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Title: Subacute AMD3100 treatment is not efficient in neonatal hypoxic-ischemic rats

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Subacute AMD3100 treatment is not efficient in neonatal hypoxic-ischemic rats

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Abstract

**Background and Purpose:** Despite the advances in treating neonatal hypoxic-ischemic encephalopathy (HIE) with induced hypothermia, the rates of severe disability are still high among survivors. Preclinical studies have indicated that cell therapies with hematopoietic stem/progenitor cells (HSPC) could improve neurological outcomes in HIE. In this study, we investigated whether the administration of AMD3100, a CXCR4 antagonist that mobilizes HSPC into the circulation, has therapeutic effects in HIE.

**Methods:** P10 Wistar rats of both sexes were subjected to right common carotid artery occlusion or sham procedure, and then were exposed to hypoxia for 120 minutes. Two subcutaneous injections of AMD3100 or vehicle were given on the third and fourth day after HIE. We first assessed the inter-individual variability in brain atrophy after experimental HIE and vehicle treatment in a small cohort of rats. Based on this exploratory analysis, we designed and conducted an experiment to test the efficacy of AMD3100. Brain atrophy on day 21 after HIE was defined as the primary endpoint. Secondary efficacy endpoints were cognitive (T-water maze) and motor function (rotarod) on days 17 and 18 after HIE, respectively.

**Results:** AMD3100 did not decrease the brain atrophy in animals of either sex. Cognitive impairments were not observed in the T-water maze, but male hypoxic-ischemic animals exhibited motor coordination deficits on the rotarod, which were not improved by AMD3100. A separate analysis combining data from animals of both sexes also revealed no evidence of the effectiveness of AMD3100 treatment.

**Conclusions:** These results indicate that the subacute treatment with AMD3100 does not improve structural and functional outcomes in a rat HIE model.
Abbreviations

BM: bone marrow

CXCL12: C-X-C Motif Chemokine Ligand 12

CXCR4: C-X-C chemokine receptor type 4

G-CSF: granulocyte-colony stimulating factor

HIE: hypoxic-ischemic encephalopathy

HSPC: hematopoietic stem/progenitor cells

SD: standard deviation

SDF-1: stromal-derived factor-1

UCB: umbilical cord blood
Introduction

Hypoxic-ischemic encephalopathy (HIE) is a leading cause of mortality and disability in term infants.\(^1,2\) Cell therapies using hematopoietic stem/progenitor cells (HSPC) have revealed promising results in animal models of several neurological disorders including HIE.\(^3-5\) Most of the studies have evaluated the effects of the mononuclear cell fraction (which is enriched in HSPC but mainly contains other cell types) from umbilical cord blood (UCB)\(^6\) or bone marrow (BM),\(^7\) and some showed beneficial effects when applying purified CD34\(^+\) HSPC populations.\(^8,9\)

While administration of HPSC or HPSC-containing cell populations has shown promising results in preclinical studies, clinical translation might be challenging.\(^10\) First, HIE is a severe condition requiring immediate, intensive and extended care to counter the hypoxic-ischemic insult, including in subacute and, potentially, chronic stages.\(^11,12\) Second, availability of cell products might be limited. UCB-derived cells can be obtained only once and may not be used for multiple injections over extended treatment periods. Alternative autologous cell sources such as BM might be difficult to access in critically ill newborns. Allogenic, human leukocyte antigen-matched off-the-shelf cell products might be an alternative, as cord blood is suitable for long-term cryopreservation. However, cryopreservation might affect the neuroprotective abilities of the cells.\(^13\)

Pharmaceutical interventions to mobilize endogenous HSPC from BM would be advantageous and allow repeated and continuous, well controllable HSPC recruitment into the circulation, and eventually into the brain. Importantly, such treatments would not significantly interfere with other approaches, including therapeutic hypothermia, in an intensive care setting. We decided to focus on AMD3100 as a promising drug candidate for this study since the use of
alternatives such as granulocyte-colony stimulating factor (G-CSF) has shown controversial results in both HIE and stroke, indicating potential side-effects.\textsuperscript{14-22}

AMD3100 is an antagonist of the chemokine receptor CXCR4. This receptor is activated by the stromal-derived factor-1 (SDF-1), and, together with the integrin α4β1 (very late antigen-4), plays an important role for HSPC retention in the BM hematopoietic niche.\textsuperscript{23} A single dose of AMD3100 leads to a more than 10-fold increase in the number of circulating HSPC. This increase occurs very quickly, and it can already be observed in the first hours after the administration of AMD3100, in contrast to the more delayed effects of G-CSF.\textsuperscript{24,25} Furthermore, AMD3100 treatment improved functional recovery after experimental ischemic stroke.\textsuperscript{26-29} Therefore, we hypothesized that AMD3100 could improve HIE outcome.

**Methods**

The data supporting the findings of this study are available from the corresponding author upon reasonable request. Full descriptions of Methods are available in the Data Supplement.

**Experimental Design**

We applied a two-step study design. Brain atrophy was defined as the primary endpoint for AMD3100 efficacy. Secondary efficacy endpoints were cognitive (T-water maze) and motor function (rotarod). Brain atrophy after HIE can vary considerably between individual animals. Step I assessed this inter-individual variability in brain atrophy after experimental HIE to estimate the standard deviation (SD). Step II was intended to test efficacy of AMD3100 in HIE. SD estimates derived from step I were used to calculate required group sizes in step II. Effect sizes on brain atrophy were derived from previous data in ischemic stroke published in peer-
reviewed publications. Sample size and power calculations were performed using G*Power 3.1.9.2 software.

Animals

Wistar rats were used in this study. All procedures were approved and conducted in accordance with the Animal Care and Use Committee at the Federal University of Rio de Janeiro (protocol number 172/13). Animals received humane care in compliance with the “Principles of Laboratory Animal Care” of the National Society for Medical Research and the U.S. National Academy of Sciences Guide for the Care and Use of Laboratory Animals. ARRIVE guidelines were applied.

Neonatal Hypoxia-Ischemia and Sham Surgery

The Rice-Vannucci model of neonatal hypoxia-ischemia was conducted to induce brain damage in term-equivalent P10 rat pups of both sexes (P0 = day of birth). Rats were randomly chosen to be subjected to either sham surgery or to right common carotid artery occlusion under isoflurane anesthesia.

AMD3100 and vehicle treatment

Each litter included HIE animals and littermate controls (sham). HIE animals were randomly assigned to treatment groups in step II. Sham animals received vehicle treatment.

A subcutaneous (between the scapulae) injection of sterile 0.9% saline solution (vehicle) or 5 mg/kg/d AMD3100 octahydrochloride hydrate in sterile saline (150 μL total volume each) was performed at days 3 and 4 after HIE. Ten additional naïve rats received AMD3100 (5
mg/kg/d) or vehicle treatment for 2 consecutive days, starting at P13, and were used to confirm
the mobilization of CXCR4^+ HSPC to the circulation (Supplemental Figure I).

**Primary endpoint: brain atrophy**

Seven (step I) or 21 days (step II) after HIE, animals were deeply anesthetized by
intraperitoneal injection of ketamine hydrochloride and xylazine (100/10 mg/kg), and
euthanized. Brain atrophy was evaluated in thionine-stained brain sections. We randomly
selected 20 brains for an exploratory analysis of proliferating cells in the subventricular zone and
the area covered by microglia/macrophages.

**Step I: estimation of inter-individual variability of the primary endpoint**

In the exploratory step I, male and female animals (n=6-7) were investigated, all
receiving vehicle treatment (Figure 1A). Assessment of brain atrophy 7 days after HIE revealed a
relatively high SD, ranging from 11.9 to 26.2% (Figure 2). There were no differences between
female and male animals (p≥0.5338, Mann-Whitney U test).

**Step II: AMD3100 efficacy analysis**

Based on results obtained in step I, we estimated SD for brain atrophy as 26.2%. Previous
publications in ischemic stroke showed a brain atrophy reduction of 31.3%^26 and 52.2%^29 after
AMD3100 treatment. We decided to design the step II experiments conservatively, i.e. to reveal
a 20% mean difference in brain atrophy at 26.2% SD and 80% power using a one-sided test, for
which 22 animals were required in each group. Additionally, age-matched reserve animals (n=25
in total, n=17/8 males/females) were also included on availability from our breeding colony, and
randomly (i.e. non-evenly) allocated to HIE and sham groups. This resulted in the setup depicted in Figure 1B with group sizes of n=24-30, compensating for either minimally higher SD (27.5%) or slightly smaller effect size (19%).

Secondary endpoints: cognitive and motor performance

Step II experiments included assessment of cognitive (T-water maze) and motor function (rotarod).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0.2 (GraphPad Software). The Data Supplement provides a comprehensive description of statistical methods and results.

Results

No evidence for long-term protective effects of AMD3100 in HIE

In the main study, animals were followed up for a period of 21 days after the induction of HIE or the sham procedure. Assessment of primary and secondary endpoints did not reveal any treatment effects. We did not observe intergroup differences in spatial learning performance assessed by the T-water maze. The latency to find the hidden platform decreased over the trials (F(3, 210)=20.41, p<0.0001 for females; F(3, 240)=13.50, p<0.0001 for males). However, it was indifferent between HIE and sham groups in either sex when considering each trial separately (p≥0.1028 in the training and p≥0.1463 in each testing trial; Figure 3A) or the mean of the 3 testing trials (p>0.9999; Figure 3B). Similar results were obtained when both sexes were
analyzed together ($p \geq 0.0967$ in the training, $p \geq 0.3131$ in each testing trial, $p \geq 0.7471$ for the mean of the 3 testing trials; Figure 3A-B). The rotarod test was used for motor function assessment. HIE did not affect the performance of female rats on the rotarod test ($p \geq 0.8794$; Figure 3C). In contrast, male HIE rats, regardless of treatment, performed significantly worse than sham-operated males ($p=0.0057$, Sham+Vehicle vs. HIE+Vehicle; $p=0.0159$, Sham+Vehicle vs. HIE+AMD3100; Figure 3C). However, there was no difference between AMD3100 and vehicle group ($p>0.9999$). When both sexes were combined, there were statistically significant differences between the HIE groups and the sham group ($p=0.0168$, Sham+Vehicle vs. HIE+Vehicle; $p=0.0319$, Sham+Vehicle vs. HIE+AMD3100), but not between the treatment groups ($p>0.9999$; Figure 3C). Finally, brain atrophy was neither changed by AMD3100 treatment in either sex ($p \geq 0.3409$ for females and $p \geq 0.6289$ for males; Figure 4), nor when males and females were analyzed together ($p \geq 0.3693$; Figure 4).

We also performed exploratory histological analyzes on 20 randomly chosen brains (5 males and 5 females per group). We found no evidence for anti-inflammatory AMD3100 effects, as the area covered by Iba1⁺ microglia/macrophages in the ipsilateral hemisphere was similar between the groups (Supplemental Figure II). Moreover, AMD3100 treatment had no effect on cell proliferation in the neurogenic subventricular zone (Supplemental Figure III).

**Discussion**

To date, there are no pharmacological HIE therapies. We evaluated the therapeutic potential of subacute AMD3100 treatment in a HIE model. Our results showed that AMD3100 does not reduce brain atrophy in animals of either sex, and that it did not prevent motor deficits in male HIE rats.
Considering a type II error

A type II error, i.e. false-negative or -neutral results, must be excluded. To avoid a type II error, we performed a preliminary study (step I) to estimate SD being required for sample size calculation for the main trial (step II). SD was estimated to be 26.2%. The assumed effect size of 20% was well in range of recommendations for effect size estimations from neighboring research areas such as stroke (10 to 20%).\(^3^2\) Indeed, much larger effect sizes were reported for AMD3100 treatments in stroke. Huang et al.\(^2^8\) described a 31.3% reduction of lesion size after ischemic stroke in adult animals whereas Wu et al.\(^2^9\) even reported an effect size of 52.2% on that endpoint. Moreover, performing separate investigations in both sexes preserved the option to analyze groups jointly.

Of note, SD was markedly larger than estimated in step II, reaching up to 46%, for unknown reasons. No protocol or setting changes were made, and experimenters were the same, conducting identical tasks. Post-hoc power calculations considering 46% SD as a worst-case scenario were performed. Combining male and female animals, resulting in group sizes of n=44 (n=51-54 including reserve animals), still allows to detect an effect size of 24.5% for a one-sided and 27.5% for a two-sided test (23%/26% including reserve animals) at 46% SD with 80% power. Although this is slightly above recommended effect sizes, it is still below reported ones for AMD3100. Nevertheless, a therapeutic effect was also missing in these composite analyses. In case a type II error occurred in our investigation, the real effect size of AMD3100 treatment should be well below 30%. A type II error based on a smaller effect size being ‘masked’ by a larger SD is nevertheless unlikely, as there have not been noticeable mean differences between
the treatment groups. We therefore should consider other potential reasons for the observed neutral outcome.

225 **AMD3100 dose**

It might be argued that the AMD3100 dose used in our study was insufficient. Our hypothesis was that AMD3100-mediated mobilization of HSPC from BM would have neuroprotective effects, comparable to what was reported after G-CSF administration or transplantation of UCB-derived HSPC/mononuclear cells in HIE. We evaluated the effects of two subcutaneous AMD3100 injections (5 mg/kg/d). A single subcutaneous injection of 5 mg/kg is sufficient to induce the mobilization of HSPC into the blood in rodents but other authors did not observe HSPC mobilization when using a lower dose of 1 mg/kg/d administered intraperitoneally. In addition, efficacy of lower doses in models of experimental stroke revealed mixed results depending on the timing of AMD3100 administration. The double injection regimen was chosen with the intention of boosting HSPC mobilization in two waves, also considering that HSPC mobilization is not altered after up to 3 consecutive daily AMD3100 injections.

239 **Timing of AMD3100 administration**

Another important aspect might be the timing of AMD3100 administration. A limitation of our study is that we only tested one therapeutic time window: AMD3100 was delivered on days 3 and 4 after HIE. This approach was based on the hypothesis that mobilized HSPC would exert neuroprotective effect on the ‘secondary’ phase of brain damage (delayed cell death), as well as a potential neurorestorative action, in a similar way to what has been reported for BM-
and UCB-derived cell therapies. UCB cells were shown to provide neuroprotection even when the treatment was delayed for 2 to 9 days after the insult in rabbits, fetal sheep, and rodents.

We also considered that the subacute treatment would be more feasible, taking in account the time required for the diagnosis of HIE, and for the inclusion of newborns in clinical trials.

Importantly, the subacute treatment with AMD3100 has already been demonstrated to exert beneficial effects after experimental stroke. Walter et al. and Ruscher et al. showed clear improvement of neurological outcome after treating adult mice and rats twice daily with intraperitoneal AMD3100 injections (0.5 mg/kg) for several days, starting on the second day following stroke. However, they did not detect any changes in the infarct volume. Substantial differences in lesion size and brain atrophy were observed in other studies, but the treatment was started earlier. Huang et al. as well as Wu et al. reported a reduction of infarct volume in mice treated by intraperitoneal injections of AMD3100 (1 mg/kg) starting immediately after the ischemic insult. Moreover, it was also shown that two intracerebroventricular AMD3100 injections 12 and 18 h after stroke decreased the migration of M1 “pro-inflammatory” microglia to the perilesional site, attenuated the infarct size, and induced functional recovery. The subacute treatment (later than 24 h) did not affect the infarct size or the functional recovery following stroke in rats.

Thus, it may be assumed that our treatment was just too late to provide beneficial effects. However, all previous studies in ischemic stroke reported clear improvements in functional outcome, with or without reduction in lesion size. We could not detect such effects in our secondary endpoints despite much larger group sizes, indicating that timing may also not be the primary reason for our neutral study results. Although we have detected deficits in motor coordination in male animals that were not reversed by AMD3100, we also recognize that the
absence of cognitive deficits in the T-water maze task in animals of both sexes and the lack of motor coordination deficits in females are limitations of our study. To overcome these limitations, using more advanced neurobehavioral tests or a more severe HIE model is recommended in future studies.

**Therapeutic mechanism**

Previous studies reported anti-inflammatory effects of AMD3100 treatment, suggesting that the beneficial effects of lower AMD3100 doses are attributed to its function as a CXCR4 antagonist in immune cells. In fact, AMD3100 is not only a potent HSPC mobilizer but also a widely applied CXCL12/CXCR4 blocker. A potential therapeutic mechanism postulated for AMD3100 is the reduction of neuroinflammation in the post-ischemic brain by preventing the egress of some immune cell populations from the circulation. AMD3100 could also counter the recruitment of HPSC into the ischemic brain. CXCL12/CXCR4 signaling was recently shown to be important for monocyte recruitment and there is at least indirect evidence that it may also play a role for HSPC migration into the brain. It is therefore not unreasonable to assume that the dose of AMD3100 applied in our study may have induced the mobilization of HPSC but, at the same time, prevented HPSC brain entry. This may counteract beneficial effects, although the entry of HSPC into the brain does not seem to be necessary for their therapeutic effects in models of stroke and HIE.

**Potential adverse effects of AMD3100 treatment in hypoxic-ischemic injury**

We cannot rule out the possibility that detrimental side effects have confounded beneficial AMD3100 actions. The dose applied in our study can mobilize other cell types, such
As endothelial progenitor cells, monocytes, neutrophils, and lymphocytes, resulting in their redistribution to secondary lymphoid organs.44-46 Lymphocytes and neutrophils have been shown to play detrimental roles in HIE,33,47-49 which could explain the lack of efficacy of our treatment. Similar issues were observed for G-CSF, and Doycheva et al.33 reported that anti-neutrophil antibodies improved the neuroprotective effects of G-CSF in HIE. Since AMD3100, but not G-CSF, effectively blocks the CXCL12/CXCR4 axis, what is even believed to be a potential AMD3100 mechanism of therapeutic action,26,27 we assume that AMD3100 could have at least counter-balanced the mobilization of proinflammatory cells by blocking their entry into the brain.

It is also possible that the lack of therapeutic effects reported here could be related to the antagonization of some of the potential beneficial aspects of SDF-1/CXCR4 signaling in the brain, such as the guidance of neural progenitors and new neurons towards the ischemic boundary.50,51 Zhao et al. have shown that a chronic intracerebroventricular treatment with AMD3100 reversed the positive effects of force limb-use on neurogenesis and behavioral recovery after stroke.52 In addition, the chronic subcutaneous infusion of AMD3100 abrogated remyelination in a model of demyelination by decreasing the differentiation of oligodendrocyte progenitor cells into oligodendrocytes.53 We have not addressed these processes and thus cannot exclude such detrimental side effects of AMD3100 in HIE, especially at the relatively high dose applied. However, our results showed that AMD3100 did not change the number of proliferating cells in the subventricular zone.

Finally, a recent study showed that CXCR4 deficiency was associated with reduced numbers of monocyte-derived macrophages in the ischemic brain and a poorer outcome after stroke.41 We found that the area covered by Iba1+ cells (which includes monocyte-derived
macrophages) was unchanged in the ipsilateral hemisphere following AMD3100 treatment, and that cognitive and motor functions were not negatively affected.

**Conclusions**

Subacute AMD3100 treatment was not capable of improving structural and functional outcomes after HIE in rats. Further studies are required to investigate the efficacy of other doses and timing of AMD3100 administration in HIE, but using a 5 mg/kg/d dose in the subacute time window might not be recommended. Interestingly, a publication becoming available after our study was concluded did not show a beneficial impact of AMD3100 on lesion size in a HIE model in mice. Treatment was initiated on the third day after the insult, at 5 mg/kg/d for 3 days. Moreover, therapeutic impact on motor function was only seen when applying a combination treatment with insulin-like growth factor 1. Another strategy deserving further investigation is the pharmacological combination of AMD3100 with cobalt chloride or β3 adrenergic agonists in order to increase the mobilization of mesenchymal stromal cells from BM, a cell population with a promising protective role in HIE. Thus, it may be assumed that the subacute treatment with AMD3100 alone, despite clear evidence for therapeutic benefits in other forms of adult hypoxic-ischemic brain injury, may not be ideally suited for the treatment of HIE for so far unknown reasons.

**Acknowledgments**

DAS, RMPC, LC and ND performed experiments, analyzed the data, discussed results, corrected and reviewed the manuscript. JB designed and discussed the experimental plan, discussed results, and wrote the manuscript. RM-O provided financial support, discussed the
experimental plan, discussed results, corrected and reviewed the manuscript. PMP-C conceived and designed the study, performed experiments, analyzed data, provided financial support, supervised the work and wrote the manuscript.

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Conflict(s)-of-Interest(s)/Disclosure(s)

None.

Supplemental Materials

- Supplemental Methods
- Supplemental Figures I-III
- Detailed Statistical Results
- Supplemental Tables I-VI
- Preclinical Checklist

References


44. Capoccia BJ, Shepherd RM and Link DC. G-CSF and AMD3100 mobilize monocytes into the blood that stimulate angiogenesis in vivo through a paracrine mechanism. _Blood._ 2006;108:2438-2445.


Figure Legends

Figure 1. Experimental design. (A) In step I, hypoxic-ischemic animals were treated with two subcutaneous injections of the vehicle (HIE + Vehicle) and their brains were analyzed 7 days after the injury. (B) In Step II, hypoxic-ischemic animals were treated with two subcutaneous injections of AMD3100 or with the vehicle (HIE + AMD3100 or HIE + Vehicle, respectively), whereas animals from the sham group were treated with the vehicle (Sham + Vehicle). Behavioral assessments were performed on days 17 (T-water maze task) and 18 (Rotarod) after surgery, and the brains were analyzed on day 21.

Figure 2. Exploratory step I. Hypoxic-ischemic animals were treated with two subcutaneous injections of the vehicle and their brains were analyzed 7 days after the injury. (A) Graphs showing the quantification of brain atrophy in five rostrocaudal levels in the coronal plane. Data shown in the graphs are individual values and means ± SD; n=6-7 animals per group. Males: light symbols; females: dark symbols. (B) Representative photomontages of coronal brain sections stained with thionine. Scale bar: 5 mm.

Figure 3. Step II: Cognitive and motor outcomes. Hypoxic-ischemic animals were treated with two subcutaneous injections of AMD3100 or with the vehicle (HIE + AMD3100 or HIE + Vehicle, respectively), whereas animals from the sham group were treated with the vehicle (Sham + Vehicle). (A) Graphs showing the latency to find the hidden platform during each trial of the T-water maze task performed 17 days after the sham procedure or the hypoxic-ischemic insult. (B) Graphs showing the latency to find the platform in the testing trials of the T-water maze task (mean of trials 2-4). (C) Graphs showing the latency to fall in the rotarod test.
performed 18 days after the sham procedure or the hypoxic-ischemic injury. Motor coordination
deficits in male hypoxic-ischemic rats were not prevented by AMD3100. Two-way repeated
measures ANOVA with Tukey's post-hoc test (A), and Kruskal-Wallis test with Dunn's multiple
comparisons test (B, C) were used. Data shown in the graphs are means ± SD; *p<0.05; n=23-30
animals per group. Males: light symbols; females: dark symbols.

Figure 4. Step II: AMD3100 does not change brain atrophy in hypoxic-ischemic rats.
Hypoxic-ischemic animals were treated with two subcutaneous injections of AMD3100 or with
the vehicle (HIE + AMD3100 or HIE + Vehicle, respectively) and their brains were analyzed 21
days after the injury. Graphs show the quantification of brain atrophy in five rostrocaudal levels
in the coronal plane. Data shown in the graphs are individual values and means ± SD; Mann-
Whitney U test was used; n=24-30 animals per group. Males: light symbols; females: dark
symbols. Representative photomontages of coronal brain sections stained with thionine are
shown. Scale bars: 5 mm.
**A**

- **surgery**
- daily injection of the vehicle
- transcardial perfusion

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**B**

- surgery
- daily injection of AMD3100 or of the vehicle
- T-water maze
- rotarod
- transcardial perfusion

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