

Title Page

Ketamine pharmacokinetics and pharmacodynamics
are altered by Pgp and Bcrp efflux transporters in mice

Samit Ganguly, John C. Panetta, Jessica K. Roberts and Erin G. Schuetz

Department of Pharmaceutical Sciences, St Jude Children's Research Hospital, Memphis, TN,
(S.G., C.P., J.K.R., E.G.S.), Cancer & Developmental Biology Track, University of Tennessee
Health Science Center, Memphis, TN (S.G.)

Running Title Page

Running Title: Pgp and Bcrp alter Ketamine's PK and Pharmacodynamics

Corresponding Author: Erin G. Schuetz, Ph.D., Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, TN 38105; PH: (901) 595-2205; Fax: (901) 595-3125; Email: erin.schuetz@stjude.org

Number of text pages:

Number of tables: 1

Number of figures: 4

Number of references: 38

Number of words in the *Abstract*: 211

Number of words in the *Introduction*: 588

Number of words in the *Discussion*: 1904

Abbreviations: dLORR, duration of loss-of-righting reflex; RUV, residual unexplained variability; BSV, between subject variability; dKO, double knock-out; WT, wild type; IP, intraperitoneal; PO, oral; PK, pharmacokinetic.

Abstract

To understand the systemic impact of Bcrp and Pgp deletion, untargeted metabolomics was performed on CSF and plasma of wild-type (WT) and Pgp and Bcrp double knockout (dKO) rats anesthetized with ketamine-xylazine. We unexpectedly found elevated ketamine levels in both CSF and plasma of dKO versus WT rats. Therefore, the effect of these transporters was investigated on ketamine's (i) oral (PO) and intraperitoneal (IP) serum pharmacokinetics (PK), using a liquid chromatography (HPLC-UV) method, and (ii) anesthetic effect using a duration of loss of righting reflex (dLORR) test in WT, Bcrp KO, Pgp KO, and Pgp-Bcrp dKO mice. The PK data demonstrated a significantly increased oral bioavailability and serum exposure of ketamine in the dKO > Pgp KO > Bcrp KO compared to the WT mice. IP Ketamine-induced dLORR was significantly longer in the dKO > Pgp KO > Bcrp KO > WT mice compared to the WT mice. Inhibition of Bcrp and Pgp in WT mice using the dual Pgp/Bcrp inhibitor elacridar increased the ketamine-induced dLORR compared to the vehicle-treated mice. Ketamine intracellular concentration was significantly decreased in MDCKII BCRP/PGP cells compared to the parental cells. In total, these results demonstrate that ketamine appears to be a dual Pgp/Bcrp substrate whose pharmacokinetics and pharmacodynamics are affected by Pgp and Bcrp-mediated efflux.

Introduction

Ketamine is a clinically useful anesthetic that is used either alone or in combination with other drugs in different surgeries (Sleigh J, 2014). It is also useful for the management of neuropathic and postoperative pain (Niesters et al., 2014; Barrett and Schrager, 2016). Recent literature reports have identified a long-lasting antidepressant activity of ketamine at sub-anesthetic doses (Machado-Vieira et al., 2009). In addition to its clinical use, ketamine is also a recreationally abused drug and chronic ketamine use causes kidney pathology, ulcerative cystitis (Shahani et al., 2007) and bladder toxicity (Chu et al., 2008).

Pharmacologically, ketamine is a N-methyl-D-aspartate (NMDA) receptor antagonist that binds to the phencyclidine binding pocket of the receptor (Mion and Villevieille, 2013) to elicit anesthesia. However, ketamine is also known to interact with other pharmacological targets (Sleigh J, 2014). For example, ketamine was shown to have rapid onset antidepressant activity following metabolism to (2R,6R)-hydroxynorketamine that activates α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA receptors) (Zanos et al., 2016).

Ketamine pharmacokinetics (PK) in humans are well-described by a two or three compartment PK model (Fanta et al., 2015; Sherwin et al., 2015) with a high volume of distribution, high clearance and low plasma protein binding (Mion and Villevieille, 2013). Ketamine is metabolized primarily by CYP2B6 and CYP3A4 to form norketamine, which is further metabolized to hydroxynorketamine and its glucuronide (Hijazi and Boulieu, 2002). Ketamine has a low oral bioavailability that can be partially explained by its high metabolism observed by the oral route compared to intravenous or intramuscular routes (Fanta et al., 2015).

In addition, modifications in ketamine metabolism have been reported to cause potential drug-drug interactions (Peltoniemi et al., 2012).

Breast cancer resistant protein (Bcrp/Abcg2) and P-glycoprotein (Pgp/Abcb1a) are membrane-bound efflux transporters that are present in a wide variety of tissues such as blood-brain barrier and blood-cerebrospinal fluid barrier in the brain, intestinal epithelial cells, and bile canalicular membrane and kidney tubules. These transporters recognize a wide variety of chemicals as their substrates and reduce substrate systemic exposure by decreasing absorption (intestine) and increasing elimination (liver and kidney). Pgp and BCRP expression at the BBB and BCSFB decreases substrate movement from blood to brain and to CSF, respectively. Thus, inhibition of Bcrp and/ or Pgp can lead to systemic overexposure of their substrates and cause toxicity. A wide variety of drugs as well as chemicals from food or drinks are known to inhibit these transporters, and can therefore lead to potential drug-drug or drug-food interactions (Varma et al., 2003; Kusuhara et al., 2012). Moreover, the BCRP Q141K inactivating allele is common in some populations with as many as 6% of Asians homozygous for this loss of function allele (Zamber et al., 2003).

Screening drugs in development for Bcrp and Pgp transport and inhibition capability is one of the FDA required drug approval criteria (Tweedie et al., 2013) because of the large number of Bcrp and Pgp substrates and inhibitors, and the potential for some chemicals to be dual substrates for both transporters. Some *in vivo* studies in rodents as well as in humans have suggested a potential interaction of ketamine with Bcrp and/or Pgp (Sato et al., 2007; Lilius et al., 2015). Given the clinical and recreational use of ketamine, and the significant interest in ketamine as an effective anti-depressant, we sought to identify if ketamine is a substrate of Bcrp and/or Pgp. Using mice with and without these two transporters, we report for the first time that

ketamine is an apparent dual Pgp/Bcrp substrate whose pharmacokinetic and pharmacodynamic properties are affected by both Pgp and Bcrp.

Materials and Methods

Drugs and Chemicals. Ketamine hydrochloride injection USP (100 mg/ml) was purchased from JHP Pharmaceuticals (Rochester, MI), elacridar was purchased from Astatech Inc. (Bristol, PA), Hydroxypropylmethylcellulose (HPMC) and Tween 80 were purchased from Sigma-Aldrich (St Louis, MO), Promega Reporter Lysis buffer (5X), PierceTM BCA protein assay reagent (A and B), bupropion, acetonitrile, formic acid and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). ³H-ketamine was obtained from American Radiolabeled Chemicals (St.Louis, MO).

Animals and cell lines. Wild-type (WT) and Bcrp/Pgp (Abcg2-Abcb1a) double-knockout (dKO) male Sprague-Dawley rats, weighing 200 – 250 g, were obtained from SAGE[®] Labs (Horizon Discovery – St Louis, MO). Male Bcrp (Abcg2) KO, Pgp (Abcb1a/b) KO, Pgp/Bcrp (Abcb1a/1b/ Abcg2) double KO (dKO), and Wild-type Friend virus B (FVB) mice were obtained from Taconic farms (Germantown, NY). Rats used in the experiments were 12 weeks old and mice were 10-16 weeks old. All animals were provided water and food ad libitum. Ketamine hydrochloride stock solution (100 mg/ml) was diluted with filtered autoclaved water to the appropriate dilution for dosing. Elacridar (10mg/ml) was formulated by suspending elacridar in a solution of 0.5 % Hydroxypropylmethylcellulose (HPMC) with 1% Tween 80. A 1.5 inch 20 GA feeding needle (Fisher Scientific Co., Pittsburgh, PA) was used to perform oral dosing. The Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital in accordance with the U.S. National Institutes of Health guidelines approved all experimental procedures.

MDCKII parental cells and derivative cells stably overexpressing both human PGP and BCRP were kindly provided by Dr. Alfred H. Schinkel's lab (The Netherlands Cancer Institute, Amsterdam) and cultured as described (Poller et al., 2011).

Drug formulations for animal experiments. Ketamine hydrochloride stock solution (100 mg/ml) was diluted with filtered autoclaved water to appropriate dilution for oral (PO) or intraperitoneal (IP) dosing. Elacridar (solution) formulation was prepared by suspending the appropriate amount of elacridar in 0.5 % Hydroxypropylmethylcellulose (HPMC) with 1% Tween 80 solution to achieve a final concentration of 10 mg/ml.

Estimation of ketamine exposure in Rat CSF and Plasma. Male WT and Pgp/Bcrp dKO rats (n= 8 per group) were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) formulated in water, at a dose volume of 10 ml/ kg of body weight. Cerebrospinal fluid (CSF) was collected from the cisterna magna immediately after the anesthesia was achieved (~7 minutes), followed by blood collection by cardiac puncture. Blood samples were centrifuged and plasma separated from the blood. Both plasma and CSF samples were immediately frozen on dry ice, stored at -80 °C, and shipped to Metabolon® (Research Triangle Park, NC) for metabolomic analysis. LC-MS/MS sample analysis, data normalization and analysis were performed at Metabolon® as described in other reports (Evans et al., 2009). Normalized ketamine peak area intensity values were compared between the WT and dKO rats in both CSF and Plasma.

Pharmacokinetic study of ketamine in WT, Bcrp KO, Pgp KO and dKO mice after oral and intraperitoneal dosing at 100 mg/kg. A serum pharmacokinetic study was conducted following ketamine intraperitoneal and oral dosing (IP and PO PK) of WT, Bcrp KO, Pgp KO and dKO mice at 100 mg/kg dose of ketamine. For IP PK, six mice from each genotype were divided into two groups of three mice each. After the animals were dosed IP, blood was collected from one group of mice at 2, 10 and 40 minutes, and from another group of mice at 5, 20 and 60 minutes. At each time point, 50 μ L blood was withdrawn from each mouse from the saphenous vein into microvette capillary blood collection tubes (Sarstedt, obtained from Fisher Scientific, Pittsburgh, PA). Samples were kept at room temperature and were centrifuged at 10,000 rpm for 5 minutes to obtain serum, which was immediately separated and stored at -80 $^{\circ}$ C until further analysis. For the PO PK study, six mice from each genotype were separated into two groups (three mice in each group) and after oral dosing blood samples were collected at alternative time points from each group similar to the IP study. After a two week washout period, the same oral study was repeated by altering the groups and time points such that all time points were collected from each mouse. The two PO PK data sets were first analyzed separately and compared to identify any significant inter-day variation, and, after confirming no inter-day difference in PK profile, the data from the two collection dates were merged to obtain complete PK profiles for each mouse.

Determination of ketamine concentration in mouse serum by HPLC/UV. Serum samples from the intraperitoneal ketamine PK studies in mice were treated with chilled acetonitrile (1:5); samples were vortexed and centrifuged for 15 minutes at 4000 rpm to precipitate the protein and extract ketamine. After centrifugation, the supernatant was collected and further centrifuged for

sample cleaning. The resultant final supernatant was then diluted 1:1 with water: acetonitrile (1:1) and a 10 μ L sample was injected onto the HPLC for further analysis. For PO PK, a 1:2.5 initial dilution with chilled acetonitrile was used, followed by centrifugation and protein precipitation and re-centrifugation of the supernatant for sample cleaning. The final supernatant was injected onto the HPLC system (Shimadzu Prominence, Kyoto, Japan) consisting of a LC-20AD binary high pressure gradient pump, SIL-20AC_{HT} auto sampler, and SPD-20AV UV-detector. Bupropion (100 μ g/mL) was added as an internal standard to all the samples prior to extraction and was extracted along with ketamine. Chromatographic separation of ketamine along with the internal standard was achieved using Hypersil ODS C18 column (150X4 mm; 5 μ m particle size) fitted with Hypersil ODS (C18) Javelin Guard Column (10X4 mm; 5 μ m particle size) and a gradient elution method with a run time of 10 minutes. The mobile phase for elution was 60:40 ammonium formate buffer (20 mM): acetonitrile (Moore et al., 2001) and the gradient used was: 0 to 3 minutes 40% acetonitrile, 3 to 8 minutes 60% acetonitrile, 8 to 9 minutes 40% acetonitrile, and the run stopped at 10 minutes. Retention times for ketamine and bupropion were 5.2 and 7.1 minutes, respectively. The ketamine peak was identified and absorbance was measured at 210 nm (Bolze and Boulieu, 1998). Analyte/IS height ratio was used for preparation of calibration and measurement of unknown concentrations. Extraction efficiency was >90% throughout the analysis. The calibration range for IP PK and PO PK analysis were 0.39 μ g/ml-200 μ g/ml and 0.19 μ g/ml-100 μ g/ml, respectively.

Measurement of Ketamine Brain/serum ratio following IP Ketamine. Male FVB WT, Pgp KO and Pgp/Bcrp dKO mice were dosed with IP Ketamine (100 mg/kg) + 1 μ Ci ³H-ketamine tracer 1 hr before and again 15 min before sacrifice. Mice were euthanized by CO₂ inhalation,

blood was collected and serum separated, and brain tissue was weighed and mixed with an equivalent volume of phosphate buffer saline (PBS) and homogenized. Brain homogenate and serum were mixed with scintillation fluid and total radioactivity determined by liquid scintillation counting. The ^3H -ketamine brain-to-serum ratio is dpm/gram brain tissue homogenate divided by dpm/ml of serum.

In another similar study, FVB male mice (WT, Pgp KO and Pgp/Bcrp dKO) were sacrificed 15 minutes after dosing 100 mg/kg ketamine intraperitoneally. Brain and blood were collected from each mouse after sacrifice. Brain tissues were weighed, homogenized with 20 mM ammonium formate buffer, and 9 μL brain tissue homogenate + 1 μL IS was extracted using chilled acetonitrile. Supernate from the extracted samples were mixed with equal volume of acetonitrile/water (1:1) and injected in HPLC-UV for measurement of ketamine against standards prepared similarly from untreated mouse brain homogenate. Serum was separated from blood samples and ketamine concentration was measured by HPLC-UV based method as described previously. Brain/ serum ratio was obtained by dividing weight normalized brain tissue concentration with the serum concentration of ketamine.

Pharmacokinetic data analysis and interpretation. (a) Non-compartment Analysis. Initial analysis of both IP and PO PK study data was performed by a non-compartment approach using PK package on R statistical software (Jaki and Wolfsegger, 2011) to determine whether there were significant differences in ketamine serum exposure between WT and Bcrp KO, Pgp KO and dKO mice. A serial sacrifice design was assumed to compute the area under time – concentration curve extrapolated to infinity (AUC_{inf}) and the associated variability as described (Jaki and Wolfsegger, 2011).

(b) **Nonlinear mixed effect modeling.** Upon identification of significant exposure difference between genotypes in the PO PK study by non-compartment analysis, the PO and IP PK data were combined and analyzed with nonlinear mixed effect modeling implemented in Monolix 4.3.3 (Lixoft, France) using a stochastic approximation expectation maximization algorithm (Kuhn and Lavielle, 2005) combined with a Markov chain Monte Carlo procedure. Population analysis of our PK data did not find any difference in ketamine serum PK between WT and Bcrp KO mice and thus, these two genotypes were combined in the final model. Out of 144 data points in the PO PK study, there were four concentrations that were below the lowest calibration standard concentration (0.19 µg/mL) and these were removed from the analysis. One- and two-compartment models were evaluated. The goodness-of-fit-criteria and minimization of Akaike information criterion were used to select the base model. The effect of over-parameterization was also assessed using the condition number and Bayesian information criterion. Covariate analysis included the effect of genotype as a categorical covariate following an exponential model. The effect of genotype on individual parameters was determined by a forward addition process with a decrease in OFV ≥ 3.84 considered significant at $p = 0.05$ for one degree of freedom, based on χ^2 (chi-square) distribution. Variability in observed data from the model predicted population mean data is considered to be due to between-subject variability (BSV) and residual unexplained variability (RUV). The following equation was used to describe BSV,

$$P_i = \theta_{pop} \times \exp(\eta_{pop}) \quad (1)$$

where, P_i = PK parameter of the i^{th} individual; θ_{pop} = population mean for P ; η = normally distributed between-subject random effect with a mean of zero and variance of ω^2 . Constant, proportional and combined constant and proportional error models were tested for RUV, and the final model used the proportional error model that follows the following equation,

$$Y_{ij} = \hat{Y}_{ij} + Y_{ij} * \epsilon \quad (2)$$

where Y_{ij} = observed concentration of i^{th} individual at time j ; \hat{Y}_{ij} = individual predicted concentration and ϵ is the proportional error that is normally distributed with a mean of zero and variance of σ^2 .

Diagnostic plots were used for the final assessment of the model. Correlation between the observed and model predicted concentrations were evaluated and used as screening criteria for model selection. Population weighted residuals or individual weighted residuals were plotted against time and population or individual predicted ketamine concentration. Normalized prediction distribution error plotted against time and population predicted ketamine concentration was also evaluated for model misspecification assessment. Apart from the diagnostic plots, final model selection also included comparison of objective function values and variability associated with parameter estimates.

Comparison of ketamine-induced duration of Loss of Righting Reflex (dLORR) between WT, Bcrp KO, Pgp KO and dKO mice. Ketamine-induced dLORR was studied as described (Sato et al., 2004). Briefly, animals were administered ketamine (50, 100 or 200 mg/kg) intraperitoneally or by oral gavage, and after four minutes waiting time (to reduce manual stimulation), mice were placed on their back. Loss of righting reflex was defined to have taken place when the mouse could not right itself for at least 20 seconds. The dLORR was then measured until the mouse woke up spontaneously and the dLORR was compared between WT, Bcrp KO, Pgp KO and dKO mice. In a separate study WT mice were orally administered

elacridar (ECD) (100 mg/kg) or vehicle 1.5 hours prior to IP administration of 50 or 100 mg/kg ketamine and dLORR was measured as described above.

Intracellular uptake of radiolabeled ketamine in MDCKII cells overexpressing human PGP and BCRP. Madine-Darby canine kidney II (MDCKII) cells +/- BCRP and PGP were plated at 0.2×10^6 cells/ well in a 24 well plate. The next day media was removed and cells were incubated with ^3H -ketamine at 37°C . At the selected time points, cells were washed with chilled PBS, incubated 1 hr with lysis buffer, cell lysates collected, and the intracellular concentration of ^3H -ketamine and protein concentration determined. Protein concentration was determined in the lysate by BCA protein assay. Results were expressed as pmole of ketamine per milligram of protein lysate. First, we determined the time to reach an equilibrium of intracellular uptake by incubating both WT and transfected cells with $0.5 \mu\text{M}$ ^3H -ketamine for one hour and measuring the intracellular radioactivity at 2, 5, 15, 30 and 45 minutes. In a separate assay, the concentration-dependent uptake of ^3H -ketamine up to the time of equilibrium was determined in MDCKII and BCRP-PGP-MDCKII cells by incubating the cells with different concentrations of ^3H -ketamine until they reached equilibrium and then measuring intracellular concentration of ^3H -ketamine. The intracellular ketamine concentration was calculated and compared between the MDCKII and BCRP-PGP-MDCKII cells.

Statistical analysis. GraphPad QuickCalcs was used to perform Grubb's outlier test to remove outlier data from the analysis. Statistical analysis of significance for CSF and plasma ketamine exposure data in rats, all pharmacodynamic (dLORR) data, *in vitro* cell uptake study data, and

non-compartment analysis were performed in GraphPad Prism software version 5.02.

Ketamine's CSF and plasma peak intensity in the dKO rats and WT rats was compared using the unpaired t-test with Welch's correction. All dLORR study data was analyzed using Mann-Whitney U test. Intracellular uptake study results were analyzed using an unpaired t-test. The non-compartment-determined AUC_{inf} was analyzed using a one-way ANOVA with Newman-Keuls Multiple Comparison Test. Significance for all analyses was equal to $p < 0.05$.

Results

Deletion of both Bcrp and Pgp results in greater ketamine exposure in the CSF and plasma

of dKO rats. We performed a metabolomic comparison analysis of the CSF and plasma from WT and Bcrp-Pgp dKO rats anesthetized with a cocktail of ketamine-xylazine. A full report of the metabolomics results will be published elsewhere. Ketamine levels (measured by LC-MS/MS) were found to be significantly higher in the CSF (1.74 times) and plasma (1.93 times) of dKO compared to WT rats (Fig 1). These results suggested that ketamine might be a substrate of Bcrp and/or Pgp. The metabolomic analysis did not identify the presence of xylazine in either the rat CSF or plasma from either WT or Pgp/Bcrp KO rats.

Ketamine oral (PO) serum exposure is higher in mice lacking Bcrp and Pgp. To determine if Bcrp and Pgp can affect the PK of ketamine, we collected serum following a 100 mg/ kg IP dosing to WT, Bcrp KO, Pgp KO, and dKO mice. Serum concentrations of ketamine at the selected time points were found to be similar between all four genotypes (Fig 2A). Ketamine serum exposure, as measured by area under time – concentration curve (AUC_{inf}) by a non-compartment analysis (NCA), was also found to be similar between all the genotypes (Fig 2B).

Since Bcrp and Pgp have a higher impact on their substrates serum/ plasma exposure after oral compared to IP dosing (Lagas et al., 2009), we collected serum following a 100 mg/kg oral dose of ketamine in WT, Bcrp KO, Pgp KO and dKO mice. Ketamine serum concentrations were significantly higher in the dKO mice compared to the WT mice (Fig 2C). Ketamine serum exposures (AUC_{inf}), calculated by the non-compartment analysis method, were found to be significantly higher in dKO (1.96-fold) and Pgp KO (1.52-fold) mice and slightly higher in Bcrp

KO (1.20-fold) mice compared to the WT mice (Fig 2D). Ketamine serum concentration at the 60 minute time point could be detected in only two out of six WT mice, but in all of the Bcrp KO, Pgp KO and dKO mice. Thus, the last time point for observed concentration is different between WT and other knockout mice. Hence, AUC_{inf} was used to compare the exposure between all genotypes. Both Pgp KO as well as Bcrp KO showed higher concentrations of ketamine than WT mice at earlier time points, indicating a possible alteration in absorption, and possibly driving the observed difference in AUC_{inf} with the WT mice. Detailed non-compartment analysis parameters for both IP and PO PK studies are listed in Supplemental Table S1. While the non-compartment analysis allowed comparison of concentration-time curve properties, there was no assumption of the compartment, and no information about which PK parameters could be causing the difference in AUC_{inf} , although there was the suggestion of an alteration in bioavailability because dividing the oral AUC_{inf} by the IP AUC_{inf} gave an (F) for the mice that was greatest in the dKO (~27%) > Pgp KO (~24%) > Bcrp KO (~17%) ~ WT (~14%).

Population pharmacokinetic analysis demonstrates an increase in ketamine oral bioavailability in Pgp/Bcrp dKO and Pgp KO mice compared to WT and Bcrp KO mice.

To better understand the impact of Bcrp and Pgp mediated efflux on PK properties of ketamine, we combined both PO and IP PK data in a simultaneous model using a population pharmacokinetic approach. This model also assumed that bioavailability after IP dosing was equal to one. The final model was a one-compartment distribution with zero order absorption for PO and first-order absorption for the IP dosing and first-order elimination from the central compartment (Supplemental Figure S1). The one compartment model was sufficient to describe

these data (Supplemental Figures S2, S3 and S4). The final parameters of population analysis are shown in Table 1. Transporter genotypes had no effect on clearance or volume of distribution. The small difference in ketamine PK between Bcrp KO versus WT mice observed by non-compartment analysis was not observed with the population approach likely due to variability in the result. However, both Pgp KO and dKO genotypes had a significant impact on population bioavailability ($p < 0.001$). Oral bioavailability (F) for the WT population was 17.5%, which significantly increases to 22% in the Pgp KO mice, and increases further to 29% in the dKO mice. Hence, altered ketamine PK were observed following oral administration to mice lacking Pgp singly or in combination with Bcrp. This difference translates to a difference in the post-hoc estimated AUC between these groups.

Ketamine-induced duration of loss of righting reflex (dLORR) was significantly increased in mice lacking Bcrp and Pgp. The difference in PO PK between WT and Bcrp and/or Pgp knockout mice, and higher plasma and CSF abundance of ketamine in the dKO compared to WT rats led us to investigate if ketamine pharmacodynamics are also affected by Bcrp and Pgp. We used the ketamine-induced loss of righting reflex (LORR) model in the mouse to study the effect of Bcrp and Pgp on ketamine pharmacodynamics. Following intraperitoneal ketamine, the median dLORR in the dKO mice was longer than in WT (2.6-fold), Bcrp KO (2.1-fold), and Pgp KO (1.3-fold) mice (Fig 3A and Supplemental Table S2). In addition, the median dLORR was 2-fold longer in the Pgp KO compared to the WT mice. These results indicate that both Pgp and Bcrp influence ketamine pharmacodynamics as measured by ketamine-induced dLORR.

To determine whether ketamine brain concentrations were different in the transporter KO vs. WT mice we first administered IP ketamine and measured brain and serum concentrations at 15 minutes after dosing (n=3 in each genotype group). Our HPLC-UV analysis shows a median brain/serum ratio of 1.32 in all genotypes and there is no significant difference in brain/serum ketamine concentration between genotypes (Supplemental Fig S5). To understand if the total concentration of ketamine and its metabolites are higher in the knockout mice compared to WT mice, we performed a whole brain homogenate ^3H -ketamine radioactivity study. We administered IP the standard anesthetic dose of ketamine (100 mg/kg) spiked with ^3H -ketamine and measured the ^3H -ketamine brain/serum ratio at 1.25 hr. Brain/serum ^3H -ketamine radioactivity ratio was not significantly different between genotypes with a median ratio of 1.45 in all mice. This result is similar to what we observed with the HPLC-UV analysis. Although there was a measurable increase in the level of ^3H -ketamine in the brains of Pgp and Pgp/Bcrp dKO mice, the difference between genotypes did not reach statistical significance (Supplemental Fig. S5), despite the remarkable impact of the transporters on ketamine's pharmacodynamic effect (Fig 3A), and the significant increase in CSF ketamine (measured by LC-MS/MS) in Pgp/Bcrp dKO rats (Fig 1). Although Pgp KO vs dKO brain ^3H - ketamine radioactivity difference reached statistical significance, the median dKO/ Pgp KO radioactivity ratio is 0.85, which could be due to differences in metabolite concentration between these two genotypes or even variability associated with analysis, dosing or other reasons.

Pharmacological inhibition of Bcrp and Pgp by elacridar increased IP ketamine-induced dLORR in WT mice. Since we observed altered PK and PD of ketamine in mice with genetic deletion of Bcrp and/or Pgp, we investigated whether ketamine PD were also altered by

pharmacological inhibition of these efflux transporters by using elacridar as a dual inhibitor of Bcrp and Pgp. Elacridar dosing and time schedules for pretreatment were based on previous reports that clearly demonstrated loss of Bcrp and Pgp function at the dose selected (Durmus et al., 2012; Sane et al., 2012; Hendrikx et al., 2014). Wild-type mice were pretreated with vehicle or 100 mg/kg elacridar and 1.5 hrs later ketamine was dosed IP at 50, 100 and 200 mg/kg (n=6 per dose group) and duration of LORR measured. The results in WT mice of ketamine treatment alone (Fig. 3B, Supplemental Table S3) demonstrated a dose-proportional increase in drug-induced dLORR from 50 to 100 mg/kg, but a disproportionately longer increase in dLORR at 200 mg/kg. Pretreatment with elacridar significantly increased dLORR in the WT mice treated with either 50 or 100 mg/kg ketamine. Based on the exceedingly long dLORR with 200 mg/kg ketamine alone, co-treatment at this dose with elacridar was not attempted to avoid toxicity. These results show that inhibition of Bcrp and Pgp with the dual inhibitor elacridar significantly increased ketamine-mediated dLORR in WT mice.

Ketamine-induced dLORR was significantly longer in dKO vs. WT mice after oral dosing of 200 mg/kg ketamine. Because ketamine can be taken orally, we compared the effect of the drug transporters on its pharmacodynamic effect following oral administration. Oral ketamine (100 mg/kg) dosing was insufficient to cause a LORR in any of the mice (data not shown) likely because the ketamine serum concentration was lower following oral vs. IP dosing (four-fold lower in dKO mice and seven-fold lower in WT mice) (Supplemental Table S1). Following oral ketamine (200 mg/kg) treatment, WT mice were all awake within 4 minutes, while one of the dKO mice died and the others needed to be warmed to wake them after 30 minutes, preventing further analysis of this dose (not shown).

Ketamine accumulation is lower in MDCKII cells transfected with human BCRP and Pgp compared to WT MDCKII cells. The time course of ^3H -ketamine cellular uptake was first compared between MDCKII cells and MDCKII-PGP-BCRP cells stably expressing the human transporters (Poller et al., 2011). The ^3H -ketamine cellular concentration was significantly greater in MDCKII parental cells compared to cells expressing BCRP and PGP at all time points, but uptake reached equilibrium by 20 minutes (Fig 4A). Likewise, the ^3H -ketamine intracellular concentration measured at 20 minutes was higher in the parental MDCKII cells compared to the dual BCRP/Pgp expressing cells between 0.1 to 5 μM concentrations but not at 10 μM concentration (Fig 4B). The data also indicated that the magnitude of difference in intracellular ^3H -ketamine was greater at lower concentrations vs. higher concentrations between the MDCKII cells with and without drug transporters. In support of these findings, Keiser et al (2018) recently published (while this manuscript was under review) in vitro studies showing that ketamine is a Pgp substrate.

Discussion

Following the unexpected finding that the concentration of the anesthetic ketamine was elevated in the CSF and plasma of Pgp-Bcrp dKO compared to wild-type rats, this study was performed in mice to determine whether ketamine was a dual substrate for these transporters. Overall the results indicate that ketamine oral exposure increased in the following order: WT < Bcrp KO < Pgp KO < dKO (Fig 2). Likewise, the rank order for ketamine-induced dLORR was significantly longer in the dKO > Pgp KO > Bcrp KO > WT mice (Fig 3). In sum, Pgp/Bcrp decreased IP ketamine-induced dLORR (Fig 3), decreased ketamine oral clearance (Fig 2D, Table 1), and elacridar increased IP ketamine induced-dLORR in wild-type mice (Fig 3B), demonstrating ketamine is an apparent substrate for both transporters.

There is mixed evidence in the literature that ketamine might interact with Pgp or Bcrp. Ketamine was previously screened for inhibitory activity against BCRP and PGP (Tournier et al., 2010) using MDCKII cells stably transfected with human BCRP or MDR1 (PGP) and found to not inhibit either transporter. However, high concentrations of cyclosporine increased the dLORR in mice (Sato et al., 2007), which could be due cyclosporine's ability to inhibit ketamine metabolizing Cyps as well as Pgp and Bcrp. The finding that female vs. male rats show a longer ketamine induced dLORR (Winters et al., 1986) might also reflect the fact that there is significant sexual dimorphism in rodent Bcrp expression with males vs. females having significantly higher levels of Bcrp in liver, intestine and kidney (Merino et al., 2005). Co-administration of morphine (a Pgp substrate) and ketamine increased ketamine's brain concentration (Lilius et al., 2015), which would be consistent with the ketamine competing with morphine for Pgp transport.

We used dLORR to compare the ketamine-induced anesthetic/hypnotic effect in mice with different transporter genotypes as dLORR can be used to assign dose-dependent pharmacodynamic effects (Sato et al., 2004). Although we observed a significantly increased IP ketamine induced dLORR in the dKO mice compared to WT, Bcrp KO and Pgp KO mice (Fig 3A), we did not find a significant increase in serum PK after IP ketamine dosing (Fig 2A) in the transporter single and dKO mice. However, it is not unprecedented for some Pgp substrates to not show systemic PK differences between WT and Pgp KO mice following IP dosing (e.g., dasatinib) (Lagas et al., 2009). Although ketamine levels were significantly higher in the plasma of Pgp/Bcrp dKO rats ~ 7 min after IP dosing (Fig 1), we were unable to detect a significant difference in ketamine PK following IP dosing of WT or single transporter KO mice (Fig 2A). This might have resulted from a number of factors including inter-species variability in metabolism/transport and the difference in blood collection time after ketamine between the two studies. PK parameters of ketamine in mice are not extensively reported in the literature. From the concentration–time data reported (Sato et al., 2004) after a 200 mg/kg IP dose, and (Zanos et al., 2016) after a 10 mg/kg IP dose in mice, ketamine’s plasma half-life ($t_{1/2}$) is approximately 25 minutes, which is similar to the results obtained in our study (Table 1). The C_{\max} after IP dosing of 10 mg/kg ketamine was reported between 0.55 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ (Can et al., 2016; Zanos et al., 2016), while we observed a C_{\max} of $7.88 \pm 1.38 \mu\text{g/ml}$ (Table S1) after 100 mg/kg IP dose of ketamine, which is within the dose linear range of the reported data. Whereas, Sato et al., after 200 mg/kg IP ketamine dosing in C57Bl/6 mice, reported a highest concentration of approximately 30 $\mu\text{g/mL}$ at 10 minutes (Sato et al., 2004), indicating a possible more than dose proportional increase in ketamine exposure beyond 100 mg/kg dose. Although ketamine bioavailability in humans after oral dosing has been reported to be between 7 and 20%

(Peltoniemi et al., 2012; Fanta et al., 2015), bioavailability after oral dosing in mice is not well established. Assuming 100% bioavailability after IP dosing, bioavailability after oral dosing in WT mice falls into the same range (F_Pop, Table S1) in our study to that observed in humans.

We did not find a significant difference in ketamine brain/ plasma ratio between the mice with different transporter genotypes (Supplemental Fig S5), and the increase of CSF concentrations in Pgp/Bcrp KO vs. WT rats can largely be explained by the increase in plasma concentrations (Fig 1). These data suggest blood brain barrier Pgp/Bcrp do not (or may not) impair ketamine brain concentration. So why are the effects of Pgp/Bcrp on ketamine's pharmacodynamics consistent following either IP or oral dosing, mirrored by their dual inhibitor elacridar, and consistent with oral ketamine PK, but *inconsistent* with ketamine's brain concentration? There are several alternative reasons for not finding a measurable effect of the transporters on ketamine brain concentration at 15 minutes (HPLC-UV) and 1.25 hr (radioactivity). First, most studies that have shown a significant effect of Pgp/Bcrp on substrate brain concentrations *in vivo* have measured drug brain levels 4-24 hrs after drug administration to allow brain accumulation and hence maximize the difference in brain substrate concentration in the KO vs. WT mice. However, because of ketamine's rapid clearance rate (estimated $t_{1/2}$ = 25 min) (Table S1), brain concentrations would not be measureable hours after dosing. Second, ketamine is a high permeability rapid clearance drug and the magnitude of effect of Pgp and Bcrp is typically greater for substrates with poor apparent permeability coefficients (Tweedie et al., 2013), so the magnitude of transporter effect on this type of substrate would be assumed to be small. Indeed, terfenadine, another drug with high passive permeability that is a Pgp substrate, showed no difference in brain concentration between WT and Pgp-KO mice (Zhao et al., 2009). Third, it was previously shown that i.v administered ketamine shows significant regional

differences in brain exposure (that are thought to be pertinent to its effect on anesthesia) during loss of righting reflex in rats, and the difference minimizes as the rats regained righting reflex (Cohen et al., 1973), which might explain its PD effect, but which would not be captured by measuring ketamine in total brain homogenate (as we did in this study). Hence, it is possible that regional concentration difference of ketamine could be driving its dLORR effect, and ketamine plasma and total brain concentration may not be a good predictor of its PK/PD relationship in the dLORR model. Fourth, we administered ketamine as the racemic mixture but it is entirely possible that the S- and R-ketamine enantiomers (that have enantiomer specific pharmacology) might interact differently with the transporters (beyond the scope of this paper). Finally, we think the striking effect of transporter genotype on ketamine PD, but not systemic or total brain distribution, for brain acting drugs like ketamine that have high passive permeability may, in fact point to the potential for missing a Pgp or Bcrp effect if only the PK is measured and used to predict whether these transporters would influence the drug's CNS pharmacodynamic activity.

A population PK model was used for simultaneous analysis of IP and PO PK data to enable estimation of fixed parameters and sources of variability. Assuming a 100% bioavailability after IP dosing ($F_2 = 1$, Table 1), population PO bioavailability in the current study was estimated to be 17.5%, which is in accordance to the published reports of ketamine bioavailability in humans after oral dosing. Population analysis of IP and PO data together identified a change in oral bioavailability in the Pgp and dKO groups. The mean population clearance for ketamine was found to be 20.2 L/hr/kg and volume of distribution 9.32 L/kg, suggesting ketamine has very high clearance and volume of distribution, which is in accordance with previously published results (Mion and Villevieille, 2013).

While our *in vivo* data clearly shows Pgp and Bcrp alter the POPK and dLORR of ketamine, we wanted to know if the same is observed *in vitro*. Previously published results show ketamine does not have any interaction with PGP, as substrate or inhibitor. However, our data clearly indicates, in a dual BCRP and PGP transfected MDCKII cell line, these transporters can effectively reduce ketamine uptake in MDCKII cells at low ketamine concentration. We think both the selection of ketamine concentration and also time of *in vitro* assay will have significant effect on the outcome. Also, our *in vitro* data suggests that effect of these transporters will be significant at ketamine exposure lower than 10 μ M or 2.4 μ g/ml. A higher clinical ketamine concentration is required for anesthetic effect of ketamine, whereas, lower concentrations are useful for antidepressant effect and also often achieved in the ketamine abusers. Thus our data warrants a possible drug-drug or food-drug interaction for antidepressant use of ketamine and in ketamine abusers.

The finding that ketamine appears to be a dual Pgp/Bcrp substrate has a number of important clinical implications. Ketamine is an anesthetic widely used in animal studies as well as human pediatric settings where a short-acting rapid-onset anesthesia is needed. Importantly, it was recently recognized that ketamine can elicit rapid-onset long-acting anti-depressant activity. However, ketamine is also a significant drug of abuse, particularly in Asian countries. Notably, reports have also appeared in the past few years that chronic ketamine abusers develop serious lower urinary tract symptoms, voiding problems, ulcerative cystitis, and significant damage to the urinary tract (Chu et al., 2008), although there may be individual differences in vulnerability. Both Pgp and Bcrp are expressed in human kidney at the proximal tubule epithelium where they move substrates into the urine, and both transporters are expressed in bladder urothelium (Bexten et al., 2015) at the apical epithelium. Presumably the urothelium expression of Pgp/Bcrp, in

addition to its waxy glycocalyx barrier layer, prevents the reabsorption of renally excreted transporter substrates. Given our findings that ketamine is an apparent Pgp/Bcrp substrate it is tempting to speculate that BCRP genetic variation, particularly the BCRP Q141K allele that leads to loss of functional BCRP protein and that has a high frequency in some populations (Zamber et al., 2003) may influence susceptibility to ketamine induced urinary tract damage. Equally important is the potential for co-administered Pgp/Bcrp substrates or inhibitors to affect both ketamine bioavailability and urinary tract damage. Indeed, our studies with the dual Pgp/Bcrp inhibitor elacridar demonstrated that pharmacological inhibition of the transporters can effect ketamine PK. Further, cyclosporine, a known Pgp/Bcrp as well as Cyp inhibitor has similarly been shown to affect ketamine dLORR in mice (Sato et al., 2007). Many therapeutically used drugs and chemicals present in food are inhibitors of Bcrp and Pgp (Kusuhara et al., 2012). Thus, our results suggest a potential interaction of ketamine with these Bcrp-Pgp inhibitors. Moreover, the proposed use of ketamine as an antidepressant may require dosing with other antidepressant drugs, some of which are known inhibitors of these transporters (Weiss et al., 2003).

We conclude that Bcrp and Pgp can affect the PK and PD of ketamine in mice. Using a pharmacological inhibitor of Bcrp and Pgp function our data clearly demonstrate DDI potential of ketamine with inhibitors of Bcrp and Pgp. Further studies should be carried out to understand the impact of these transporters on ketamine PK, PD and DDI potential in humans and whether persons with polymorphic Bcrp taking Pgp inhibitors have an increased risk of ketamine-mediated toxicity.

Acknowledgements

We gratefully acknowledge the technical support of St Jude Children's Research Hospital: the Animal Resource Center, the Hartwell Center for DNA sequencing, and the Computational Biology and Bioinformatics Core.

We acknowledge Dr. Clinton Stewart and his lab members at Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital for their assistance with HPLC analysis of Ketamine.

Author Contributions:

Participated in research design: S.G., E.G.S.

Conducted experiments: S.G.

Contributed new reagents or analytic tools: S.G.

Performed data analysis: S.G., C.P., J.K.R., E.G.S.

Wrote or contributed to the writing of the manuscript: S.G., C.P., J.K.R., E.G.S.

References

- Barrett TW and Schrager DL (2016) Move Over Morphine: Is Ketamine an Effective and Safe Alternative for Treating Acute Pain?: Answers to the September 2015 Journal Club. *Ann Emerg Med* **67**:289-294.
- Bexten M, Oswald S, Grube M, Jia J, Graf T, Zimmermann U, Rodewald K, Zolk O, Schwantes U, Siegmund W, and Keiser M (2015) Expression of drug transporters and drug metabolizing enzymes in the bladder urothelium in man and affinity of the bladder spasmolytic trospium chloride to transporters likely involved in its pharmacokinetics. *Mol Pharm* **12**:171-178.
- Bolze S and Boulieu R (1998) HPLC determination of ketamine, norketamine, and dehydronorketamine in plasma with a high-purity reversed-phase sorbent. *Clin Chem* **44**:560-564.
- Can A, Zanos P, Moaddel R, Kang HJ, Dossou KS, Wainer IW, Cheer JF, Frost DO, Huang XP, and Gould TD (2016) Effects of Ketamine and Ketamine Metabolites on Evoked Striatal Dopamine Release, Dopamine Receptors, and Monoamine Transporters. *J Pharmacol Exp Ther* **359**:159-170.
- Chu PS, Ma WK, Wong SC, Chu RW, Cheng CH, Wong S, Tse JM, Lau FL, Yiu MK, and Man CW (2008) The destruction of the lower urinary tract by ketamine abuse: a new syndrome? *BJU Int* **102**:1616-1622.
- Cohen ML, Chan SL, Way WL, and Trevor AJ (1973) Distribution in the brain and metabolism of ketamine in the rat after intravenous administration. *Anesthesiology* **39**:370-376.
- Durmus S, Sparidans RW, Wagenaar E, Beijnen JH, and Schinkel AH (2012) Oral availability and brain penetration of the B-RafV600E inhibitor vemurafenib can be enhanced by the

- P-GLYCOprotein (ABCB1) and breast cancer resistance protein (ABCG2) inhibitor elacridar. *Mol Pharm* **9**:3236-3245.
- Evans AM, DeHaven CD, Barrett T, Mitchell M, and Milgram E (2009) Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal Chem* **81**:6656-6667.
- Fanta S, Kinnunen M, Backman JT, and Kalso E (2015) Population pharmacokinetics of S-ketamine and norketamine in healthy volunteers after intravenous and oral dosing. *Eur J Clin Pharmacol* **71**:441-447.
- Hendriks JJ, Lagas JS, Wagenaar E, Rosing H, Schellens JH, Beijnen JH, and Schinkel AH (2014) Oral co-administration of elacridar and ritonavir enhances plasma levels of oral paclitaxel and docetaxel without affecting relative brain accumulation. *Br J Cancer* **110**:2669-2676.
- Hijazi Y and Boulieu R (2002) Contribution of CYP3A4, CYP2B6, and CYP2C9 isoforms to N-demethylation of ketamine in human liver microsomes. *Drug Metab Dispos* **30**:853-858.
- Jaki T and Wolfsegger MJ (2011) Estimation of pharmacokinetic parameters with the R package PK. *Pharmaceutical Statistics* **10**:284-288.
- Keiser M, Hasan M and Oswald S (2018) Affinity of ketamine to clinically relevant transporters. *Mol Pharmaceutics* **15**:326-331.
- Kuhn E and Lavielle M (2005) Maximum likelihood estimation in nonlinear mixed effects models. *Computational Statistics & Data Analysis* **49**:1020-1038.
- Kusuhara H, Furuie H, Inano A, Sunagawa A, Yamada S, Wu C, Fukizawa S, Morimoto N, Ieiri I, Morishita M, Sumita K, Mayahara H, Fujita T, Maeda K, and Sugiyama Y (2012)

- Pharmacokinetic interaction study of sulphasalazine in healthy subjects and the impact of curcumin as an in vivo inhibitor of BCRP. *Br J Pharmacol* **166**:1793-1803.
- Lagas JS, van Waterschoot RA, van Tilburg VA, Hillebrand MJ, Lankheet N, Rosing H, Beijnen JH, and Schinkel AH (2009) Brain accumulation of dasatinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by elacridar treatment. *Clin Cancer Res* **15**:2344-2351.
- Lilius TO, Jokinen V, Neuvonen MS, Niemi M, Kalso EA, and Rauhala PV (2015) Ketamine coadministration attenuates morphine tolerance and leads to increased brain concentrations of both drugs in the rat. *Br J Pharmacol* **172**:2799-2813.
- Machado-Vieira R, Salvadore G, Diazgranados N, and Zarate CA, Jr. (2009) Ketamine and the next generation of antidepressants with a rapid onset of action. *Pharmacol Ther* **123**:143-150.
- Merino G, van Herwaarden AE, Wagenaar E, Jonker JW, and Schinkel AH (2005) Sex-dependent expression and activity of the ATP-binding cassette transporter breast cancer resistance protein (BCRP/ABCG2) in liver. *Mol Pharmacol* **67**:1765-1771.
- Mion G and Villevieille T (2013) Ketamine pharmacology: an update (pharmacodynamics and molecular aspects, recent findings). *CNS Neurosci Ther* **19**:370-380.
- Moore KA, Sklerov J, Levine B, and Jacobs AJ (2001) Urine concentrations of ketamine and norketamine following illegal consumption. *J Anal Toxicol* **25**:583-588.
- Niesters M, Martini C, and Dahan A (2014) Ketamine for chronic pain: risks and benefits. *Br J Clin Pharmacol* **77**:357-367.

- Peltoniemi MA, Saari TI, Hagelberg NM, Laine K, Neuvonen PJ, and Olkkola KT (2012) St John's wort greatly decreases the plasma concentrations of oral S-ketamine. *Fundam Clin Pharmacol* **26**:743-750.
- Poller B, Wagenaar E, Tang SC, and Schinkel AH (2011) Double-transduced MDCKII cells to study human P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) interplay in drug transport across the blood-brain barrier. *Mol Pharm* **8**:571-582.
- Sane R, Agarwal S, and Elmquist WF (2012) Brain distribution and bioavailability of elacridar after different routes of administration in the mouse. *Drug Metab Dispos* **40**:1612-1619.
- Sato Y, Kobayashi E, Hakamata Y, Kobahashi M, Wainai T, Murayama T, Mishina M, and Seo N (2004) Chronopharmacological studies of ketamine in normal and NMDA epsilon1 receptor knockout mice. *Br J Anaesth* **92**:859-864.
- Sato Y, Onaka T, Kobayashi E, and Seo N (2007) The differential effect of cyclosporine on hypnotic response and pain reaction in mice. *Anesth Analg* **105**:1489-1493, table of contents.
- Shahani R, Streutker C, Dickson B, and Stewart RJ (2007) Ketamine-associated ulcerative cystitis: a new clinical entity. *Urology* **69**:810-812.
- Sherwin CM, Stockmann C, Grimsrud K, Herd DW, Anderson BJ, and Spigarelli MG (2015) Development of an optimal sampling schedule for children receiving ketamine for short-term procedural sedation and analgesia. *Paediatr Anaesth* **25**:211-216.
- Sleigh J HM, Voss L, Denny B (2014) Ketamine – More mechanisms of action than just NMDA blockade. *Trends in anesthesia and critical care* **4**:76-81.
- Tournier N, Chevillard L, Megarbane B, Pirnay S, Scherrmann JM, and Decleves X (2010) Interaction of drugs of abuse and maintenance treatments with human P-glycoprotein

- (ABCB1) and breast cancer resistance protein (ABCG2). *Int J Neuropsychopharmacol* **13**:905-915.
- Tweedie D, Polli JW, Berglund EG, Huang SM, Zhang L, Poirier A, Chu X, and Feng B (2013) Transporter studies in drug development: experience to date and follow-up on decision trees from the International Transporter Consortium. *Clin Pharmacol Ther* **94**:113-125.
- Varma MV, Ashokraj Y, Dey CS, and Panchagnula R (2003) P-glycoprotein inhibitors and their screening: a perspective from bioavailability enhancement. *Pharmacol Res* **48**:347-359.
- Weiss J, Dormann SM, Martin-Facklam M, Kerpen CJ, Ketabi-Kiyanvash N, and Haefeli WE (2003) Inhibition of P-glycoprotein by newer antidepressants. *J Pharmacol Exp Ther* **305**:197-204.
- Winters WD, Hance AJ, Cadd GC, and Lakin ML (1986) Seasonal and sex influences on ketamine-induced analgesia and catalepsy in the rat; a possible role for melatonin. *Neuropharmacology* **25**:1095-1101.
- Zamber CP, Lamba JK, Yasuda K, Farnum J, Thummel K, Schuetz JD, and Schuetz EG (2003) Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. *Pharmacogenetics* **13**:19-28.
- Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI, Alkondon M, Yuan P, Pribut HJ, Singh NS, Dossou KS, Fang Y, Huang XP, Mayo CL, Wainer IW, Albuquerque EX, Thompson SM, Thomas CJ, Zarate CA, Jr., and Gould TD (2016) NMDAR inhibition-independent antidepressant actions of ketamine metabolites. *Nature* **533**:481-486.
- Zhao R, Kalvass JC, Yanni SB, Bridges AS, and Pollack GM (2009) Fexofenadine brain exposure and the influence of blood-brain barrier P-glycoprotein after fexofenadine and terfenadine administration. *Drug Metab Dispos* **37**:529-535.

Footnotes

a) This work was supported in part by the National Institutes of Health National Cancer Institute [Cancer Center Support Grant [P30 CA21765] and the American Lebanese Syrian Associated Charities (ALSAC).

b) Person to receive reprint requests: Erin Schuetz, Ph.D. Department of Pharmaceutical Sciences, 262 Danny Thomas Place, St. Jude Children's Research Hospital, Memphis, TN-38105; Phone: (901) 595-2205; FAX: (901) 595-3125.

Figure Legends

Figure 1. Elevated ketamine CSF and plasma levels in Pgp/Bcrp dKO vs. WT rats.

Ketamine levels in male Sprague Dawley rats after IP administration of 100 mg/kg ketamine. Data is plotted as median LC-MS/MS peak intensity \pm range (n=8/genotype). The plasma and CSF median peak intensity in dKO rats was compared to the corresponding median peak intensity from WT rats using the unpaired t-test with Welch's correction. * $p < 0.05$

Figure 2. Ketamine serum exposure in Pgp/Bcrp dKO mice is significantly higher than in WT mice after oral dosing. (A) & (C): Mean ketamine plasma concentrations \pm SE over time curves after IP (n = 3/genotype) and oral (n=6/genotype) ketamine dosing, respectively of FVB WT, Pgp KO, Bcrp KO and dKO mice. (B) & (D): Ketamine plasma $AUC_{inf} \pm$ SE following IP and oral dosing of FVB WT, Pgp KO, Bcrp KO and dKO mice. The AUC was calculated by non-compartment analysis using the PK package on R. One way ANOVA with Newman-Keuls Multiple Comparison Test was used to compare AUC_{inf} . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. The duration of IP ketamine induced loss of righting reflex (dLORR) significantly increases in mice with absent or inhibited Pgp and Bcrp. (A) Comparison of the duration of ketamine (100 mg/kg body weight, IP) induced dLORR in WT, Bcrp KO, Pgp KO and dKO FVB mice. (B) The duration of ketamine (50, 100 and 200 mg/kg body weight, intraperitoneal) induced LORR in WT mice with or without 1.5 hr pretreatment with elacridar (ECD) (100 mg/kg body weight). Results are expressed as median dLORR \pm range. Significance is calculated using Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 4. ^3H -ketamine uptake is lower in the MDCKII BCRP-PGP cells compared to MDCKII WT cells. (A) Time dependent uptake of ^3H -ketamine in WT and BCRP-PGP transfected MDCKII cells. Ketamine's intracellular concentration at each time point was compared between the WT and BCRP-PGP cells using an unpaired t-test, ** $p < 0.01$. (B) ^3H -ketamine uptake in WT and BCRP-PGP transfected MDCKII cells at different ketamine concentrations. The intracellular concentration of ^3H -ketamine at each treatment was compared between the WT and BCRP-PGP cells using an unpaired t-test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table I. Population PK parameter estimates

	Mean Parameter estimates	S.E	p-value
F _{pop}	0.175	0.0072	
θ_{Pgp} KO	0.227	0.062	0.00028
θ_{dKO}	0.514	0.062	< 1e-10
Tk0 _{pop} (1/hr)	0.104	0.0092	
F2 _{pop}	1 Fixed	N.E.	
ka _{pop} (1/hr)	19.1	1.6	
V1 _{pop} (L/Kg)	9.32	0.35	
CL _{pop} (L/hr/Kg)	20.2	0.72	
ω_{BSV}			
F	0.0276	0.077	
Tk0	0.32	0.077	
F2	N.E.	N.E.	
ka	0.091	0.14	
V1	0.0857	0.061	
CL	0.0781	0.056	
σ - RUV			
Proportional	0.341	0.022	
F _(WT and Bcrp KO)	0.175	0.0072	
F _(Pgp KO)	0.22	0.011	

F_(dKO)	0.293	0.015	
----------	-------	-------	--

F – oral bioavailability; F_pop – Population mean oral bioavailability; $\theta_{\text{Pgp KO}}$ and θ_{dKO} – estimated coefficient of the effect of categorical covariate Pgp KO and dKO on the oral bioavailability; Tk0_{pop} – population mean oral zero order absorption rate constant; F2_{pop} – population mean intraperitoneal bioavailability which was assumed to be 1; ka_{pop} – population mean first order absorption rate constant for i.p route; V1_{pop} – population mean volume of distribution; CL_{pop} – population mean clearance; ω_{BSV} – between subject variability; σ - RUV – residual unexplained variability.

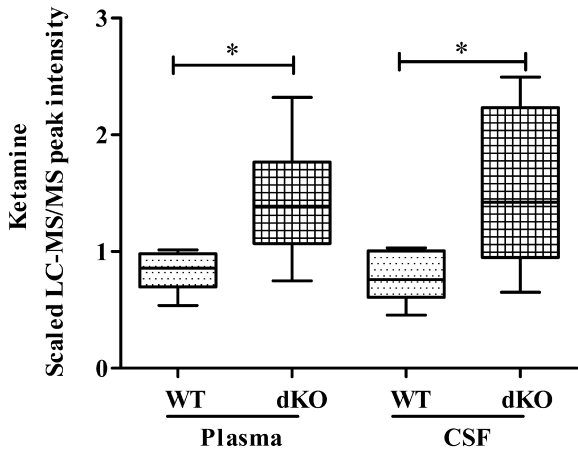


Figure 1

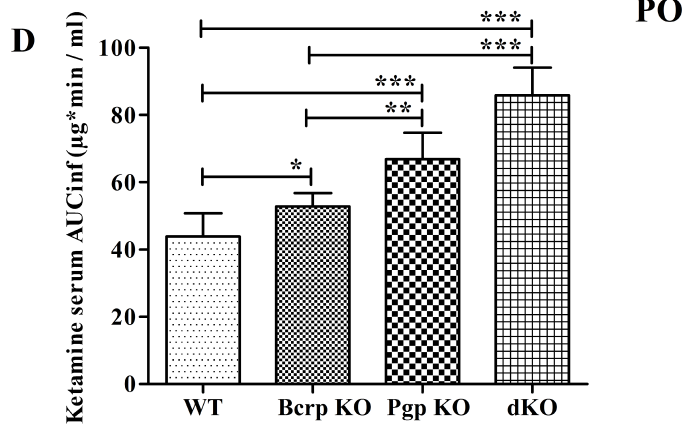
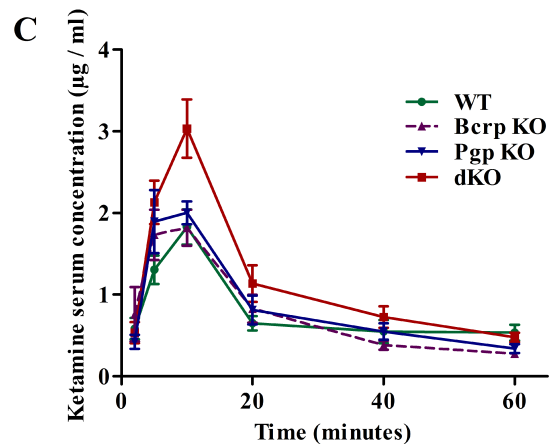
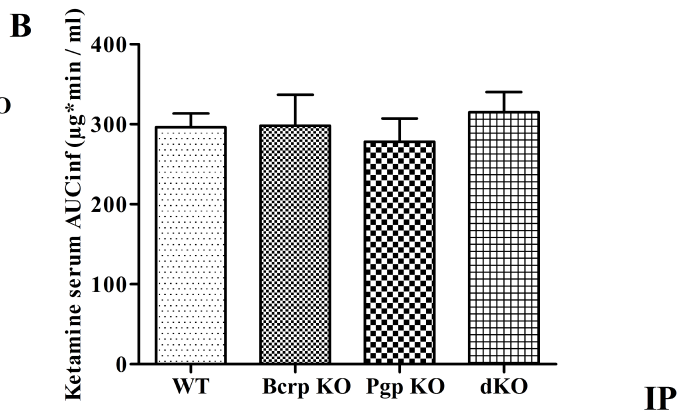
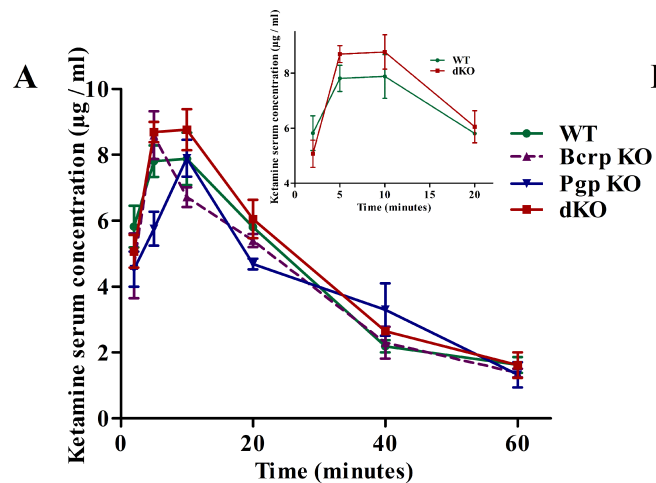
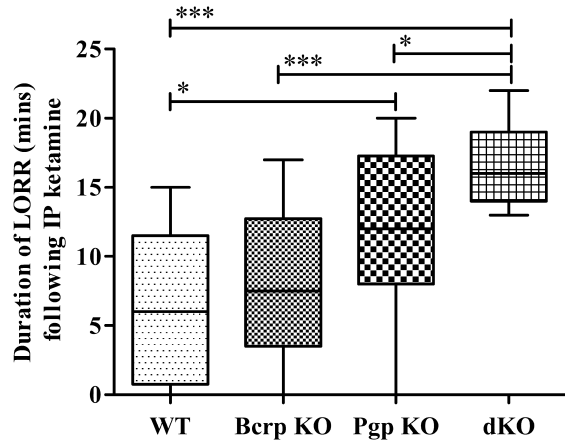
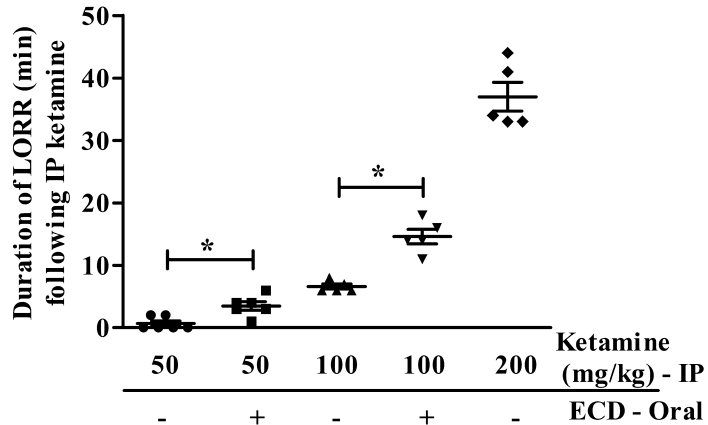


Figure 2

A**B****Figure 3**

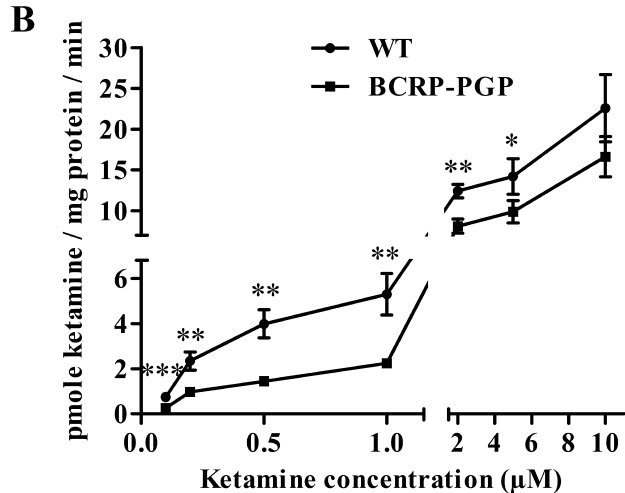
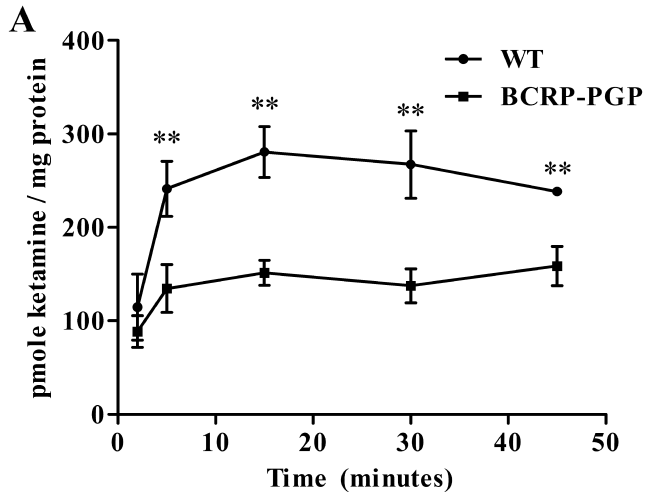


Figure 4