CNS distribution of the ATM inhibitor AZD1390: Implications for the treatment of brain tumors

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Number of text pages – 42

Number of tables – 2

Number of figures – 10

Number of references – 49

Number of words in –

1. Abstract – 231
2. Introduction – 673
3. Discussion – 1603 (after incorporating reviewers’ comments)

Non-standard abbreviations –

ABC – Adenosine tri-phosphate binding cassette, ATM – ataxia telangiectasia mutated kinase, AUC – area under the curve, Bcrp – breast cancer resistance protein, CL – clearance, CNS -

**Recommended section:** Metabolism, Transport, and Pharmacogenomics
ABSTRACT

Effective drug delivery to the brain is critical for the treatment of glioblastoma (GBM), an aggressive and invasive primary brain tumor that has a dismal prognosis. Radiation therapy, the mainstay of brain tumor treatment, works by inducing DNA damage. Therefore, inhibiting DNA damage response (DDR) pathways can sensitize tumor cells to radiation and enhance cytotoxicity. AZD1390 is an inhibitor of ataxia-telangiectasia mutated kinase (ATM), a critical regulator of DDR. Our in vivo studies in the mouse indicate that delivery of AZD1390 to the central nervous system (CNS) is restricted due to active efflux by P-gp. The free fraction of AZD1390 in brain and spinal cord were found to be low, thereby reducing the partitioning of free drug to these organs. Co-administration of an efflux inhibitor significantly increased CNS exposure of AZD1390. No differences were observed in distribution of AZD1390 within different anatomical regions of CNS, and the functional activity of P-gp and Bcrp also remained the same across brain regions. In an intracranial glioblastoma patient-derived xenograft (PDX) model, AZD1390 accumulation was higher in the tumor core and rim compared to surrounding brain. Despite this heterogenous delivery within tumor-bearing brain, AZD1390 concentrations in normal brain, tumor rim and tumor core were above in vitro effective radiosensitizing concentrations. These results indicate that despite being a substrate of efflux in the mouse brain, sufficient AZD1390 exposure is anticipated even in regions of normal brain.
SIGNIFICANCE STATEMENT

Given the invasive nature of GBM, tumor cells are often protected by an intact blood-brain barrier, requiring the development of brain penetrant molecules for effective treatment. We show that efflux mediated by P-gp limits CNS distribution of AZD1390, and there are no distributional differences within anatomical regions of CNS. Despite efflux by P-gp, concentrations effective for potent radiosensitization are achieved in GBM tumor bearing mouse brains indicating that AZD1390 is an attractive molecule for clinical development of brain tumors.
INTRODUCTION

Tumors in the CNS, either primary or metastatic, are almost uniformly lethal and have a dismal prognosis despite the aggressive treatment strategy of surgery, radiation therapy (RT), and chemotherapy. The median survival of GBM patients is approximately 15 months and is 7-8 months for patients with brain metastases (Tan et al. 2020; Moravan et al. 2020). The overall poor brain tumor control following RT for either primary or metastatic brain tumors is a primary motivation for development of novel radiosensitizing molecules for the treatment of brain tumors.

Unrepaired DNA double-strand breaks are the lethal lesion associated with RT, and ataxia telangiectasia mutated (ATM) kinase is a crucial component of DDR involved in the repair of these lesions (Figure 1) (Kastan and Lim 2000). Modulating cell cycle checkpoints, DNA repair and cell survival are key functions of ATM (Weber and Ryan 2015; Lee and Paull 2021). Therefore, inhibition of ATM, when combined with RT can lead to potent radiosensitization (Figure 1). However, patients with a homozygous mutation in ATM suffer from a rare disorder called ataxia-telangiectasia (AT) syndrome, which is characterized by profound hypersensitivity to RT (Gatti 2009; Pollard and Gatti 2009). Severe radiation toxicities have been observed in patients with AT, even leading to death (Pritchard et al. 1982; Tamminga et al. 2002). It is therefore critical to understand the potential impacts of ATM inhibitors on both tumor and normal brain tissues.

ATM inhibition has been demonstrated to effectively radiosensitize CNS metastases of breast cancer (Tew, Durant, and Salhia 2021) and GBM cells (Golding et al. 2012; Biddlestone-Thorpe et al. 2013; Karlin et al. 2018), particularly GBM stem cells that show enhanced radioresistance (Carruthers et al. 2015). AZD1390, a potent ATM inhibitor (Durant et al. 2018), is currently in
Phase I clinical trials (NCT03423628) with RT for GBM and brain metastases. A careful evaluation of mechanisms that influence the CNS delivery of AZD1390 is therefore key in understanding is optimal potential uses for brain tumor treatment.

A key limiting factor in CNS distribution of therapeutics is the presence of the BBB. The unique nature of the BBB allows selective entry of molecules to brain as needed for its nutrition, growth, and development, while restricting entry of other circulating molecules (Abbott et al. 2010). A major component of this restrictive entry is the presence of ATP binding cassette (ABC) transporters, that actively efflux molecules back into blood stream thereby preventing their entry into the CNS. Two key players of this active efflux at the BBB are P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp). These proteins in the ABC superfamily of transporters are involved in the limited CNS delivery of a multitude of anti-cancer agents for brain tumors (Löscher and Potschka 2005). Through this study, we evaluate the role of P-gp and Bcrp in limiting the CNS delivery of AZD1390. We also evaluate the functional activity of P-gp and Bcrp across different anatomical regions of the CNS.

In addition to the presence of active efflux limiting CNS delivery of chemotherapeutics, the variability in the BBB disruption in and around the tumor also limits drug delivery (Parrish, Sarkaria, and Elmquist 2015). While it has been shown that the BBB is often leaky and disrupted in case of GBM, this disruption is almost always heterogenous because of the highly infiltrative nature of GBM. With maximum leakiness at the tumor core followed by adjacent tumor rim, integrity of the BBB is intact in surrounding normal brain area (Sarkaria et al. 2018). Because of the infiltrative nature of GBM, complete surgical resection is impossible, leaving behind tumor cells in the surrounding normal brain regions, thereby leading to tumor recurrence (Agarwal et al. 2011). It is therefore important for molecules to be able to cross an intact BBB and be
delivered to the tumor cells sheltered behind it for effective treatment of GBM (Figure 2). To evaluate these differences in drug accumulation within brain tumors, we examine the spatial distribution of AZD1390 in tumor bearing mice brains.

Recently published reports indicate that AZD1390 is a brain penetrant ATM inhibitor and while it is a substrate of active efflux in rodents from in vitro studies, it has “low efflux liability” in humans (Durant et al. 2018; Jucaite et al. 2021). Our aim is to thoroughly investigate the role of active efflux by P-gp and Bcrp in limiting the CNS delivery of AZD1390 in mice, examine the functional activity of P-gp and Bcrp within different anatomical regions of the CNS and evaluate its distribution within GBM PDX tumors to determine its potential as a radiosensitizing agent in brain tumors.
MATERIALS AND METHODS

Chemicals and Reagents

AZD1390 was obtained from Astra Zeneca. Dasatinib was purchased from LC Laboratories. Elacridar was purchased from Toronto Research Chemicals. Rapid equilibrium dialysis (RED) base plate and inserts (8kDa molecular weight cutoff, cellulose membrane) were purchased from Thermo Fisher Scientific. All other reagents were high-performance liquid chromatography-grade and were purchased from Thermo Fisher Scientific or Sigma-Aldrich.

Determination of fraction unbound of AZD1390 using Rapid Equilibrium Dialysis (RED)

Binding of AZD1390 was conducted in plasma, brain homogenate and spinal cord homogenate as per the following revisions to the manufacturer’s protocol (Thermo Fisher Scientific). Mouse brain and spinal cord homogenates were freshly prepared in three volumes (w/v) of phosphate-buffered saline (PBS) (pH 7.4) on the day of experiment. Mouse plasma used was also pH adjusted to 7.4. RED base plate was washed in 70% ethanol, dried overnight, and allowed to warm up to 37°C before the experiment. RED inserts were added to the base plate and loaded with 300 microliters of AZD1390 spiked in either plasma, brain, or spinal cord homogenate in the donor compartment at a final concentration of 5 μM in 0.475% of DMSO. 500 microliters of PBS with 0.475% DMSO was loaded into the receiver compartment. An adhesive covering was used to seal the RED plate and it was allowed to equilibrate on an orbital shaker at 37°C for 24 hours at 600 rpm. At the end of 24 hours, donor and receiver samples were collected and stored at -80°C prior to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Unbound fraction (f_u) in the plasma was calculated using the ratio of the measured concentration in the buffer (receiver compartment) to the measured concentration in the plasma (donor compartment).
compartment). For brain and spinal cord, the $f_u$ was calculated considering the dilution factor (D=4) introduced by homogenizing the two tissues in PBS.

$$f_{u, \text{brain or spinal cord}} = \frac{1/D}{\left(\frac{1}{f_{u, \text{diluted}}} - 1\right) + \frac{1}{D}} \quad (Equation \ 1)$$

Where $f_{u, \text{diluted}}$ is the ratio of the measured concentration in the buffer (receiver compartment) to the measured concentration in the brain or spinal cord homogenate (donor compartment).

**Animals**

For pharmacokinetic studies, Friend leukemia virus strain B (FVB) wild-type, Bcrp1−/− (Bcrp knockout, BKO), Mdr1a/b−/− (P-gp knockout, PKO) and Mdr1a/b−/− Bcrp1−/− (P-gp and Bcrp knockout/Triple knockout, TKO) mice (equal males and females) of age 8-14 weeks were used. Breeding license was purchased from Taconic Biosciences Inc. (Germantown, NY) and animal colonies were developed and maintained as per the breeding protocol in the Research Animal Resources housing facility at the Academic Health Center, University of Minnesota (Minneapolis, MN). Animals were maintained on a 12-hour light/dark cycle with unlimited access to food and water. Animal genotypes were routinely verified using tail-snip (TransnetYX, Cordova, TN). All the pharmacokinetic studies and surgeries were approved by the University of Minnesota Institutional Animal Care and Use committee and carried out in accordance with the guidelines established by US National Institutes of Health (Bethesda, MA).

Tumor distribution studies were performed using female athymic nude mice (Hsd:athymic Nude-Foxn1nu; Envigo, Indianapolis, IN) aged 4–5 weeks. Mice were implanted with an intracranial tumor (GBM PDX G12) transduced with a lentiviral vector for expression of enhanced green fluorescent protein and firefly luciferase 2 (G12-eGFP-FLUC2). Animals were maintained on a
12-hour light/dark cycle with unlimited access to food and water. All studies using animals were approved by the Institutional Animal Care and Use Committee, Mayo Clinic (Rochester, MN).

**Drug formulation and dose**

AZD1390 was formulated at a dose of 5 mg/kg into a solution with 30% (1:1 – EtOh : Cremophor EL) and 70% Saline for intravenous administration using tail-vein injection. AZD1390 was formulated at a dose of 10 mg/kg as a suspension with 0.5% w/v HPMC and 0.1% w/v Tween 80 for oral dosing using oral gavage in pharmacokinetic studies. For oral dosing in the tumor distribution study with GBM 12 tumors, AZD1390 was given at a dose of 20 mg/kg in the same formulation. In the steady state infusion study, AZD1390 was loaded at a concentration of 10 mg/ml in DMSO and administered at a rate of 10 μg/hour using Alzet osmotic pump (1003D; Durect Corporation, Cupertino, CA). Elacridar was formulated in a microemulsion at a 10 mg/kg dose (Sane, Agarwal, and Elmquist 2012) and was administered intraperitoneally for the efflux inhibition study with AZD1390.

**CNS distribution of AZD1390 following intravenous and oral administration**

CNS distribution and systemic pharmacokinetics of AZD1390 was evaluated after an intravenous bolus dose of 5 mg/kg in FVB wild-type, BKO, PKO and TKO mice. Similarly, a single oral dose of 10 mg/kg of AZD1390 was administered to FVB wild-type and TKO mice. Blood, brain and spinal cord were harvested from 10 minutes to 12 hours (n=4 at each time point) following dose administration. Blood samples were stored on ice and immediately centrifuged at 7500 rpm for 10 mins to separate plasma. Brain and spinal cord were immediately dipped in ice-cold saline on collection and rolled on tissue paper to wipe off excess blood. Samples were stored at -80°C and analyzed using LC-MS/MS.
CNS distribution of AZD1390 with pharmacological inhibition of efflux using elacridar

Impact of pharmacological inhibition of P-gp and Bcrp efflux was evaluated using the co-administration of elacridar in FVB wild-type and TKO mice along with AZD1390. 10 mg/kg AZD1390 was administered using oral gavage along with 10 mg/kg elacridar administered intraperitoneally at the same time. Blood, brain, and spinal cord were harvested 2 hours (n=4) following AZD1390 and elacridar dose. Blood samples were stored on ice and immediately centrifuged at 7500 rpm for 10 mins to separate plasma. Brain and spinal cord were immediately dipped in ice-cold saline upon collection and rolled on tissue paper to wipe off excess blood. Samples were stored at -80°C and analyzed using LC-MS/MS.

Distribution of AZD1390 within different anatomical regions of the CNS following steady state infusion

CNS distribution of AZD1390 at steady state was evaluated by implanting Alzet osmotic pumps into the intraperitoneal cavity of FVB wild-type, BKO, PKO and TKO mice to release AZD1390 at 10 μg/hr (Agarwal et al. 2010). 24 hours after the pump implantation (greater than 8 half-lives), mice were sacrificed and blood, brain and spinal cord were collected (n=5 for each genotype). Blood samples were stored on ice and immediately centrifuged at 7500 rpm for 10 mins to separate plasma. Brain and spinal cord were immediately dipped in ice-cold saline on collection and rolled on tissue paper to wipe off excess blood. Brain was quickly dissected into the following anatomical regions: cortex, cerebellum, hypothalamus & thalamus, and spinal cord. Samples were stored at -80°C and analyzed using LC-MS/MS.
Regional distribution of AZD1390 in G12 PDX tumor bearing mouse brains

AZD1390 was dosed orally at 20 mg/kg to the G12-eGFP-FLUC2 tumor bearing mice 18 days after tumor implantation. Blood and brain were collected at 4 and 12 hours following the oral dose. Blood samples were stored on ice and immediately centrifuged at 7500 rpm for 10 mins to separate plasma. Brain was immediately dipped in ice-cold saline on collection and rolled on a tissue paper to wipe off excess blood and flash frozen. Frozen brains were sliced and punched using fluorescence-guided punch biopsy technique described earlier (Gampa et al. 2020; Talele et al. 2021). Tumor bearing brains were divided into the following regions: tumor core (tumor regions with fluorescence intensity 5-times greater than the background), tumor rim (tumor regions with fluorescence intensity 3-5 times greater than the background, and normal brain (not above background fluorescence). Samples were stored at -80°C and analyzed using LC-MS/MS.

LC-MS/MS analysis

Concentrations of AZD1390 within the plasma, brain, spinal cord, and tumor region specimens were evaluated using LC-MS/MS analysis. Brain, spinal cord, and tumor regions were homogenized in three volumes of 5% bovine serum albumin prior to analysis. 25 microliters plasma and 100 microliters of brain and spinal cord homogenate were used for analysis. AZD1390 and the internal standard, dasatinib, were extracted from each unknown and standard matrix by liquid-liquid extraction using five volumes of ice-cold ethyl acetate and one volume of ice-cold pH 11 buffer (Sodium bicarbonate-Sodium hydroxide buffer solution). Samples were then vortexed for 5 minutes followed by centrifugation at 7500 rpm for 10 minutes. Sample tubes were then cooled at -80°C for 20 minutes to conveniently separate the supernatant organic phase containing the drug. This supernatant was dried under nitrogen gas followed by reconstitution with mobile phase prior to injection. A reverse-phase liquid chromatographic separation method
was used on the ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA) paired with the Micromass Quattro Ultima mass spectrometer (Waters, Milford, MA) operated in the positive ionization mode. 7.5 microliters of the reconstituted sample was injected into the UPLC system at a flow rate of 0.5 ml/min into a Synergy 4μm Polar-RP 80Å column (75 X 2 mm; Phenomenex, Torrance, CA) for chromatographic separation. An isocratic method with a run-time of 4-minutes was developed. The mobile phase composition was 65% distilled and filtered water with 0.1% formic acid and 35% acetonitrile with 0.1% formic acid. The retention time was 0.9 minutes for AZD1390 and 0.84 minutes for dasatinib. The mass to charge (m/z) transition for AZD1390 was 478.3 → 126.06 and dasatinib was 488.21 → 400.99. MassLynx (Waters Corporation, Milford, MA) software was used to acquire and analyze the LC-MS/MS data. The LOQ was 1 ng/ml for all matrices. For every sample run, the standard calibration curve was linear from 1-2000 ng/ml (weighted 1/Y^2) with a coefficient of variation less than 15%. All the measured concentrations fell within the range of the standard curve.

**Pharmacokinetic calculations and non-compartmental analysis**

Non-compartmental analysis (NCA) was performed using Phoenix WinNonlin Version 8.3 (Certara USA Inc., Princeton, NJ), to obtain pharmacokinetic parameters from the concentration-time profiles following intravenous and oral dosing in the plasma, brain and spinal cord. Exposure following dosing, measured by area under the curve (AUC) for plasma, brain and spinal cord is calculated by the linear trapezoidal integration method. In this method, the AUC till the last measured time-point (AUC_{last}) is calculated by area integration and the AUC_{0→∞} is extrapolated by dividing the concentration at the last measured time point (C_{last}) by the first-order terminal elimination rate constant (λ_z), estimated from the log-linear portion of the
concentration-time profile by the linear regression of time versus log concentration. The
percentage AUC extrapolation from t_last to infinity was <10% in all cases indicating adequate
capture of drug exposure from our pharmacokinetic study design. Variances for AUC_last are
determined by Phoenix using the Bailer method (Bailer 1988). We determined the variances for
AUC_0→∞ using the Yuan modification of the Bailer method (Yuan 1993).

Other pharmacokinetic parameters using NCA are calculated using the following equations:

\[ \text{Half life } (t_{1/2}) = \frac{0.693}{\lambda_z} \quad (\text{Equation 2}) \]

\[ \text{Systemic clearance } (CL \text{ or } CL/F) = \frac{\text{Dose}}{AUC_{0→∞}} \quad (\text{Equation 3}) \]

\[ \text{Steady state volume of distribution } (V_{ss}) = \text{MRT}_{inf} \times CL \quad (\text{Equation 4}) \]

where MRT_{inf} is the area under the first moment curve to infinity divided by area under the curve
to infinity. V_{ss} is calculated for intravenous bolus dose. For oral administration, apparent volume
of distribution (V_d/F) is calculated by:

\[ \text{Apparent volume of distribution } \left( \frac{V_d}{F} \right) = \frac{\text{Dose}}{\lambda_z \times AUC_{0→∞}} \quad (\text{Equation 5}) \]

The brain or spinal cord tissue partition coefficients (Kp) were calculated using the following
equation for intravenous and oral administration:

\[ Kp_{brain \text{ or spinal cord}} = \frac{AUC_{(0→∞),brain \text{ or spinal cord}}}{AUC_{(0→∞),plasma}} \quad (\text{Equation 6}) \]

The steady state brain region or spinal cord tissue partition coefficient (Kp) was calculated using:

\[ Kp_{brain \text{ region or spinal cord}} = \frac{C_{steady \text{ state},brain \text{ region or spinal cord}}}{C_{steady \text{ state,plasma}}} \quad (\text{Equation 7}) \]
An instantaneous tissue partition coefficient ($K_t$) was determined for the efflux inhibition study and tumor distribution study of AZD1390 at a particular time point for brain, spinal cord, tumor core and rim as follows:

$$K_t = \frac{C_{\text{brain or spinal cord or tumor core or tumor rim}}}{C_{\text{plasma}}} \quad \text{(Equation 8)}$$

The free brain or spinal cord tissue partition coefficients ($K_{puu}$) were determined as follows:

$$K_{puu,\text{brain or spinal cord}} = K_{p\text{brain or spinal cord}} \times \frac{f_{u,\text{brain or spinal cord}}}{f_{u,\text{plasma}}} \quad \text{(Equation 9)}$$

Free distribution advantage ($DA_{\text{free}}$) was used to compare the relative unbound exposure in the brain and spinal cord for AZD1390 between the genetic knockout mice (BKO, PKO and TKO) and wild-type mice.

$$DA_{\text{free}} = \frac{K_{p_{uu,knackout}}}{K_{p_{uu,wild-type}}} \quad \text{(Equation 10)}$$

Oral bioavailability of AZD1390 was calculated using oral and intravenous doses and exposures using the following equation:

$$\text{Oral bioavailability (F)} = \left\{ \frac{\left( AUC_{(0-\infty),\text{plasma}} \right)_{\text{oral}}}{\left( AUC_{(0-\infty),\text{plasma}} \right)_{\text{IV}}} \times \left[ \frac{\text{Dose}_{\text{IV}}}{\text{Dose}_{\text{oral}}} \right] \right\} \quad \text{(Equation 11)}$$

**Statistical analysis**

Data were presented using the GraphPad Prism software (Version 8; GraphPad software, LaJolla, CA). Data are represented as mean ± standard deviation in all figures and tables. Statistical tests were also conducted within GraphPad Prism and in all cases, $P < 0.05$ was considered statistically significant. For comparisons between two groups, an unpaired Student $t$-test was
used to generate p-values. For comparisons between multiple groups, one-way analysis of variance (ANOVA) followed by multiple comparisons using the Holm-Sidak test were used to generate adjusted p-values.
RESULTS

Pharmacokinetics following intravenous administration of AZD1390

We evaluated the plasma, brain, and spinal cord distribution of AZD1390 in wild-type, BKO, PKO and TKO mice following an intravenous bolus dose of 5 mg/kg. Concentrations in plasma for all four genotypes are depicted in Figure 3A. AUC$_{0-\infty}$ plasma is not significantly different across genotypes (p > 0.05) (Table 1). This indicates that the systemic exposure, hence the clearance, of AZD1390 does not change across the four genotypes. Pharmacokinetic parameters were calculated using NCA and the elimination half-life (t$_{1/2}$) was 2.6 hours, systemic clearance (CL) was 0.95 L/hr/kg, and the volume of distribution at steady state (V$_{ss}$) was 2.7 L/kg in wild-type mice. These pharmacokinetic parameters remain similar across the four genotypes (Table 1). Concentrations in brain are depicted in Figure 3B. Brain concentrations are the highest in TKO mice, similar to PKO mice, followed by lower concentrations in BKO mice, similar to wild-type mice. The same trends are observed for concentrations in spinal cord (Figure 3C). AUC$_{0-\infty}$ brain and spinal cord are highest in TKO mice followed by PKO, BKO and wild-type mice. AUC$_{0-\infty}$ brain and spinal cord are significantly greater in the TKO, PKO and BKO mice as compared to the wild-type mice (p < 0.05) (Table 1). The change in the Kp$_{brain}$ and Kp$_{spinal\,cord}$ with respect to time is depicted in Figure 4. Kp$_{brain}$ is highest in TKO mice (3.13) and equal to PKO mice (3.13) followed by BKO (0.32) and wild-type (0.29) mice (Table 1). Similarly, Kp$_{spinal\,cord}$ is highest in TKO mice (3.03), followed by PKO (2.7), BKO (0.32) and wild-type (0.29) mice (Table 1). These results are clearly indicative of active efflux by P-gp contributing to the limited brain and spinal cord delivery of AZD1390.
Pharmacokinetics following oral administration of AZD1390

We evaluated the plasma, brain, and spinal cord distribution of AZD1390 in wild-type and TKO mice following a single oral dose of 10 mg/kg. Concentrations are highest in plasma followed by brain and spinal cord in wild-type mice (Figure 5A). Plasma concentrations reach a maximum at 1 hr, while brain and spinal cord concentrations reach a maximum at 2 hr. Conversely, in TKO mice, concentrations are highest in brain followed by spinal cord, and lowest in plasma (Figure 5B). Plasma concentrations reach a maximum at 0.5 hr, while brain and spinal cord concentrations reach a maximum at 2 hr. Systemic exposure of AZD1390, indicated by AUC$_{0-\infty}$ plasma is not significantly different between wild-type and TKO mice (p > 0.05) (Table 2). The $t_{1/2}$ was 3.4 hours, apparent systemic clearance (CL/F) was 2.2 L/hr/kg and the apparent volume of distribution (V_d/F) was 10.7 L/kg in wild-type mice. These pharmacokinetic parameters were similar in TKO mice. Comparison of the areas under the curve for plasma between intravenous and oral administration (Equation 11), indicates the oral bioavailability (F) of AZD1390 in wild-type and TKO mice. There was no apparent difference in the oral bioavailability between wild-type (0.43) and TKO mice (0.48). These results indicate that P-gp and Bcrp do not have an influence on the oral absorption and systemic clearance of AZD1390. However, AUC$_{0-\infty}$ brain and spinal cord are significantly higher in TKO mice as compared to wild-type mice (p < 0.05). The changes in $K_{p_{\text{brain}}}$ and $K_{p_{\text{spinal cord}}}$ with respect to time are depicted in Figure 5 (C, D). $K_{p_{\text{brain}}}$ and $K_{p_{\text{spinal cord}}}$ reach a distributional equilibrium at 4 hrs following which they appear to hit a plateau. $K_{p_{\text{brain}}}$ is higher in TKO mice (3.72) as compared to wild-type (0.32) mice (Table 2). Similarly, $K_{p_{\text{spinal cord}}}$ is higher in TKO mice (2.52) as compared to wild-type (0.23) mice (Table 2). These results are indicative of limited brain delivery of AZD1390 to the CNS through active efflux at the BBB.
Effect of binding on the free drug partitioning of AZD1390

We evaluated the effect of AZD1390 binding to plasma, brain homogenate and spinal cord homogenate on its free drug partitioning into the CNS. AZD1390 is highly bound to brain and spinal cord homogenate when compared to plasma. While the unbound percentage of AZD1390 is 20.3% in plasma, it is 11% in spinal cord and 7.5% in brain (Table 1). This differential binding of AZD1390 to brain and spinal cord as compared to plasma will affect the partitioning of unbound AZD1390 to these organs. Free drug partition coefficient ($K_{puu}$) is calculated by accounting for plasma and brain/spinal cord free fraction along with the $K_p$, determined from total concentrations. Due to the high extent of binding to all these tissues, the $K_{puu}$ brain and spinal cord for AZD1390 is further lowered as compared to $K_p$. $K_{puu,brain}$ is highest in the TKO (1.16) and PKO (1.16) mice, followed by a lower value in BKO (0.12) mice, similar to the wild-type (0.10) mice following intravenous administration (Table 1). Similarly, $K_{puu,spinal cord}$ is highest in TKO (1.64) mice, followed by PKO (1.46), BKO (0.18) and wild-type (0.17) mice (Table 1). Free distribution advantage ($DA_{free}$), indicative of the ability of the unbound drug to be delivered across BBB, made possible by the lack of efflux in the knockout mice, shows a similar trend to the $K_{puu}$. Highest $DA_{free}$ is observed in TKO and PKO mice followed by the BKO mice, in both brain and spinal cord. Similarly, in case of oral administration, $K_{puu,brain}$ and $K_{puu,spinal cord}$ are 1.37 in TKO mice and 0.12 in wild-type mice, leading to a $DA_{free}$ of 11.4 in TKO mice (Table 2). There are no apparent differences between $K_{puu}$ and $DA_{free}$, in the brain and spinal cord when accounting for AZD1390 binding.

Effect of administration of elacridar on the brain and spinal cord delivery of AZD1390

We evaluated the effect of efflux inhibition using elacridar, a dual inhibitor of P-gp and Bcrp, on the CNS distribution of AZD1390 in both wild-type and TKO mice. Figure 6 depicts the results
from wild-type mice. We observe that the plasma concentrations remain unchanged, however, the brain and spinal cord concentrations significantly increase (p < 0.05) upon the co-administration of elacridar (Figure 6A). This translates into an approximately 7-fold increase in the Kp2hr of both brain and spinal cord on elacridar co-administration (Figure 6B). Kp2hr for brain and spinal cord are depicted in Figure 6C. These results clearly indicate that inhibition of P-gp mediated efflux at the BBB improves the CNS delivery of AZD1390. However, a single 2-hour time point cannot entirely characterize the time course of AZD1390 concentration changes to the brain on the co-administration of elacridar. This single time-point study has been conducted to demonstrate the effect a pharmacological inhibitor of P-gp can have on the brain delivery of AZD1390, and thorough characterization is necessary before recommending the use of elacridar to increase AZD1390 brain delivery. Figure 7 describes the effects of elacridar co-administration in TKO mice. There are no significant changes in plasma, brain, and spinal cord concentrations, and neither in the Kp and Kp2hr values (Figure 7). This clearly indicates that in mice lacking both P-gp and Bcrp, there is no effect on the CNS distribution of AZD1390 with the co-administration of elacridar.

**Regional distribution within the CNS following a steady state infusion of AZD1390**

We examined differences in AZD1390 concentrations within different anatomical regions of the CNS following a steady-state infusion in wild-type, BKO, PKO and TKO mice. We observed that within the same genotype, there were no significant differences (p > 0.05) between these different anatomical regions, namely the cortex, cerebellum, hypothalamus & thalamus, brain stem and spinal cord (Figure 8A). However, as we observed in our intravenous administration study, the CNS regional concentrations were lowest in the wild-type and BKO mice followed by increased concentrations in the PKO and TKO mice, confirming P-gp mediated active efflux at
the BBB. Partitioning of AZD1390 within different anatomical regions of the CNS showed no significant differences except $K_{p_{\text{spinal cord}}}$, that was lower than other brain regions in the wild-type, PKO and TKO mice ($p < 0.05$) (Figure 8B). However, on accounting for free fraction of brain and spinal cord, $K_{p_{uu}}$ was not significantly different across different anatomical regions of the CNS within the same genotype ($p > 0.05$) (Figure 8C). $K_{p_{uu}}$ was the lowest in wild-type mice and not different than the BKO mice ($p > 0.05$), followed by a significant increase in the PKO and TKO mice ($p < 0.05$) for all the regions. Our regional distribution studies strongly indicate that AZD1390 distribution is not different among different anatomical regions of the CNS. In addition, the impact of efflux activity on AZD1390 distribution is functionally similar across different regions of the CNS.

**Distribution of AZD1390 to GBM 12 PDX tumor model in mice**

We evaluated the distribution of AZD1390 in GBM 12 tumor bearing mouse brains to examine if effective concentrations were achieved within different regions of the tumor bearing brain at the dose of 20 mg/kg. Figure 9A is a brain slice image from the GBM 12 tumor bearing mouse brain depicting tumor core, tumor rim and the surrounding normal brain. These regions were evaluated for differences in AZD1390 concentration at 4 and 12 hrs after dosing. We observe that AZD1390 distribution varies depending on the region and the region with the greatest accumulation among the different regions is the tumor core at both time points (Figure 9B). Concentration in the tumor rim is not significantly different from the tumor core ($p > 0.05$) (Figure 9B). However, concentration in the normal brain is significantly lower than the tumor rim and tumor core at both the time points ($p < 0.05$) (Figure 9B). This heterogeneity in AZD1390 distribution within tumor bearing brain is reflected in the $K_p$ values, where $K_{p_{\text{normal brain}}}$ is significantly lower than the $K_{p_{\text{tumor rim}}}$ and $K_{p_{\text{tumor core}}}$ at both time points ($p < 0.05$) (Figure
According to a previously published report, a total concentration 30 nM AZD1390 in media shows effective radiosensitization in GBM tumor cells (Chen et al. 2020). Despite these differences in AZD1390 accumulation within the tumor core and rim as opposed to the normal brain, we see that total concentrations are above 30 nM in all these tumor and brain regions as well as the plasma. These results indicate that despite lower concentrations in the normal brain as opposed to tumor core and rim, at the examined dose, AZD1390 might be able to showcase potent radiosensitizing activity in GBM.
DISCUSSION

The discovery and development of brain penetrant molecules for GBM is crucial to achieve a breakthrough treatment. Surgical resection, whenever possible, is not able to effectively rid the brain of tumor because of highly infiltrative nature of the disease (Figure 2). Invasive tumor cells find sanctuary behind an intact BBB in the surrounding normal brain region, and continue to proliferate, leading to tumor recurrence (Sarkaria et al. 2018). However, RT, a cornerstone of GBM treatment, when combined with a potent and brain penetrant radiosensitizing agent, may be an important step forward (Figure 2).

AZD1390 is a novel, potent ATM inhibitor developed by structurally modifying a potent ATM inhibitor, AZD0156, for enhanced brain penetration (Durant et al. 2018). In vitro studies with human MDR1 and BCRP showed that AZD1390 is not a significant substrate of human P-gp and Bcrp. However, a low brain penetration was observed in rats and mice with Kpuu of 0.17 and 0.04, respectively (Durant et al. 2018). Our studies were designed to thoroughly investigate CNS delivery of AZD1390 in mice using transporter knockout models lacking P-gp and Bcrp.

Pharmacokinetic studies following an intravenous dose indicate that AZD1390 is a substrate of P-gp in mice, however Bcrp does not limit brain or spinal cord delivery. As such, there is no functional compensation by P-gp in the Bcrp knockout mice, as have been previously observed for several targeted anti-cancer agents (Kodaira et al. 2010; Agarwal et al. 2012). The current study examined the influence of two important efflux systems at the BBB and reports significant effects by P-gp. Given the variety of factors involved in determining the brain distribution of AZD1390, efflux is one mechanism limiting the brain delivery of AZD1390. The scope of the current study was to examine the impact of active efflux by P-gp and Bcrp on the CNS delivery of AZD1390. Our results indicate that efflux mechanism for limiting AZD1390 is due to P-gp.
CNS exposure following oral administration was determined to inform pre-clinical in vivo efficacy studies. Oral bioavailability showed no apparent differences between wild-type and TKO mice. This lack of effect of P-gp on the systemic absorption of AZD1390 can be attributed to the possibility of saturation of intestinal P-gp efflux by high concentrations of AZD1390 in the intestinal lumen (Lin and Yamazaki 2003; Oostendorp et al. 2009). This lack of effect on oral absorption contrasts with the significant limiting effect of P-gp on delivery of AZD1390 across the BBB. The tissue partition coefficients of total drug to the brain and spinal cord were 0.32 and 0.23, respectively in transporter intact mice.

Importantly, this total CNS partitioning provides an incomplete picture of the CNS exposure available for eliciting an effect at the site of action. According to free drug hypothesis, only the free or unbound drug is available to cross membranes and interact with the target to provide an effect (Schanker 1962). Hence, CNS delivery of molecules should be estimated based on unbound concentrations. AZD1390 is highly bound to plasma, brain and spinal cord, and has a $K_{puu}$ of 0.12 in brain and spinal cord. This $K_{puu}$ estimation from our studies is higher than the $K_{puu,brain}$ of 0.04 estimated by Durant et. al from their investigations (Durant et al. 2018). A possibility for this discrepancy might be attributed to the use of two different methods to determine the binding of AZD1390 in tissues. While we used the RED technique to quantify $f_u$ for brain and spinal cord in mice, Durant et al. used $f_u$ determined by the brain slice method in rats to apply to concentration data from mice (Durant et al. 2018). Previous reports have indicated that $f_u$ determination from brain slice method versus RED method, have varying level of differences for the same compound, particularly for basic drugs like AZD1390 (Fridén et al. 2011). These data highlight the importance of drug binding determinations for the calculation of $K_{puu}$. This is especially true if $K_{puu}$ is used as a key parameter in the choice of a brain penetrant
drug. However, low $K_{pu}$ values from both studies, point to the same conclusion, that CNS delivery of AZD1390 is restricted in mice. Our studies further indicate that this limited delivery is by active efflux mediated by P-gp.

The impact of inhibiting P-gp mediated efflux by co-administration of the dual P-gp and Bcrp inhibitor, elacridar, was examined. Co-administration of elacridar with AZD1390 significantly increased the brain and spinal cord delivery of AZD1390 in wild-type mice. However, the effect of inhibition of efflux by elacridar is lower than the complete knockdown of these efflux transporters as seen in TKO mice. This can be explained by the incomplete inhibition of P-gp and Bcrp at the administered dose of elacridar (Talele et al. 2022). The extent of inhibition will depend on the free concentration of elacridar in the brain and the affinity of the transporter systems for elacridar.

We also evaluated the distribution of AZD1390 within different anatomical regions of the CNS. Regional distribution of AZD1390 was studied in wild-type, PKO, BKO and TKO mice to evaluate any regional differences in transporter function at the BBB within CNS. This is particularly important to evaluate in the context of patients receiving whole brain radiation and AZD1390. Evaluating if AZD1390 is disproportionately distributed within different anatomical regions of the brain can provide critical insights into possible toxic side effects of drug/RT combinations. However, no regional differences within the CNS were observed in wild-type, PKO, BKO and TKO mice. This indicates that efflux activity by P-gp and Bcrp is not functionally different across different anatomical regions, reducing possibilities of enhanced localized toxicities within CNS.

Finally, examining focal AZD1390 exposure in and around the tumor is critical for determining effective concentrations and limiting untoward effects. As discussed earlier, in case of GBM,
effective agents must be successfully delivered even to the tumor bearing rim and normal brain regions that can lead to tumor recurrence (Figure 2). Our data indicate that, even though AZD1390 accumulation is higher in the tumor core as opposed to normal brain and rim, total concentrations in all these brain regions are above the 30 nM effective in vitro radiosensitizing concentration (Chen et al. 2020). At the studied dose of 20 mg/kg, AZD1390, in conjunction with RT, showed efficacy in intracranial GBM tumor models (Durant et al. 2018). Therefore, although AZD1390 is a substrate of P-gp mediated efflux in mice, effective total concentrations are achieved in the normal brain for a radiosensitizing effect. While effective delivery of AZD1390 across an intact BBB may effectively target isolated nests of tumor cells, careful investigation of normal brain tissue toxicity in combination with RT is warranted in pre-clinical efficacy studies (Dragojevic et al. 2021).

A positron emission tomography (PET) micro dosing study of AZD1390 in humans (Jucaite et al. 2021), indicated that the Kpuu,brain was 0.24, which is higher than the Kpuu,brain in mice from our studies. This discrepancy points to possible interspecies differences in the CNS distribution of AZD1390. A previous report from Syvanen et al. has also reported species differences in the brain delivery of three P-gp substrates, with higher Kpbrain in humans as compared to rodents (Syvänen et al. 2009). The expression of P-gp at the BBB is approximately 3-fold higher in mice as compared to humans (Uchida et al. 2011; Chu, Bleasby, and Evers 2013). However, this quantification is of total P-gp protein expression and not its functional form available for efflux within cell membranes. There is still a gap of knowledge about interspecies differences in the functionally active form of P-gp among different species. Multiple other reports also indicate differences in substrate affinities across various species for P-gp. For example, in vitro studies using cells transfected with human and mouse P-gp have shown interspecies differences in
affinities of various compounds (Yamazaki et al. 2001; Takeuchi et al. 2006; Katoh et al. 2006; Baltes et al. 2007). However, there have also been contrasting reports where a correlation was reported between P-gp transfected human and mouse permeability studies using MDCK assays for various CNS marketed compounds (Feng et al. 2008). More recently, there also has been a report of similarity in the ligand binding sites of human and mouse P-gp (Jain, Grandits, and Ecker 2018). Therefore, improved understanding about interspecies differences between mouse models used for clinical translation of molecules for CNS delivery is important. Tools like humanized P-gp mouse models can be particularly useful in bridging this gap (Yamasaki et al. 2018). Additionally, pre-clinical studies in large-animal models that are more representative of human anatomy and physiology are of greater relevance (Shen, Yang, and Rodrigues 2021). Canine, porcine and non-human primate models have shown promise in GBM, and will be particularly useful transitional models to bridge the translational gap from mice to humans (Hicks et al. 2021).

In conclusion, our studies elucidated mechanisms limiting CNS distribution of AZD1390 in mice and distribution of AZD1390 in a GBM PDX model. Even though the $K_{pu}$ in mice was 0.12 due to active efflux by P-gp and high binding in brain, AZD1390 levels in the normal brain were above the minimum effective radiosensitizing concentrations. This brings forward an important question of determining a cut-off for $K_{pu}$ values to be deemed high enough to declare agents to be “brain penetrant”. While there is no clear consensus on a target $K_{pu}$ value, it is important to appreciate that unbound brain exposure is just one aspect governing the efficacy of a drug in the CNS. In addition to brain penetrability, it is also important to recognize how potency, target engagement, and distribution to potential sites of toxicity may impact the eventual use of a compound for a CNS disorder (Figure 10). For AZD1390, at a $K_{pu}$ of 0.12, high enough drug
levels were achieved for the 20 mg/kg dose in mice that were adequate for effective pharmacodynamic target engagement and thereby improved efficacy was observed in intracranial models of GBM (Durant et al. 2018). AZD1390 has also demonstrated radiosensitizing activity in brain metastatic PDX models of breast cancer (Tew, Durant, and Salhia 2021). Therefore, developing potent and brain penetrant ATM inhibitors may significantly improve the treatment of patients with brain tumors. While these ATM inhibitors might be particularly effective in enhancing tumor control, potentially heightened risks of radiation injury to normal brain tissues need to be carefully studied for their safe clinical utilization (Figure 10).

ACKNOWLEDGEMENTS

The authors would like to thank James Fisher, Clinical Pharmacology Analytical Services, University of Minnesota for help with the development of AZD1390 LC-MS/MS assay.

AUTHORSHIP CONTRIBUTIONS

Participated in study design – Talele, Sarkaria, Elmquist

Performed experiments – Talele, Zhang, Chen

Performed data analysis – Talele, Gupta, Burgenske, Elmquist

Contributed to the writing of the manuscript – Talele, Sarkaria, Elmquist
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FINANCIAL DISCLOSURE

No author has an actual or perceived conflict of interest with the contents of this article.

FOOTNOTES

This work was supported by the National Institutes of Health [Grants U19 CA264362, U54 CA210180, U01 CA227954, R24 NS092940 and P50 CA108961]. Surabhi Talele was supported by the Rory P. Remmel and Cheryl L. Zimmerman fellowship in Drug Metabolism and Pharmacokinetics, Edward G. Rippie fellowship, Bighley Graduate fellowship, Ronald J. Sawchuk fellowship in Pharmacokinetics, and Doctoral Dissertation fellowship.
FIGURE LEGENDS

Figure 1. Rationale for ATM inhibition using AZD1390 for radiosensitization of tumor cells

Figure 2. Need for the development of brain penetrant ATM inhibitors for radiosensitization in GBM.

(A) Incomplete surgical resection due to invasive nature of GBM leads to tumor recurrence.

(B) Ability to target tumor cells residing behind a relatively intact BBB in normal brain for effective treatment.

Figure 3. Pharmacokinetics of AZD1390 following intravenous administration. Data represents mean ± S.D., n = 4.

Concentration-time profiles of AZD1390 in (A) Plasma, (B) Brain, and (C) Spinal cord following a single intravenous bolus dose of 5 mg/kg in FVB wild-type, BKO, PKO and TKO mice.

Figure 4. Brain and Spinal Cord to Plasma ratios following intravenous administration. Data represents mean ± S.D., n = 4.

(A) Brain-to-plasma ratio, and (B) Spinal cord-to-plasma ratio over time following a single intravenous bolus dose of 5 mg/kg in FVB wild-type, BKO, PKO and TKO mice.

Figure 5. Pharmacokinetics of AZD1390 following oral administration. Data represents mean ± S.D., n = 4.

Plasma, brain, and spinal cord concentrations of AZD1390 in (A) FVB wild-type, and (B) TKO mice; Brain-to-plasma and Spinal cord-to-plasma ratios of AZD1390 with time in (C) FVB wild-type, and (D) TKO mice; following a single oral dose of 10 mg/kg.
Figure 6. Impact of efflux inhibition using elacridar in FVB wild-type mice. Data represents mean ± S.D., n = 4. * P < 0.05

(A) Plasma, brain and spinal cord concentrations; (B) Kp_{brain} and Kp_{spinal cord}; and (C) Kp_{uu,brain} and Kp_{uu,spinal cord} at 2 hours following co-administration of 10 mg/kg oral AZD1390 and 10 mg/kg intraperitoneal elacridar in wild-type mice.

Figure 7. Impact of efflux inhibition using elacridar in TKO mice. Data represents mean ± S.D., n = 4. * P < 0.05

(A) Plasma, brain and spinal cord concentrations; (B) Kp_{brain} and Kp_{spinal cord}; and (C) Kp_{uu,brain} and Kp_{uu,spinal cord} at 2 hours following co-administration of 10 mg/kg oral AZD1390 and 10 mg/kg intraperitoneal elacridar in TKO mice.

Figure 8. CNS regional distribution of AZD1390 following steady-state infusion. Data represents mean ± S.D., n = 4-5. * P < 0.05

(A) Concentration of AZD1390 within the cortex, cerebellum, hypothalamus & thalamus, brain stem, spinal cord and plasma; (B) Regional Kp, and (C) Regional Kp_{uu} of AZD1390 within the cortex, cerebellum, hypothalamus & thalamus, brain stem, and spinal cord in FVB-wild type, BKO, PKO and TKO mice following a steady state infusion of 10 μg/hr for 24 hours.

Figure 9. Tumor distribution of AZD1390 in GBM 12 PDX mouse brains. Data represents mean ± S.D., n = 4-5. * P < 0.05

(A) Brain slice used for tumor carving, (B) Concentration in plasma, tumor core, tumor rim and normal brain; (C) Kp in tumor core, tumor rim and normal brain in GBM 12 bearing mouse brains dosed with 20 mg/kg of AZD1390 and harvested at 4 and 12 hours.
**Figure 10.** Key factors to be considered in the development of ATM inhibitors for brain tumors.
LIST OF TABLES

Table 1. Summary of pharmacokinetic parameters in plasma, brain and spinal cord in FVB wild-type, BKO, PKO and TKO mice following an intravenous bolus dose of 5 mg/kg AZD1390

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Plasma</th>
<th>Brain</th>
<th>Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
<td>Bcrp1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Mdr1a/b&lt;sup&gt;+/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;</td>
<td>hour</td>
<td>2.6</td>
<td>2.4</td>
<td>2.8</td>
</tr>
<tr>
<td>CL</td>
<td>L/hr/kg</td>
<td>0.95</td>
<td>0.88</td>
<td>0.97</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>L/kg</td>
<td>2.7</td>
<td>2.7</td>
<td>3.0</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt;</td>
<td>hr*ng/ml</td>
<td>5261 ± 295</td>
<td>5690 ± 197</td>
<td>5178 ± 361</td>
</tr>
<tr>
<td>K&lt;sub&gt;p&lt;/sub&gt;</td>
<td></td>
<td>0.29</td>
<td>0.32</td>
<td>3.13</td>
</tr>
<tr>
<td>f&lt;sub&gt;u&lt;/sub&gt;</td>
<td></td>
<td>0.203 ± 0.025</td>
<td></td>
<td>0.075 ± 0.002</td>
</tr>
<tr>
<td>K&lt;sub&gt;p,u&lt;/sub&gt;</td>
<td></td>
<td>0.1</td>
<td>0.12</td>
<td>1.16</td>
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<tr>
<td>DA&lt;sub&gt;free&lt;/sub&gt;</td>
<td></td>
<td>1</td>
<td>1.2</td>
<td>11.6</td>
</tr>
</tbody>
</table>
Table 2. Summary of pharmacokinetic parameters in plasma, brain and spinal cord in FVB wild-type and TKO mice following a single oral dose of 10 mg/kg AZD1390

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Brain</th>
<th>Spinal cord</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
<td>Mdr1a/b-/-</td>
<td>Bcrp1-/-</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>hour</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
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<tr>
<td>$C_{\text{max}}$</td>
<td>ng/ml</td>
<td>1523</td>
<td>1367</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 435</td>
<td>± 207</td>
<td>± 11</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>hour</td>
<td>3.4</td>
<td>3.3</td>
<td>3.4</td>
</tr>
<tr>
<td>CL/F</td>
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<td>1.9</td>
<td>-</td>
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<tr>
<td>$V_d/F$</td>
<td>L/kg</td>
<td>10.7</td>
<td>9.0</td>
<td>-</td>
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<tr>
<td>$AUC_{0-\infty}$</td>
<td>hr*ng/ml</td>
<td>4543</td>
<td>5313</td>
<td>1440</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 538</td>
<td>± 795</td>
<td>± 131</td>
</tr>
<tr>
<td>Oral bioavailability</td>
<td></td>
<td>0.43</td>
<td>0.48</td>
<td>-</td>
</tr>
<tr>
<td>Kp</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.32</td>
</tr>
<tr>
<td>$Kp_{uu}$</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.12</td>
</tr>
<tr>
<td>DAfree</td>
<td></td>
<td>1</td>
<td>11.4</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

(A) Concentration (nM) vs. Time (hours)

(B) Concentration (nM) vs. Time (hours)

(C) Kp vs. Time (hours)

(D) Kp vs. Time (hours)
Figure 6.
Figure 7.
Figure 8.
Figure 9.
Figure 10.