity assays, we found that both dLop and Lop inhibited P-gp

function in a concentration-dependent manner but had no effect on Mrp1 or BCRP. In the

inhibition assay, expressed relative to the low uptake of fluorescent substrate in resistant cells,

the two highest concentrations (20 and 50 µM) of both dLop and Lop increased uptake by 4- and

12-fold, respectively (Figure 5). At these same concentrations, neither dLop nor Lop inhibited

Mrp1 or BCRP function (Figure 5). Inhibitors specific for each ABC transporter were used as a

positive control to demonstrate inhibition of efflux in each resistant cell line. Cyclosporin A

increased accumulation of the P-gp substrate 30-fold; MK-571 increased the accumulation of the

Mrp1 substrate 5-fold; and fumitremorgin C increased the accumulation of the BCRP substrate

6-fold. In fact, fumitremorgin C increased uptake of the fluorescent substrate in *ABCG2*-

expressing cells higher than that in untreated parental H460 cells. This result was not anomalous

because H460 cells are known to express a small amount of functional BCRP that is not

detectable by Western analysis (Henrich et al., 2007). Furthermore, when H460 cells were

treated with fumitremorgin C, they accumulated the same amount of fluorescent substrate as the

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resistant cells (data not shown). Parental cell lines were used as an additional control to show accumulation of each fluorescent substrate without (Figure 5) and with modulators (data not shown).

In the cytotoxicity assay, we found that dLop (20 µM) and Lop (20 µM) sensitized the *ABCB1*-expressing cell line to doxorubicin by ~4 fold ($P < 0.001$) and ~6 fold ($P < 0.001$), respectively (Table 1). However, neither compound significantly modulated the resistance of the *ABCC1*- or *ABCG2*-expressing cell lines to etoposide or to mitoxantrone, respectively. When assayed in the absence of cytotoxic agents, neither dLop nor Lop were toxic ($IC_{50} > 50$ µM) by themselves to any cell line (data not shown).

DISCUSSION

In both human and mouse tissues, dLop is selective for P-gp among these three most prevalent efflux transporters at the blood-brain barrier. However, the activity of dLop is dependent on its concentration. At low concentrations, dLop acts as only a substrate; at high concentrations, it acts as both a substrate and an inhibitor (i.e., a competitive substrate). In human cells, the selectivity of dLop for P-gp was demonstrated because accumulation of [^{3}H]dLop significantly differed between the parental and resistant cells for only the *ABCB1* pair, but not for the *ABCC1* or *ABCG2* pairs. The selectivity measured *in vitro* using human cells paralleled that *in vivo* using transgenic mice, since the concentration of radioactivity in brain after injection of [^{11}C]dLop was at least 2.5-fold higher in *abcb1a/b*-knockout mice than in *abcc1a*-knockout, *abcg2*-knockout, or wild-type mice.

High concentrations of both dLop and Lop selectively inhibit P-gp as competitive substrates, but not Mrp1 or BCRP. Selectivity at high concentrations was demonstrated in two ways. First, both dLop and Lop ($\geq$ 20 µM) inhibited the function of only P-gp, but not that of Mrp1 or BCRP. Our results also suggest that Lop is a more avid substrate than dLop because Lop inhibited P-gp function at a lower concentration than dLop. Second, both dLop and Lop (20 µM) significantly decreased the resistance of only the *ABCB1*-expressing cell line to its cognate cytotoxic substrate. The ability of dLop and Lop to inhibit P-gp function as competitive substrates is consistent with that of other substrates of ABC transporters. For example, the P-gp substrate verapamil also inhibits the function of P-gp as a competitive substrate (Ambudkar et al., 1999). Although high concentrations (micromolar) of both dLop and Lop inhibit P-gp function, the low concentrations (nanomolar) typically used for PET radiotracers would reflect substrate activity and not competitive inhibition of the transporter's function.

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Our method of using both cultured human cells and knockout mice to measure the

selectivity of substrates for ABC transporters is valuable for two reasons. First, since selectivity

for human ABC transporters was measured using human cells, our results were not confounded

by species differences in substrate selectivity that is known for ABC transporters (Syvanen et al.,

2009). Second, we showed that our results from the *in vitro* model extended to an *in vivo* model,

which consisted of knockout mice that selectively lack one of the three ABC transporters. This *in*

vivo model was also advantageous because it avoided the use of pharmacological inhibitors for

ABC transporters. Although some inhibitors are reported to be selective for one ABC transporter

at low concentrations, they may lose that selectivity at high concentrations. For example, low

concentrations ($\leq$ 100 nM) of tariquidar appear to be selective for P-gp (Martin et al., 1999; Fox

and Bates, 2007), but higher concentrations (> 1 μM) show cross reactivity for BCRP (Robey et

al., 2004). Therefore, the use of both human cells and knockout mice is advantageous because

the two systems allow selective interrogation of a single ABC transporter.

One limitation of our imaging studies is the possibility that the *abcc1a-* and *abcg2-*

knockout mice had altered peripheral metabolism of [^{11}C]dLop to make it appear that dLop is not

a substrate for these two transporters. For example, these mice might have metabolized the

radioligand so quickly that little was available for uptake into brain. However, we think

this possibility is highly unlikely. We previously studied the metabolism of [^{11}C]dLop in

abcb1a/b-knockout and wild-type mice by measuring the metabolic profile of radioactivity in

extracts of plasma and brain at 30 min after injection of [^{11}C]dLop (Lazarova et al., 2008). The

concentrations of parent radioligand and radiometabolites in plasma were similar for both

abcb1a/b-knockout and wild-type mice. However, the concentrations in brain differed, with

several fold higher concentration of [^{11}C]dLop in brains of *abcb1a/b*-knockout than of wild-type

mice (Lazarova et al., 2008). Since the knockout mice for *abcc1a* and *abcg2* derive from the same genetic background as the other two strains, they are highly unlikely to have unusually fast or altered metabolism to cause a false negative result.

Will [¹¹C]dLop be useful to measure P-gp in neuropathological conditions associated with both decreased and increased function? [¹¹C]dLop has low brain uptake at baseline, and P-gp inhibition increases brain uptake 2 to 4 fold (Kreisl et al., 2009). These characteristics suggest that [¹¹C]dLop will be useful to measure P-gp in pathophysiological conditions associated with decreased function, such as Alzheimer's disease. That is, decreased function of P-gp in Alzheimer's disease would be predicted to increase brain uptake of [¹¹C]dLop. However, the uptake [¹¹C]dLop into brain at baseline is so low that it may not be useful to measure P-gp in pathological conditions associated with increased function, such as drug-resistant epilepsy. In contrast, a substrate radiotracer, such as [¹¹C]verapamil, that has moderate brain uptake at baseline might be capable of measuring increased P-gp function. However, a recent study by Langer and colleagues (2007) found no significant decrease of [¹¹C]verapamil uptake in drug-resistant epilepsy (2007). Further studies are required to determine the relative utility of substrates, like [¹¹C]verapamil, that have moderate brain uptake at baseline compared to those, like [¹¹C]dLop, that have low brain uptake at baseline.

In conclusion, our results show that dLop is selective for P-gp both in human cells and in live mice and that its activity is dependent on concentration. At low concentrations, dLop acts as only a substrate; at high concentrations, it acts as both a substrate and an inhibitor (*i.e.*, a competitive substrate). Since low concentrations of radiotracer are used for PET imaging, [¹¹C]dLop acts selectively and only as a substrate for P-gp at the blood-brain barrier.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Expression of P-glycoprotein (P-gp), multidrug resistance protein 1 (Mrp1), and breast cancer resistance protein (BCRP) in three pairs of cell lines as determined by Western blot analysis. The three pairs are (listed as parental and resistant, respectively): MCF-7 and MCF-7/VP16 (*ABCC1*); KB-3-1 and KB-8-5-11 (*ABCB1*); H460 and H460/MX20 (*ABCG2*). A protein ladder was loaded with each Western blot to mark the bands for Mrp1 (190kDa), P-gp (160 kDa), BCRP (70kDa), and GAPDH (40 kDa). Cell lysates were probed for GAPDH as a control for the amount of protein loaded. A non-specific band appears in all the lanes when probed against BCRP.

Figure 2. Time-dependent accumulation of [^{3}H]*N-desmethyl*-loperamide (dLop) in parental cells of the *ABCB1* line. Cells were incubated with [^{3}H]dLop (1 nM), lysed at the times indicated, and assayed for radioactivity. Data represent mean ± SD of three observations.

Figure 3. Accumulation of [^{3}H]dLop by three pairs of parental (white bar) and drug-resistant (black bar) cells. Accumulation was significantly different between the parental and resistant cells of only the *ABCB1* pair. The amount of [^{3}H]dLop in cells was assayed 45 min after the addition of [^{3}H]dLop (1 nM). Data represent mean ± SD of three observations. ***$P < 0.001$ by unpaired Student's two-tailed *t*-test (α = 0.05).

Figure 4. Concentration of radioactivity (%SUV) measured by PET in brains of four strains of mice after injection of [^{11}C]dLop. Three strains of mice were selectively knocked out for the gene that encodes either P-gp (○), Mrp1 (□), or BCRP (■); the fourth strain was wild-type (●).

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Concentration of radioactivity in *abcb1a/b*-knockout mice was at least 2.5 times higher ($P <$ 0.0001 by two-way ANOVA) than that in other three strains of mice. Symbols represent mean $\pm$ SD values from four mice per strain. For clarity, the concentration of radioactivity taken up into brain in the first three minutes is shown as inset. Conc = concentration.

Figure 5. The ability of dLop and loperamide (Lop) to inhibit (as competitive substrates) the function of P-gp, Mrp1, and BCRP. Both dLop and Lop selectively inhibited only P-gp function. The cellular accumulation of transporter-specific fluorescent substrate is shown as a bar that represents the mean fluorescence intensity normalized to accumulation of untreated parental cells for a total of 10,000 cells. For each cell line pair, accumulation is shown for: untreated parental and resistant cells (white bar), inhibitor-treated resistant cells (black bar), and dLop- or Lop-treated resistant cells (shade-gradated bars). Fluorescent substrates with activity for each transporter were used: rhodamine 123 (Rh123) for P-gp, calcein-AM (Ca-AM) for Mrp1, and mitoxantrone (MTX) for BCRP. In addition to substrate, an inhibitor was used for each ABC transporter: cyclosporin A (CsA; 10 µM) for P-gp, MK-571 (50 µM) for Mrp1, and fumitremorgin C (FTC; 5 µM) for BCRP. Bars represent mean $\pm$ SD of three observations. Note that some error bars are smaller than the line thickness of the bars.

Table 1. Effect of dLop and Lop to modulate the cytotoxicity of drugs preferentially effluxed by P-gp, Mrp1, and BCRP

Cell line	Cytotoxic drug	Cytotoxicity					
				dLop (20 µM)		Lop (20 µM)	
		IC_{50}	RR	IC_{50}	RR	IC_{50}	RR
B1 resistant	Doxorubicin	846 ± 68 nM	71	234 ± 57 nM	20[***]	144 ± 15 nM	12[***]
B1 parental	Doxorubicin	12 ± 4 nM					
C1 resistant	Etoposide	79 ± 20 µM	79	140 ± 41 µM	140[†]	169 ± 73 µM	169[†]
C1 parental	Etoposide	1 ± 1 µM					
G2 resistant	Mitoxantrone	223 ± 54 nM	37	256 ± 56 nM	42	367 ± 118 nM	61
G2 parental	Mitoxantrone	6 ± 1 nM					

IC_{50} values are mean ± SD from three independent experiments.

RR = resistance ratio, which is the quotient of the IC_{50} value of the resistant cell line to that of the parental line.

***$P < 0.001$ ($\alpha = 0.05$, from initial IC_{50} value of resistant cell line) by Student's two-tailed t-test.
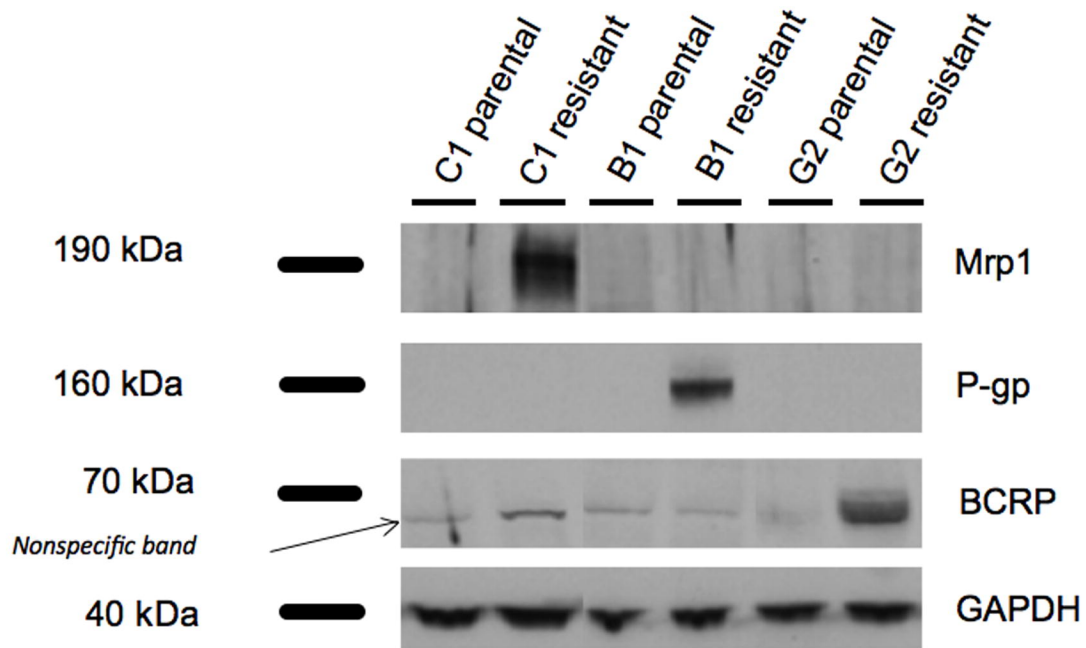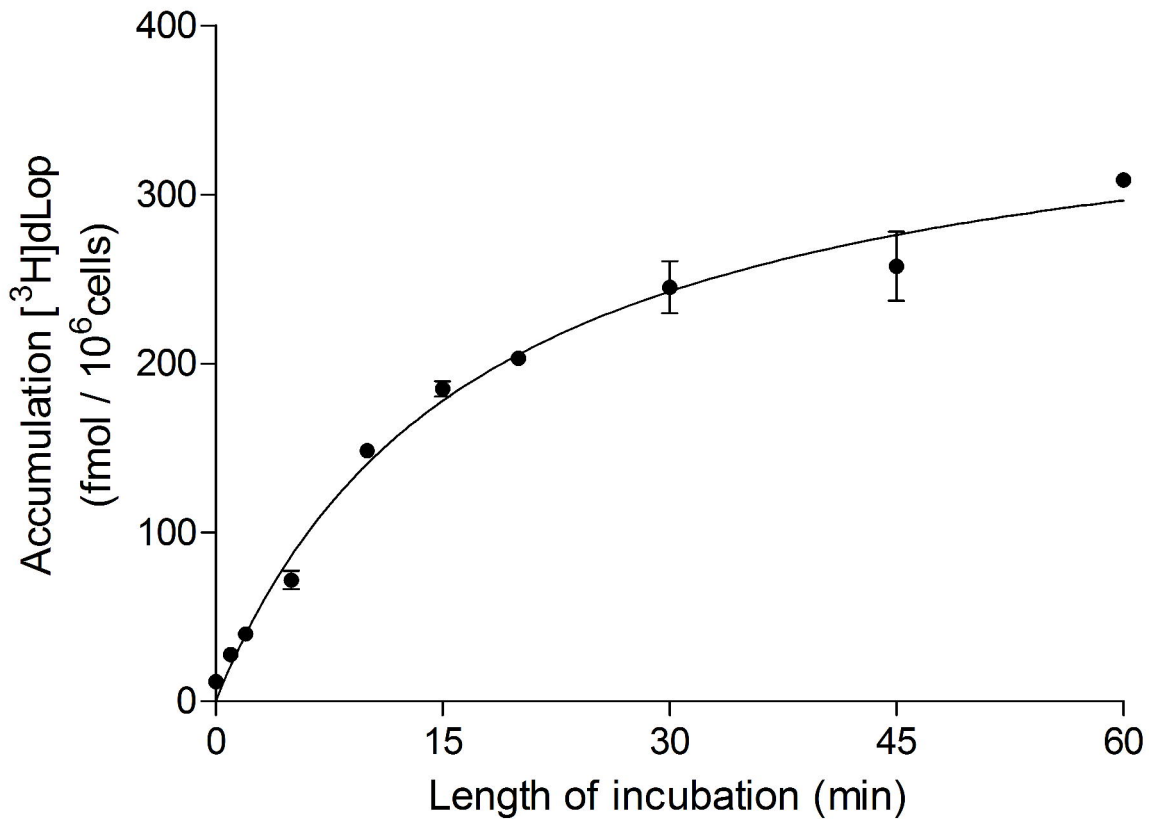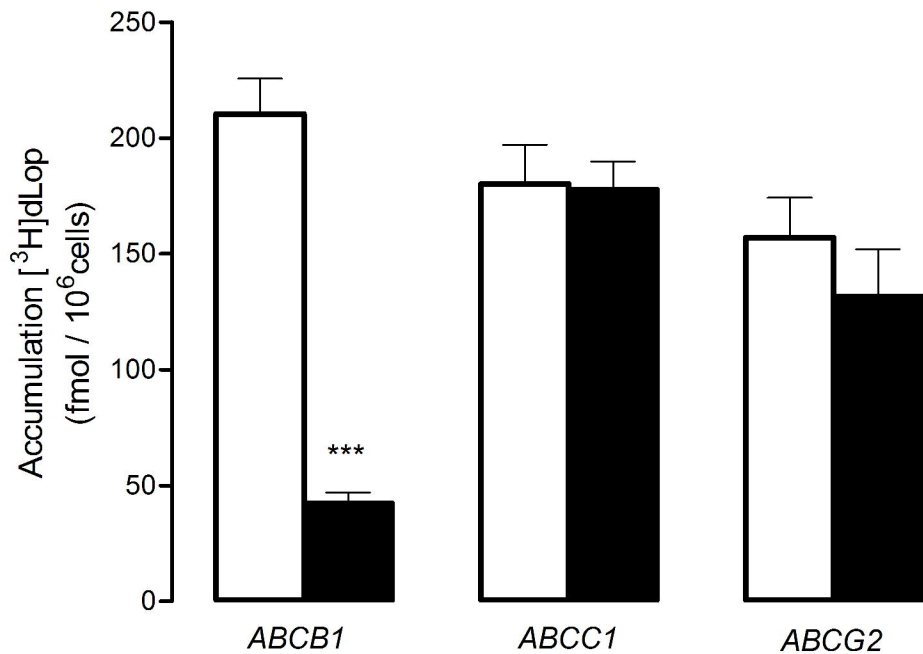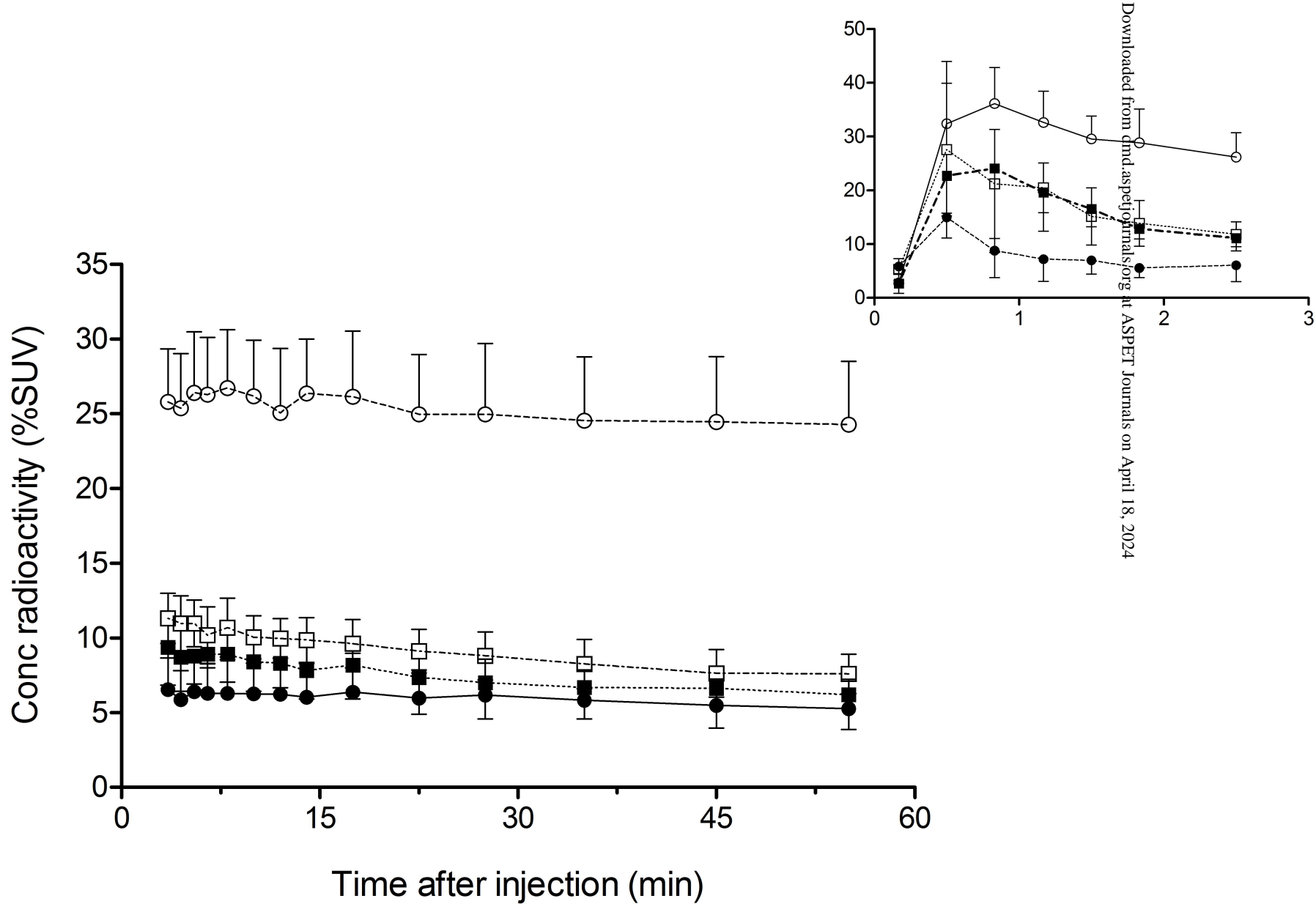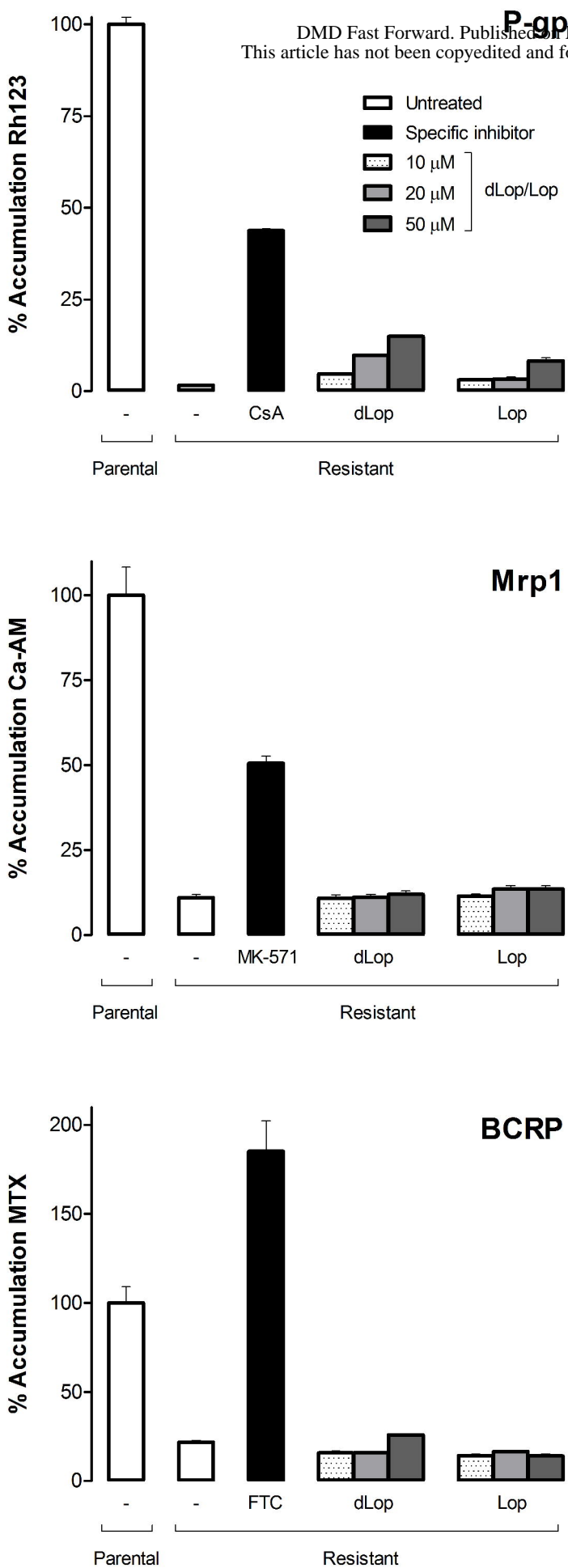
[†] $P > 0.18$

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.