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Influence of hydroxyurea on imatinib mesylate (Gleevec®) transport at the mouse blood-brain barrier

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Running title: interaction of hydroxyurea and imatinib mesylate at the BBB

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Abbreviations: BBB, blood-brain barrier; Bcrp1, Breast cancer resistance protein 1; EIAEDs, enzyme-inducing antiepileptic drugs; GBM, glioblastoma; K_{net} , brain uptake clearance; K_p , brain-to-blood concentration ratio at steady-state; PDGFR, platelet-derived growth factor receptor; P-gp, P-glycoprotein; SD, standard deviation.

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Abstract

The combination of imatinib mesylate and hydroxyurea provides a therapeutic benefit in patients with glioblastoma, although each drug is not effective when used alone. The increase of brain delivery of one or both drugs has been suggested to be a potential cause of this therapeutic benefit. The cross-influence of hydroxyurea and imatinib on their respective brain distribution was examined in mice and rats. We used *in situ* brain perfusion in mice to determine whether these two drugs have an influence on their respective initial transport across the blood-brain barrier. The brain penetration of hydroxyurea, assessed by its brain uptake clearance K_{net} , was low in mice ($\sim 0.10 \mu\text{L/g/s}$) and not modified by co-perfusion of imatinib ($0.5\text{-}500 \mu\text{M}$). Likewise, the brain penetration of imatinib was low ($K_{net}, 1.39 \pm 0.17 \mu\text{L/g/s}$) and not modified by direct co-perfusion of hydroxyurea ($0.2\text{-}1000 \mu\text{M}$) or by intravenous pre-treatment with 15 or 1000 mg/kg hydroxyurea. We also examined a potential time-dependent influence of hydroxyurea on imatinib brain distribution after sustained subcutaneous administration in rats using an implantable osmotic pump. The brain penetration of imatinib in rats increased with time, ~ 1.6 -fold ($p < 0.01$) following 7 and 14 days infusion of imatinib (3 mg/day) with or without hydroxyurea (15 mg/d), and was not influenced by hydroxyurea. The results of these two sets of experiments indicate that hydroxyurea has no significant influence on the brain distribution of imatinib in mice and rats.

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Introduction

Gliomas are the most frequent primary brain tumors in adults and are associated with poor prognosis. The majority of patients with glioblastoma multiform (GBM), the most aggressive form of malignant glioma, experience fatal disease progression within the first 24 months following the initial diagnosis. The optimal treatment currently involves surgical resection, external beam radiotherapy associated with temozolomide-based chemotherapy and results in a median survival time of around 12 months (Stupp et al., 2005).

With the increasing understanding of the molecular basis of GBM oncogenesis, new therapeutic targets have been identified. There is a considerable body of evidence implicating the platelet-derived growth factor receptor (PDGFR) autocrine stimulation in the pathogenesis of gliomas (Board and Jayson, 2005). Imatinib mesylate (Gleevec®, formerly STI571, 4-[(4-methylpiperazin-1-yl)methyl]-N-[4-methyl-3-[(4-pyridin-3-yl)pyrimidin-2-yl]amino]phenyl]benzamide methanesulfonate), an ATP competitive inhibitor, blocks the tyrosine kinase activity of various proteins, including ABL, BCR-ABL, c-KIT and PDGFRs. This drug is used in treatment of chronic myeloid leukaemia and gastrointestinal stromal tumors (Dagher et al., 2002; Cohen et al., 2005). Preclinical studies have also suggested that imatinib could be effective for treating GBM (Kilic et al., 2000; Buchdunger et al., 2002).

Recently, two phase II studies evaluated the efficacy and safety of an innovative strategy for GBM treatment using a combination of imatinib and hydroxyurea, a ribonucleotide reductase inhibitor (Dresemann, 2005; Reardon et al., 2005). This combination seemed to be well tolerated and provided a durable antitumor activity in the patients included in these studies (n=63 in total). As both imatinib and hydroxyurea had poor efficacy when used alone in the treatment of GBM (Wen et al., 2002; Raymond et al., 2004), Dresemann and Reardon highlighted the unexpected therapeutic benefit of this combination and speculated about the mechanism(s) involved in this interaction. Of the various hypotheses, they suggested that cross-interactions on the passage of these drugs across the cerebral barriers could enhance the drug delivery into the brain, thereby their efficacy. The brain penetration of imatinib is low in human and mouse (Dai et al., 2003; Le Coutre et al., 2004). In a previous study, we showed that the transport of imatinib across the mouse blood-brain barrier (BBB) is limited by active efflux transporters: P-glycoprotein (P-gp, Abcb1a/1b) and probably Breast cancer resistance protein 1 (Bcrp1, Abcg2) (Bihorel et al., submitted for publication to Clinical Cancer Research). Likewise, hydroxyurea poorly enters the brain of guinea-pigs and seems to be substrate of a digoxin-sensitive efflux transport (Dogruel et al., 2003). Thus, P-gp was suggested to restrict the passage of hydroxyurea into the brain of guinea-pigs.

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In this study, the potential pharmacokinetic interaction between hydroxyurea and imatinib on their respective brain delivery, with a special attention turned to the influence of hydroxyurea on the brain penetration of imatinib, was investigated in mouse and rat. *In situ* brain perfusion was used in mice to assess brain penetration of hydroxyurea and imatinib in presence of the corresponding competitor. This very sensitive method is designed to specifically assess the kinetics and the mechanisms of transport of solutes at the luminal side of the BBB. It avoids any peripheral distribution or metabolic interference, as the blood circulation to the brain is taken over by a perfusion fluid containing the solute of interest and administered by catheterization of the carotid artery. This method also respects the physiological properties of the BBB. Finally, we investigated a potential time-dependent effect of hydroxyurea on the brain penetration of imatinib in rats following a 2-weeks continuous subcutaneous administration of imatinib alone or in combination with hydroxyurea.

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Materials and Methods

Chemicals and reagents.

[¹⁴C]Imatinib (1.94 GBq/mmol) and unlabeled imatinib (both as mesylate salts) were kindly provided by Novartis Pharma (Basel, Switzerland). [¹⁴C]Hydroxyurea (1.98 GBq/mmol) was purchased from American Radiolabeled Chemicals Inc (Saint-Louis, MO). [³H]sucrose (0.43 TBq/mmol) and unlabeled hydroxyurea were purchased from Sigma-Aldrich (Buchs, Switzerland). All other chemicals were of analytical grade. The doses or concentrations mentioned in the following text refer to the base form of compounds.

Animals.

All experiments were performed on adult male FVB mice (weighing 20 to 30 g) and Hanover Wistar rats (weighing 270 to 310 g) supplied by Charles River (L'Arbresles, France). The animals were kept under standard conditions of temperature and lighting with free access to food and water. These studies complied with the Swiss Federal Act on Animal Protection (revised 2003) and with the Swiss Animal Protection Ordinance (revised 2001).

In situ brain perfusion procedure in mice.

The transport of imatinib and hydroxyurea into the brain compartment was assessed by *in situ* brain perfusion. The details of this model have been described previously (Dagenais et al., 2000; Smith and Allen, 2003). Briefly, mice were anaesthetized with a mixture of xylazine (8 mg/kg, Bayer, Leverkusen, Germany) and ketamine (140 mg/kg, Graeb, Bern, Switzerland) given i.p. The right common carotid artery was exposed and ligated on the heart side. The external carotid artery was ligated rostral to the occipital artery at the bifurcation of the common carotid artery with the internal carotid. Then the right common carotid artery was catheterized with polyethylene tubing (0.28 mm i.d. × 0.61 mm o.d.; Smiths Medical, Hythe, United Kingdom) containing 25 units/mL heparin. The thorax of the mouse was opened and the heart cut immediately before starting perfusion (flow rate: 2.5 mL/min). The perfusion fluid consisted of bicarbonate-buffered physiological saline (mM): 128 NaCl, 24 NaHCO₃, 4.2 KCl, 2.4 NaH₂PO₄, 1.5 CaCl₂, 0.9 MgSO₄ and 9 D-glucose. The solution was gassed with 95% O₂ - 5% CO₂ for oxygenation and pH control (7.4) and warmed to 37°C. All mice were perfused with the test compounds, [¹⁴C]imatinib (0.6-1.4 kBq/mL) or [¹⁴C]hydroxyurea (0.9-1.8 kBq/mL), plus [³H]sucrose (6.5-30 kBq/mL) as a vascular marker. [¹⁴C]Hydroxyurea was dissolved in 0.9% NaCl whereas [¹⁴C]imatinib and [³H]sucrose were dissolved in a 1:1 and 1:9 mixture of ethanol:water v/v, respectively. The perfusions were terminated by decapitation after 60 and 120 seconds for [¹⁴C]imatinib and [¹⁴C]hydroxyurea uptake experiments, respectively. The brain was quickly extracted from the skull and dissected on ice. The right cerebral hemisphere

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was sampled and weighed in a tared vial. Finally, aliquots of the perfusion solution were collected from the catheter and weighed to determine the concentration of tracers in the perfusate. All samples were digested with 1 mL Biolute-S (Zinsser Analytic, Frankfurt, Germany) over night at room temperature and mixed with 500 μ L of 2N HCl and 10 mL IrgaSafe-Plus (Zinsser). ^3H and ^{14}C labels were counted simultaneously in a Tricarb 2500TR Liquid Scintillation Analyzer (Packard, Meriden, CT).

The brain uptake of hydroxyurea was examined over a large concentration range (0.5-500 μM). We also studied the influence of imatinib co-perfusion (0.5-500 μM) on the brain penetration of hydroxyurea (1 μM), as well as the influence of hydroxyurea co-perfusion (0-1000 μM) on the brain penetration of imatinib (0.5 μM). Finally, the brain penetration of 0.5 μM imatinib was measured in mice having received, 15 min or 1 h before starting perfusion, an intravenous administration (5 mL/kg) of 0.9% NaCl, hydroxyurea 15 mg/kg or 1000 mg/kg (both dosages prepared in 0.9% NaCl) in the exposed saphenous vein.

All calculations of BBB transport parameters were done as previously described (Takasato et al., 1984; Smith, 1996). The brain vascular volume (V_{vasc} , $\mu\text{L/g}$) was estimated from the brain distribution volume of [^3H]sucrose using the following equation:

$$V_{\text{vasc}} = X^* / C_{\text{perf}}^*$$

where X^* (dpm/g) is the concentration of [^3H]sucrose in the right cerebral hemisphere and C_{perf}^* (dpm/ μL) the concentration of [^3H]sucrose in the perfusion solution.

The transport of imatinib or hydroxyurea towards brain is expressed as the net transport coefficient (K_{net} , $\mu\text{L/g/s}$), which corresponds to the brain uptake clearance. It was calculated using the following equation which includes a correction for vascular contamination:

$$K_{\text{net}} = \frac{X_{\text{tot}} - V_{\text{vasc}} \times C_{\text{perf}}}{C_{\text{perf}} \times T}$$

where X_{tot} (dpm/g) is the concentration of radiolabeled test drug found in the brain tissue sample, *i.e.* microvasculature plus extravascular material, C_{perf} (dpm/ μL) the concentration of radiolabeled test drug in the perfusate and T the perfusion time (s).

Impact of hydroxyurea on the brain penetration of imatinib at steady-state in rats.

Hanover Wistar rats received subcutaneously either 3 mg/day imatinib (1.4 MBq/d) alone or in combination with 15 mg/d hydroxyurea, both released by 2ML2 Alzet osmotic pumps (delivery flow: 4.5 $\mu\text{L/h}$; Charles River). The pumps were inserted subcutaneously in the back of each rat anesthetized by inhalation of isoflurane (Abbott AG, Baar, Switzerland). The solution containing the test compounds were prepared in 0.9% NaCl to achieve appropriate concentrations and sterilized by filtration on 0.2 μm polyethersulfone-based membrane (Waters AG,

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Rapperswil, Switzerland) just before filling the pumps. The animals were sacrificed 24, 192 (Day 7) or 336 h (Day 14) after implantation of the pump (n=3 per time point). Blood (~10 mL) was collected by puncture into the *vena cava*. Brain was sampled and quickly cleaned to remove the largest blood vessels. Blood and brain samples were immediately stored at – 20°C pending analysis. Brain samples were homogenized just before analysis in 10 mL demineralized water.

The concentrations of unchanged imatinib in blood (200 µL) and brain (500 µL of homogenate) were determined by liquid chromatography as previously described (Bihorel et al., 2006, submitted for publication). The brain penetration of imatinib was estimated by the brain-to-blood concentration ratios at steady state (K_p).

Statistical analysis.

All values are presented as means \pm standard deviations (SD) for three-five mice in *in situ* brain perfusion experiment or three rats in the steady-state pharmacokinetic study. K_{net} values, obtained by *in situ* brain perfusion, were compared by one way analyses of variance followed by multiple comparisons to the control group using Dunnett's tests (SigmaStat 3.11, SPSS Inc, Chicago, IL). Imatinib K_{net} obtained in mouse pre-treated with hydroxyurea were compared using a two way analysis of variance, to investigate the influence of the pre-treatment dose and time. Imatinib blood concentrations and K_p values observed at different time points after continuous administration of imatinib were compared by two way analyses of variance on log-transformed values, followed by multiple comparisons using Tukey's tests (SigmaStat 3.11). In all analyses, the statistical significance was set at $p < 0.05$.

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Results and Discussion

Various concentrations of hydroxyurea and imatinib were perfused into the brains of mice with or without the corresponding competitor, and always in the presence of the vascular space marker [³H]sucrose. The brain distribution volume of [³H]sucrose was similar in all experiments (overall mean of $17.4 \pm 1.8 \mu\text{L/g}$, data not shown) and consistent with the values ($13\text{--}17 \mu\text{L/g}$) usually reported for sucrose (Dagenais et al., 2000; Cisternino et al., 2004). This suggests that the mouse BBB remained intact during all perfusion experiments with hydroxyurea and imatinib.

K_{net} of hydroxyurea (MW = 76.06, log P = -1.27) after 120 s perfusion was low ($0.10 \mu\text{L/g/s}$) and concentration-independent ($0.5\text{--}500 \mu\text{M}$; Fig. 1A). Using the same technique, previous studies have shown that K_{net} of drugs can range from $\sim 0.03 \mu\text{L/g/s}$ for morphine-6-glucuronide (MW = 462.14, log P = -2.4), a compound with very low brain penetration, to more than $40 \mu\text{L/g/s}$ for drugs that can freely cross cell membranes, like diazepam (MW = 284.74, log P = 2.8) (Dagenais et al., 2000; Bourasset and Scherrmann, 2006). Hydroxyurea K_{net} was not significantly increased by simultaneous perfusion of $0.5\text{--}500 \mu\text{M}$ imatinib (Fig. 1B). These data indicate that the initial transport of hydroxyurea across the mouse BBB is low, independent of any saturable mechanism and not enhanced by imatinib. Recently, Dogruel et al. showed that [¹⁴C]hydroxyurea crosses the brain barriers of guinea-pigs at a slow rate and that its distribution in cerebrum can be significantly increased by co-perfusion of $200 \mu\text{M}$ hydroxyurea or $25 \mu\text{M}$ digoxin after 20 min perfusion (Dogruel et al., 2003). This suggests that a digoxin-sensitive efflux mechanism transports hydroxyurea out of guinea-pig brain, after long exposure. Therefore, we cannot exclude that a similar mechanism, perhaps influenced by imatinib, removes hydroxyurea from the brain of mice when the drug distribution equilibrium is reached into the whole brain. Unfortunately, our study was not expanded to investigate this issue, as the *in situ* brain perfusion procedure in mouse is not designed to perform long perfusion (Dagenais et al., 2000).

In a previous work, we examined the brain penetration of imatinib in wild-type and P-gp or Bcrp1 knockout mice and found that it was limited by efflux transporters, P-gp and probably Bcrp1 (Bihorel et al., submitted for publication). In the present study, the brain of wild-type mice were perfused with $0.5 \mu\text{M}$ imatinib and increasing concentration of hydroxyurea ($0.2\text{--}1000 \mu\text{M}$), to study the potential effect of hydroxyurea on P-gp and Bcrp1-mediated efflux. The imatinib K_{net} value in absence of hydroxyurea ($1.39 \pm 1.7 \mu\text{L/g/s}$) was in line with our previous experiments. Moreover, co-perfusion of hydroxyurea did not significantly modify the brain penetration of imatinib (Fig. 1C).

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The drug-binding sites on P-gp are known to lie within the transmembrane domains of the protein (Ambudkar et al., 2003); less is known about the molecular interactions between Bcrp1 and its substrates. Considering the low brain penetration of hydroxyurea after 120 s perfusion, the amounts of drug that had access to the drug-binding sites on P-gp and Bcrp1 after 1 min perfusion might have been very low and insufficient to influence the efflux of imatinib. Therefore, we measured imatinib K_{net} in mice pre-treated with hydroxyurea: physiological 0.9% NaCl, low (15 mg/kg) or high doses (1000 mg/kg) of hydroxyurea were administered intravenously in FVB mice 15 min or 1 h before starting perfusion. The brain penetration of imatinib was not significantly increased under these conditions. Together, these results suggest that hydroxyurea does not influence the transport of imatinib at the mouse BBB (Fig. 1D).

The patients, whose GMB positively responded during the two abovementioned phase II studies, received oral dosages of imatinib and hydroxyurea on a continuous daily schedule (Dresemann, 2005; Reardon et al., 2005). Therefore, it is possible that one drug had a time-dependent influence on the systemic or brain pharmacokinetics of the other. In one of these studies, the pharmacokinetics of imatinib and hydroxyurea were examined after one day or 28 days of treatment in patients co-medicated or not with enzyme-inducing antiepileptic drugs (EIAEDs) (Reardon et al., 2005). At day 1 or at steady-state, the kinetic parameters of imatinib in patients, not treated with EIAEDs, were similar to those obtained in patients only treated with a similar daily dosage of imatinib (Le Coutre et al., 2004; Peng et al., 2004). Likewise, there was no significant difference between imatinib plasma protein binding obtained in presence or absence of hydroxyurea (Kretz et al., 2004; Reardon et al., 2005). This suggests that hydroxyurea does not significantly modify the systemic pharmacokinetics of imatinib. In contrast, the pharmacokinetics of hydroxyurea at steady-state seem to be slightly altered in patients co-medicated with imatinib (with or without EIAED co-medication) (Tracewell et al., 1995; Villani et al., 1996; Reardon et al., 2005). The variations include a modest 2-fold increase in the apparent clearance of hydroxyurea, a 2-fold decrease in plasma exposure and lower peak concentrations in plasma. Therefore, they should not result in any improvement in the brain penetration of hydroxyurea. Furthermore, the extent of hydroxyurea binding to plasma proteins has not been published. Finally, these data indicate that the combination of hydroxyurea and imatinib is unlikely to enhance the brain delivery of one or both drugs by an increase in their systemic concentration.

We expanded our study to investigate the influence of hydroxyurea on the brain penetration of imatinib at steady-state after chronic administration. As hydroxyurea has a very short half-life in the systemic circulation of mice and rats (< 1 h), a resource-intensive study design would be required to maintain significant hydroxyurea blood levels by standard administration routes (Philips et al., 1967; Iyamu et al., 2001). Therefore, imatinib was given subcutaneously in rat with or without hydroxyurea over 2 weeks, both drugs being administered using a

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sustained zero-order delivery system, *i.e.* Alzet osmotic mini-pumps. The blood concentrations of imatinib were significantly lower on Day 7 and Day 14 than after one day ($p < 0.01$) but not significantly influenced by hydroxyurea (Fig. 2A). The levels of drug reached in blood, *i.e.* 0.2-0.5 μM , were roughly those tested in our *in situ* brain perfusion studies. Therefore, imatinib did most likely not reach saturating concentration. Imatinib K_p in brain ranged from 0.15 ± 0.02 to 0.28 ± 0.07 and were significantly increased at late time point ($p < 0.001$) but not by the co-administration of hydroxyurea (Fig. 2B). These data indicate that hydroxyurea had no time-dependent influence on the brain penetration of imatinib in rats.

In summary, the results reported here in healthy rodents suggest that hydroxyurea does not modify the brain penetration of imatinib after short or sustained co-administration. Likewise, we found that imatinib had no influence on the initial transport of hydroxyurea in the mouse BBB. Whether imatinib inhibits the activity of an efflux transport of hydroxyurea at brain distribution equilibrium remains unknown. Additional studies should further characterize this interaction on preclinical tumor models, as the permeability properties of the cerebral endothelium are probably modified in capillaries that irrigate the GBM tumor. Lastly, the uptake of imatinib and hydroxyurea into tumor cells should be examined, as well as their potential cross-influence.

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Footnotes

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Legends for figures

Figure 1. *A*, brain transport coefficient (K_{net} , $\mu\text{L/g/s}$) of [^{14}C]hydroxyurea in mice measured after *in situ* brain perfusion for 120 s. *Points*, means; *bars*, SD for 4-5 animals. *B*, [^{14}C]hydroxyurea K_{net} measured after *in situ* brain perfusion (120 s) of the drug (1 μM) with various concentrations of imatinib in mice. *Columns*, means; *bars*, SD for 4-5 animals. *, $p < 0.05$, control mice perfused with 0.5 μM hydroxyurea *versus* mice co-perfused with 0.5 μM hydroxyurea and imatinib. *C*, [^{14}C]imatinib mesylate K_{net} measured after *in situ* brain perfusion (60 s) of the drug (0.5 μM) with various concentrations of hydroxyurea in mice. *Columns*, means; *bars*, SD for 3-5 animals. *D*, [^{14}C]imatinib mesylate K_{net} , measured after *in situ* brain perfusion (60 s) in mice; the mice received 0.9% NaCl, 15 mg/kg or 1000 mg/kg hydroxyurea 15 min (open columns) or 1h before starting perfusion (closed columns). *Columns*, means; *bars*, SD for 4-5 animals.

Figure 2. Blood concentrations (part *A*) and brain-to-blood concentration ratios (brain K_p , part *B*) of imatinib in rats at various time points; the animals subcutaneously received either 3 mg/day [^{14}C]imatinib mesylate (open columns) or 3 mg/day [^{14}C]imatinib mesylate plus 15 mg/day hydroxyurea (closed columns) released by 2ML2 Alzet osmotic pumps.

Figure 1

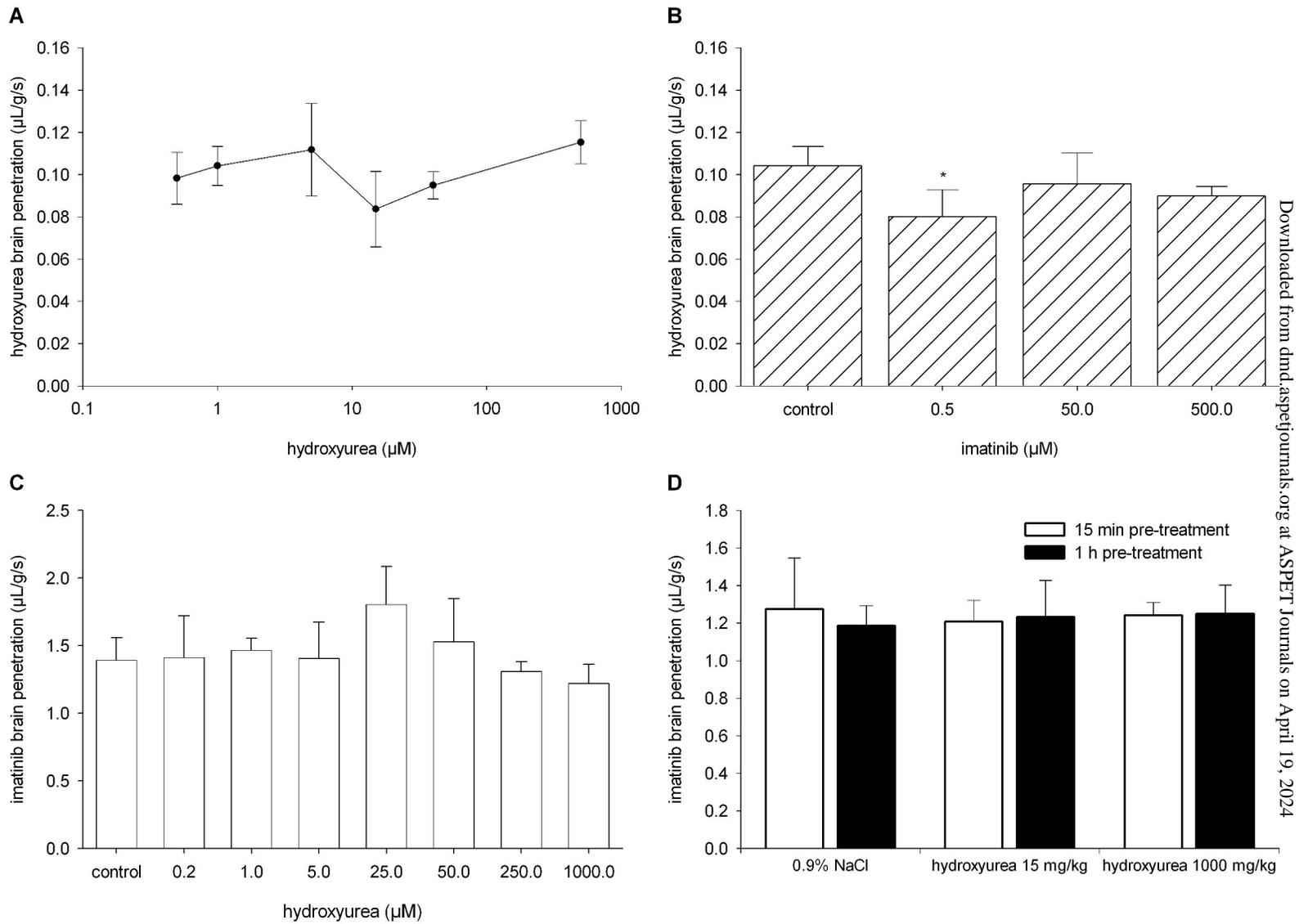


Figure 2

