Adenosine A1R/A3R Agonist AST-004 Reduces Brain Infarction in a Nonhuman Primate Model of Stroke

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ABSTRACT

Background and Purpose Treatment with adenosine A1 or A3 receptor (A1R/A3R) agonists in rodent models of acute ischemic stroke (AIS) results in significantly reduced lesion volume, indicating activation of adenosine A1R or A3R is cerebroprotective. However, dosing and timing required for cerebroprotection has yet to be established, and whether adenosine A1R/A3R activation will lead to cerebroprotection in a gyrencephalic species has yet to be determined.

Methods The current study employed clinical study intervention timelines in a nonhuman primate model of transient, 4 hour middle cerebral artery occlusion (tMCAO) to investigate a potential cerebroprotective effect of the dual adenosine A1R/A3R agonist AST-004. Bolus and then 22 hours intravenous infusion of AST-004 was initiated 2 hours after tMCAO. Primary outcome measures included lesion volume, lesion growth kinetics, penumbra volume as well as initial pharmacokinetic-pharmacodynamic relationships (PK/PD) measured up to five days after tMCAO. Secondary outcome measures included physiological parameters and neurological function.

Results Administration of AST-004 resulted in rapid and statistically significant decreases in lesion growth rate and total lesion volume. In addition, penumbra volume decline over time was significantly less under AST-004 treatment compared to vehicle treatment. These changes correlated with unbound AST-004 concentrations in the plasma and cerebrospinal fluid as well as estimated brain A1R and A3R occupancy. No relevant changes in physiological parameters were observed during AST-004 treatment.

Conclusions These findings suggest that administration of AST-004 and combined A1R/A3R agonism in the brain are efficacious pharmacological interventions in AIS and warrant further clinical evaluation.

Nonstandard Abbreviations and Acronyms

A1R, adenosine A1 receptor
A2aR, adenosine A2a receptor
A2bR, adenosine A2b receptor
A3R, adenosine A3 receptor
AAALAC, American Association for Accreditation of Laboratory Animal Care
ADC, apparent diffusion coefficient
AIS, acute ischemic stroke
AR, adenosine agonist
ASL, arterial spin labeling
CBF, cerebral blood flow
COMP, composite of all drug-treated groups
CSF, cerebrospinal fluid
Cmax, maximum concentration
DWI, diffusion-weighted imaging
Emax, maximum effect
FLAIR, fluid-attenuated inversion-recovery
HE, hemotoxylin-eosin staining
HERMES, Highly Effective Reperfusion Using Multiple Endovascular Devices
MABP, mean arterial blood pressure
MRI, magnetic resonance imaging
MCA, middle cerebral artery
MCAO, middle cerebral artery occlusion
MRA, magnetic resonance angiography
NDS, neurological deficit score
NHP, non-human primate
PEG, polyethylene glycol
PI, prediction interval
PK/PD, pharmacokinetics/pharmacodynamics
r-tPA, recombinant tissue plasminogen activator
RO, receptor occupancy
SEM, standard error of the mean
SMTP-7, Stachybotrys microspora triprenyl phenol-7
STAIR, Stroke Treatment Academic Industry Roundtable
tMCAO, temporary middle cerebral artery occlusion

INTRODUCTION

Current acute ischemic stroke (AIS) therapy is limited to recanalization by thrombolysis or thrombectomy.[1] These therapies focus on restoring blood flow and oxygenation of hypoperfused tissue. Thrombolytics, however, can only be given to <5% of AIS patients within a limited time window post-occlusion, while thrombectomy requires access to the site of occlusion and is currently utilized in less than 20% of AIS patients.[2]

Pharmacotherapy that is both cerebroprotective and administerable to a majority of AIS patients in conjunction with recanalization is a major unmet need.[3] The vast majority of previous preclinical neuroprotection programs focused on efficacy evaluations in rodent models, with limited insights into drug distribution, target engagement and pharmacological response, perhaps inevitably leading to neutral or negative findings during clinical trials.[4] For these reasons, the international Stroke Treatment Academic Industry Roundtable (STAIR) has issued detailed guidelines on preclinical testing of potential AIS therapies, including evaluation of efficacy in both lyssencephalic and gyrencephalic species.[5] To date, the authors are only aware of the postsynaptic density protein-95 inhibitor nerinetide and the plasminogen modulator SMTP-7 that fulfilled STAIR guidelines, being evaluated in both rodent and nonhuman primate (NHP) stroke models, prior to initiation of clinical trials.[6-9]

In the current study, we followed STAIR recommendations in evaluating the effect of AST-004, a combined adenosine A1R/A3R receptor agonist, in a cynomolgus monkey model of transient middle cerebral artery occlusion (tMCAO).[10-12] In rodent stroke models, activation of central adenosine A1R or A3R leads to robust cerebroprotection, as defined by smaller brain lesion volumes compared to vehicle treatment.[13-20] However, previous rodent studies have utilized treatment prior to initiation of the occlusion which questions translatablity of the findings to the clinical setting.[13]

The current study was designed to closely follow the average timelines for clinical study intervention in human AIS patients described in the HERMES (Supplementary Figure IA).[21] Significant efficacy in terms of lesion growth rate inhibition and overall lesion size reduction were observed while no adverse effects on core physiological parameters became evident. Furthermore, clear relationships were observed between the observed efficacy and AST-004 cerebrospinal fluid and plasma drug concentrations as well as estimated brain A1R/A3R receptor occupancy.

METHODS

Comprehensive details for all methods are provided in the online supplements. Study design is depicted in Supplementary Figure IB.

Animals

All procedures were approved by the Hamamatsu Pharma Research, Inc. Animal Care and Use Committee (approval number: HPRIRB-444) and were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Science, 2011). The study facility is accredited by AAALAC International. A total of 25 adult male *Macaca fascicularis* macaques were used in this study, with ages ranging from 47-83 months and weights ranging from 2.7-5.4 kg (Supplementary Table I).

The study was carried out in completely randomized (despite replacement subjects, see below) and blinded fashion. Subjects were randomized prior to treatment induction. Allocation concealment was maintained throughout study as separate blinded investigators were utilized for MCAO surgery, treatment induction and subsequent imaging and efficacy endpoint analyses. This was the first efficacy study of its type utilizing this primate model of transient MCAO with extensive imaging endpoints. Thus, we could not anticipate the size of potential treatment outcomes that would be required for a priori power and sample size calculations. The data generated in this exploratory study will allow us to conduct power and size calculations for future efficacy studies.

Physiological parameter assessment

Body weight, core body temperature, mean arterial blood pressure (MABP), heart rate, pO_2, pCO_2, sO_2 and blood pH were assessed prior to and after tMCAO at designated intervals (Supplementary Tables II-VIII) in relation to reported normal physiological ranges for each parameter.[22, 23]

Transient middle cerebral artery occlusion and reperfusion

Transorbital transient MCA occlusion (tMCAO) was performed using 2 microvascular clips, one placed on the proximal part of the main MCA trunk and the other on the distal-to-orbitofrontal branch (Supplementary Figure II).[10, 11, 24] Four hours after MCA occlusion, these clips were removed for recanalization. After visual confirmation of restituted MCA blood flow, the burr hole was closed using Clearfil New Bond (Kuraray Noritake Dental, Inc., Tokyo, Japan) and the orbital cavity was closed according to best veterinary practice.

Magnetic resonance imaging

Serial coronal magnetic resonance imaging (MRI) of the brain (3 mm slice thickness) was performed 0.5, 1.5, 1.8, 3.5, 6.0, 24 and 120h post-occlusion. Imaging sequences were (i) diffusion-weighted imaging (DWI), arterial spin labeling (ASL), (ii) magnetic resonance angiography (MRA), and (iii) fluid-attenuated inversion-recovery (FLAIR) T2-weighted imaging. Apparent diffusion coefficient (ADC) maps, cerebral blood flow (CBF), and perfusion deficit were generated with FuncTool Performance (GE Healthcare, Milwaukee, WI, USA) available on the MRI scanner console. Inhibition of lesion volume was considered the primary efficacy endpoint. Penumbral volume (mm^3) was calculated by subtracting the lesion volume delineated from the DWI diffusion maps from the total calculated perfusion deficit.

Drug administration and pharmacokinetic sampling

Macaques received a bolus dose of vehicle (40% PEG400 in 0.9% saline) or AST-004 into a saphenous vein 2h after occlusion (2h prior to restoration of MCA blood flow), followed immediately by a 22h intravenous infusion. This dosing regimen was designed to rapidly achieve and maintain pre-determined plasma and cerebrospinal fluid (CSF) steady-state concentrations of AST-004 based on its pharmacokinetics previously determined in naïve and MCA occluded macaques (Table 1, Supplementary Tables IX and X). During MRI, vehicle or AST-004 were infused into the saphenous vein via a syringe pump (TOP5500E, TOP Corp., Tokyo, Japan). Infusion following MRI was maintained via a portable, programmable iPrecio® Dual infusion pump (DMP-100; Primetech Corp., Tokyo, Japan) ported into a jugular venous line, both being secured in a jacket. Methods for pharmacokinetic sampling and bioanalytical analysis of plasma and CSF are described in the Supplement.

Neurologic deficit assessment

Twenty-four hours and 5 days after occlusion, neurologic deficits were scored using the Neurologic Deficits Score (NDS, Supplementary Table XI) as described elsewhere.[25] The NDS was considered as a secondary efficacy endpoint.

Exclusion criteria and replacement subjects

Exclusion criteria are fully described in Supplemental Methods and based on comparison of infarct volumes to 90% prediction intervals (PI) generated from lesion volumes in preliminary studies. Based on these exclusion criteria, two macaques were found to have infarct volumes outside the 90% PI and were excluded and replaced. Subjects that died during the study were also replaced. Three subjects died following complications from MCAO surgery and were replaced (Supplemenatary Table XII). Other exclusion criteria comprised general health limitation prior to study induction and violation of species-specific ranges of physiological parameters on 3 consecutive time points. No animals needed to be excluded based on these criteria.

Statistics

The mean and standard error of the mean (SEM) were calculated using Microsoft Excel 2016 (Microsoft Corporation). Statistical analyses were performed using SAS Analytical Pro version 9.4 (SAS Institute, Tokyo, Japan) and EXSUS version 8.1 (CAC Croit Corp., Tokyo, Japan). Additional statistical analyses were performed with Prism 4.02 (GraphPad Software, San Diego, CA). Please see Supplemental Material for details. Prior to the t-test of the composite and control groups, the F-test was conducted to confirm equal variances between the control and composite groups. Then the t-test, equal variances, was conducted on the composite versus the control group and if positive for the endpoint, the individual dose groups were examined and discussed for relevance. These individual analyses will not alter a conclusion about the statistical significance of the composite and are considered descriptive analyses, not tests of hypotheses. Statistical significance was set at $p<0.05$, with trends towards statistical significance defined as $0.05 \leq p < 0.1$.

RESULTS

Physiological parameters and baseline measurements

Physiological parameters were measured prior to tMCAO (baseline) and throughout the study. There were no clinically relevant differences between vehicle-treated and AST-004-treated groups for any parameter at baseline or during the study period (Supplementary Tables I-VIII). All parameters predominantly stayed within normal physiological ranges with occasional minor and transient deviations, not triggering pre-set exclusion criteria.

AST-004 slows ischemic lesion growth

AST-004 administration resulted in a rapid decrease in lesion growth rate (i.e. decreased slope) compared to both vehicle and pre-AST-004 treatment growth rates. The slope of lesion growth was calculated as a measure of lesion growth rate, comparing the linear phases of the lesion growth curve during the pre-drug initiation (0.5-1.8h) and post-drug initiation (1.8-6.0h) periods (Figures 1A, B). During the pre-drug initiation period, slopes of lesion growth were not different between vehicle- and AST-004-treated groups. However, after initiation of AST-004 treatment, the composite group slope was less than that of the vehicle group ($p=0.004$). The slopes of lesion growth for AST-004 dose groups were smaller than that of the vehicle-treated group with statistical significance achieved in the Mid and High dose groups ($p=0.02$), and with a trend ($p=0.06$) observed in the Low dose group. In addition, when the post-drug slopes of lesion growth were compared to their own pre-drug slopes, the rate of increase of the composite group after AST-004 treatment was significantly less than the rate of increase before AST-004 treatment ($p<0.0001$). Similar results were observed for the Mid and High dose groups ($p<0.002$ and $p<0.009$, respectively; Figure 1C). These data suggested that AST-004 activation of the adenosine A1 and A3 receptors led to a significant reduction in the rate of lesion growth following tMCAO.

AST-004 preserves the penumbra and reduces overall stroke volumes

Initial measures of cerebral perfusion deficits were determined at 0.5h post-occlusion (Supplementary Figure III). As assessed by either <30% or <50% contralateral cerebral blood flow[11, 26-28], initial MCAO perfusion deficits were not significantly different between vehicle-treated or any AST-004-treated dose group ($p=0.63$-0.97).

In general, the penumbra volume (as calculated by 30% perfusion deficit minus lesion volume) decreased during the ischemic period across all groups (Figure 2). In the vehicle group, penumbra volume decreased by an average of 71% (at a rate of 604.5 mm^3/h). In contrast, the AST-004-treated composite decreased by 48% (at a rate of 296.0 mm^3/h; $p=0.01$). Retention of penumbra volume was greatest in the Low dose group ($p=0.001$) compared to the vehicle group. The other AST-004 treatment groups showed lower mean penumbra decrease rates compared to the vehicle group but high intersubject variability may have prevented statistical significance in the Mid and High dose groups ($p\geq0.3$).

The reduction in the rate of lesion growth with AST-004 treatment resulted in a significant inhibition of overall lesion volume as measured by DWI (Figures 3A, B). At 24h post-occlusion, overall lesion volume tended to be 20% smaller in the AST-004 composite compared to vehicle treatment ($p=0.07$; Figure 3C). However, overall lesion volume of the AST-004 composite was 30% smaller than that of the vehicle group 120h post-occlusion ($p=0.05$). Furthermore, the greatest inhibition of overall lesion volume was observed with Mid ($p=0.04$) and High ($p=0.02$) treatment

(Figure 3D). Overall lesion volume findings at 120h were confirmed by histological lesion volume assessed in HE-stained brain sections (Figure 4, Supplementary Figure IV).

AST-004 plasma and CSF pharmacokinetics and pharmacodynamics

The AST-004 dose levels in this study were designed to target specific multiples of plasma and CSF concentrations of AST-004 and associated estimated brain adenosine A1 and A3 receptor occupancy, based on previous analyses of the pharmacokinetics of AST-004 in naïve and tMCAO monkeys (Supplementary Table IX). Using the combined bolus/infusion regimen, average plasma and CSF concentrations of AST-004 were within 2-fold of targets at all dose levels (Table, Figures 5A, B and Supplementary Table X) and remained within throughout the infusion period. Plasma concentration-time analyses confirmed the advantage of this dosing regimen to maintain targeted concentrations compared to a single, intravenous bolus in which AST-004 plasma concentrations were below bioanalytical limits of quantitation 8h post-dose. There was a good correlation between AST-004 plasma and CSF concentrations, indicating a plasma/CSF ratio of approximately 10 (Figure 5C).

Comparisons of average AST-004 plasma and CSF concentrations to the primary efficacy endpoint of %inhibition of lesion volume demonstrated a linear relationship when analyzed by semi-logarithmic E_{max} plots (Figure 5D). These analyses also demonstrate a linear relationship between lesion volume inhibition and brain A1R/A3R occupancy when plotted on an E_{max} curve (Figure 5E).

Secondary endpoint

The current study did not have the appropriate power and duration to statistically assess neurological deficits following tMCAO. However, preliminary assessments were performed 1 and 5 days post-occlusion to identify any potential trends in neurological function. From study Days 1 to 5, improved but statistically non-signicant (p=0.11) neurological function was observed in the AST-004-treated composite compared to the vehicle-treated group (Supplemental Figure V).

Discussion

The current study demonstrated cerebroprotective efficacy of AST-004, a dual agonist for the human A1R and A3R[12], in a NHP model of 4h transient cerebral ischemia. Compared to vehicle treatment, AST-004 treatment reduced total infarct volume 24h and 5 days after MCA occlusion. In addition to reduced total infarct volume, AST-004 treatment reduced the rate of expansion of the infarct volume over time. Our findings suggest a cerebroprotective effect of AST-004, supported by the finding that penumbra volume decline was reduced under AST-004 treatment. In summary, the current findings suggest activation of A1R/A3R as a potential cerebroprotective strategy that could be utilized to prevent brain tissue necrosis and ultimately enhance functional outcome following an AIS.

Timely reperfusion of an occluded vessel will minimize brain tissue death and neurological impairment following AIS.[29] Although recanalization approaches such as thrombolysis by recombinant tissue plasminogen activator (r-tPA) or mechanical thrombectomy have revolutionized AIS treatment, they are restricted to relatively narrow time windows (less than 4.5 hours for r-tPA) and are restricted to selected patient populations exhibiting a large penumbra/core mismatch and accessible clots in operable large blood vessels.[29] A significant risk of cerebral hemorrhage is associated with delayed r-tPA treatment, and r-tPA is contraindicated for use in non-thrombotic strokes. This limits usage to a small percentage of stroke patients. A treatment that protects brain tissue from hypoxic insult and is not restricted to these narrow time windows would be of immense value in the treatment of stroke. Moreover, a treatment that has the potential to immediately slow penumbra decline before recanalization would widen the therapeutic time window for both thrombolysis and thrombectomy, increasing the number of eligible patients for these interventions and lowering the severity of AIS[3].

Basic considerations and pharmacokinetics

Extracellular brain adenosine concentrations significantly increase after the onset of ischemic stroke.[30, 31] Activation of the four G-protein-coupled adenosine receptors, A1R, A2aR, A2bR and A3R, plays important roles in both neuroprotection and neurodegeneration.[32] Activation of A2aR can lead to neurodegeneration through a glutamate receptor-mediated pathway. Activation of A2bR can lead to neurodegeneration through promotion of neuroinflammation, although research to date for this receptor is contradictory, with examples of both A2bR agonism and antagonism leading to cerebroprotection.[33, 34] Neuroprotective effects are observed following activation of A1R and A3R.[13-20, 35] Thus far, there has been limited progress in developing therapeutics based on high-affinity adenosine receptor agonism for AIS, for several reasons. First, data suggests A1R and A3R are susceptible to rapid desensitization by potent agonists including their endogenous ligand adenosine.[36, 37] Second, high-affinity, full A1R agonists have unacceptable peripheral cardiovascular side effects, including bradycardia and hypotension, due to vascular A1R activation.[38, 39] Moreover, clinical evaluations of high-affinity A3R agonists in AIS were likely limited by problematic chemical properties of previously synthesized nucleoside ligands, including poor brain distribution[40-43], low unbound brain concentrations preventing adequate target engagement[44, 45], as well as the aforementioned tendency to rapidly desensitize the receptor. An ideal AR agonist should exhibit excellent distribution in brain tissue and avoid potential adverse cardiovascular effects, for example with either lower-affinity or partial agonism, attributes that could also decrease the potential for receptor desensitization. Thus, the current study not only evaluated a potential cerebroprotective effect of AST-004, but also carefully monitored subjects for any possible adverse cardiovascular side effects following systemic administration.

AST-004 demonstrated good brain distribution, plus a high free fraction in both plasma and brain tissue. Cerebrospinal fluid drug concentration is an established proxy for unbound drug brain concentration that interacts with central receptor targets.[46, 47] The unbound brain concentrations and resulting brain receptor occupancy at the A1R and A3R can be estimated using receptor affinity data and simple mass action equations.[48, 49] In previous studies with neonatal pigs, we demonstrated that AST-004 CSF concentrations were equivalent to unbound AST-004 brain extracellular fluid concentrations as determined via in situ equilibrium dialysis probes.[35] Accordingly, during the 22h infusion of AST-004, sufficient CSF concentrations were available to provide coverage of central A1R and A3R in the macaque. Measurable concentrations of AST-004 were found in plasma 24h following termination of Mid and High dose infusions, suggesting prolonged presence of significant AST-004 concentrations in the brain.

AST-004 administration did not result in significant alterations in MABP or heart rate as would be expected with systemic administration of a full A1R agonist.[38, 39] In addition, no preset physiological criteria were violated for changes to body temperature, MABP pressure, heart rate, pO_2, pCO_2, sO_2 or pH. Occasional deviations of these parameters from the normal range could have been stroke- or procedure-related as such deviations were observed in both vehicle- and AST-004-treated macaques. These data suggest a lack of significant adverse side effects following sustained activation of A1R and A3R and further suggests that AST-004 can be safely evaluated in human patients.

AST-004-mediated cerebroprotection

The penumbra eventually turns into infarcted tissue if timely revascularization does not occur. Thus, preservation or decelerating the loss of viable tissue in the penumbra during occlusion is a key objective of contemporary cerebroprotective agents.[3] Potential cerebroprotective agents have been previously tested in rodent models of tMCAO, but few have examined the effects of these agents on the penumbra, due in part to limited access to small animal MRI needed to visualize the diffusion-perfusion mismatch. Studies in large animals, including NHPs, have mostly relied on final lesion volume as a neuroanatomical indicator of neuroprotection.[7, 24] The current study utilized the change in penumbra volume over time as an indication of cerebroprotection. The smaller infarct volumes observed at 24h and 5 days following AST-004 treatment appear as reductions in the rate of penumbra volume loss over time. Indeed, reduced penumbra volume loss in the composite and Low dose groups ($p<0.01$) were observed. While there were lower mean differences in penumbra volume loss in all other AST-004-treated groups, these differences were not statitistically significant. Nonetheless, the data obtained are very encouraging, but could require larger cohorts for verification.

Decreased lesion growth rates are strong indicators of cerebroprotection. After occlusion but prior to treatment (0.5h to 1.8h post-occlusion, before infusion), the rates of lesion growth between vehicle- and AST-004-treated groups were similar. However, compared to vehicle treatment, the rates of lesion growth after the onset of AST-004 treatment were significantly lower. Decreased rates of infarct growth, in turn, resulted in significantly smaller infarct volumes at Day 5. Future studies using higher resolution in vivo imaging studies or absolute quantification of cerebral blood flow could identify specific neuroanatomical regions in the penumbra during tMCAO that benefit from therapeutic intervention.[50]

There is high sequence homology between NHP and human A1R and A3R[51], and the affinity of AST-004 for the human A1R and A3R is similar[12], so it is expected that the calculated receptor occupancy values should be highly similar for both adenosine receptors in the NHP and human

brain. On E_{max} plots of effect (%inhibition of lesion volume) versus matrix concentrations or estimated receptor occupancy, a linear relationship was observed across the AST-004 dose regimens utilized in this study. Since E_{max} relationships are typically sigmoidal, these linear relationships present the intriguing possibility that the maximum efficacy of AST-004 was not achieved in this study. Further studies will be needed to determine whether even higher levels of AST-004 efficacy can be achieved in the NHP tMCAO model and to establish at what matrix concentrations and receptor occupancies E_{max} is reached.

Limitations

Several limitations of the current study should be noted. First is the relatively small sample size. We chose the minimum sample in an attempt to reduce the number of animals used. Nevertheless, statistically significant positive outcomes were achieved in the primary outcome measure, suggesting a relatively large effect size. Second, there was high intersubject variability in many readout parameters. This may be due to the small sample size in treatment subgroups in combination with the relatively large scatter of individual data that may have 'masked' statistical significance. A larger scatter of data is typical for large animal models, which draw from a mixed population, and well reflects clinical reality.[52] Thus, we may assume that external validity of our study was good, although this means at the same time that certain endpoints may require larger group sizes for thorough assessment. Third, the overall observation period was relatively short. Additional changes may have occurred in some endpoints including the NDS in the long-term, but primary and other MRI-based key efficacy outcomes have benefitted from the chosen setup. Confirmation of our results in a larger cohort and primarily targeting functional outcome and long-term lesion organization may be warranted.

Conclusion

To summarize, the current study investigated the effect of A1R/A3R activation via AST-004, a novel adenosine A1R/A3R receptor agonist, in a NHP model of AIS with recanalization. Efficacy was observed in key outcome measures including rate of infarct volume growth, total lesion volume and retention of penumbral volume. Efficacy observed in these neuroanatomical outcome measures paralleled AST-004 concentrations in plasma and CSF and, furthermore, align with estimated brain A1R/A3R receptor occupancy. Importantly, the receptor occupancy estimates associated with efficacy in this nonhuman primate model can be utilized to identify human clinical trial dose levels that yield similar levels of receptor occupancy, thus increasing the potential for translation in human stroke trials and ensuring that the pharmacological approach has been fully evaluated. These findings warrant further preclinical and clinical investigation of A1R and A3R activation as a novel cerebroprotective strategy. The positive outcome regarding safety parameters also warrant early-stage clinical safety and efficacy testing in human patients.

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DISCLOSURES

T.E.L., R.B.P. and W.S.K are employees of Astrocyte Pharmaceuticals, Inc. J.D.L. is a co-founder of Astrocyte Pharmaceuticals, Inc. A.H., I.H., N.T. and H.T. are employees of Hamamatsu Pharma Research. J.B. received compensation for consultancy services during the planning of the study and interim data analyses.

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Figures legends

Figure 1. Effect of AST-004 treatment initiation on the slope of DWI lesion growth following tMCAO in nonhuman primates.
(A) Slopes of MCAO lesion growth prior to initiation of vehicle or AST-004 treatment. **(B)** Slopes of MCAO lesion growth following vehicle or AST-004 treatment. **(C)** Comparison of lesion growth slopes before and after initiation of vehicle or AST-004 treatment 2 hours post-occlusion. Results represent mean±SEM, n=4/dose group and n=16 for the composite ("Comp")of all AST-004-treated groups combined. Pre-treatment slopes of lesion growth were determined for each subject from 0.5-1.8 hours post-occlusion. Initial post-treatment slopes were determined from 1.8-6.0 hours post-occlusion to provide sufficient timepoints for slope determination. Unpaired t-test comparing each AST-004 treatment group to vehicle group (Panels A, B) and paired t-test comparing each group to itself before and after treatment.

Figure 2. Percent change in penumbra volume following tMCAO and treatment with vehicle or AST-004.
Penumbra volume was calculated from the difference between cerebral perfusion deficit and lesion volume at a threshold of <30% contralateral cerebral blood flow from 0.5h to 3.5h post-occlusion. Results represent mean±SEM, n=4/dose group and n=16 for the composite of all AST-004-treated groups. Unpaired t-test comparing each AST-004 treatment group to vehicle group.

Figure 3. Comparison of DWI lesion volume growth between vehicle- and AST-004-treated subjects following tMCAO.
(A) Comparison of DWI lesion size and growth between vehicle-treated and a composite of all AST-004-treated subjects. **(B)** Comparison of DWI lesion size and growth between vehicle-treated and each AST-004-treated dose group. **(C)** Comparison of percent inhibition of DWI lesion volume at 24 hours post-occlusion. **(D)** Comparison of percent inhibition of DWI lesion volume at 120 hours post-occlusion. Results represent mean±SEM, n=4/dose group and n=16 for the composite of all AST-004-treated groups. Unpaired t-tests.

Figure 4. Comparison of representative DWI and HE-stained lesion images from vehicle- and AST-004-treated subject.
(A) Representative DWI images of lesions at 0.5h s and 120h post-occlusion in each vehicle- and AST-004 dose group. **(B)** Representative HE stained brain sections 120h post-occlusion. Shaded regions denote infarcted areas.

Figure 5. AST-004 plasma and CSF pharmacokinetics in non-human primates, and relationships between DWI lesion volume inhibition and average AST-004 unbound plasma concentrations, total CSF concentrations and estimated A1R/A3R brain receptor occupancy following MCAO.
(A) Plasma and **(B)** CSF pharmacokinetics. **(C)** Correlation between CSF and unbound plasma concentrations. Pharmacokinetics were determined following initiation of bolus/infusion regimen and compared to a reference intravenous bolus dose. **(D)** Relationship between %inhibition of lesion volume at final DWI measurement (120h) and unbound plasma concentrations (red), total CSF concentrations (blue). **(E)** Relationship between %inhibition of lesion volume at final DWI

measurement and estimated AST-004 brain receptor occupancy of adenosine A1 and A3 receptors (green). Results represent mean±SEM, n=4/dose group.

Table. Dose regimens, predicted plasma concentrations and resulting measured average plasma and CSF concentrations of AST-004.

Dose group	bolus/infusion dose regimen	predicted AST-004 *[Plasma] (ng/mL)	measured AST-004 *[Plasma] (ng/mL)	Measured AST-004 *[CSF] (ng/mL)
Very Low	0.11 mg/kg; 0.06 mg/kg/h, 22h	35	32±11	2.1±0.4
Low	0.47 mg/kg; 0.25 mg/kg/h, 22h	150	186±24	8.8±2.6
Mid	1.7 mg/kg; 0.9 mg/kg/h, 22h	540	483±23	16.8±5.7
High	5.2 mg/kg; 2.8 mg/kg/h, 22h	1660	1127±246	108±35

*Plasma and CSF concentrations are means±SEM of average concentrations over 22-hour infusion period, n=4/ dose group.

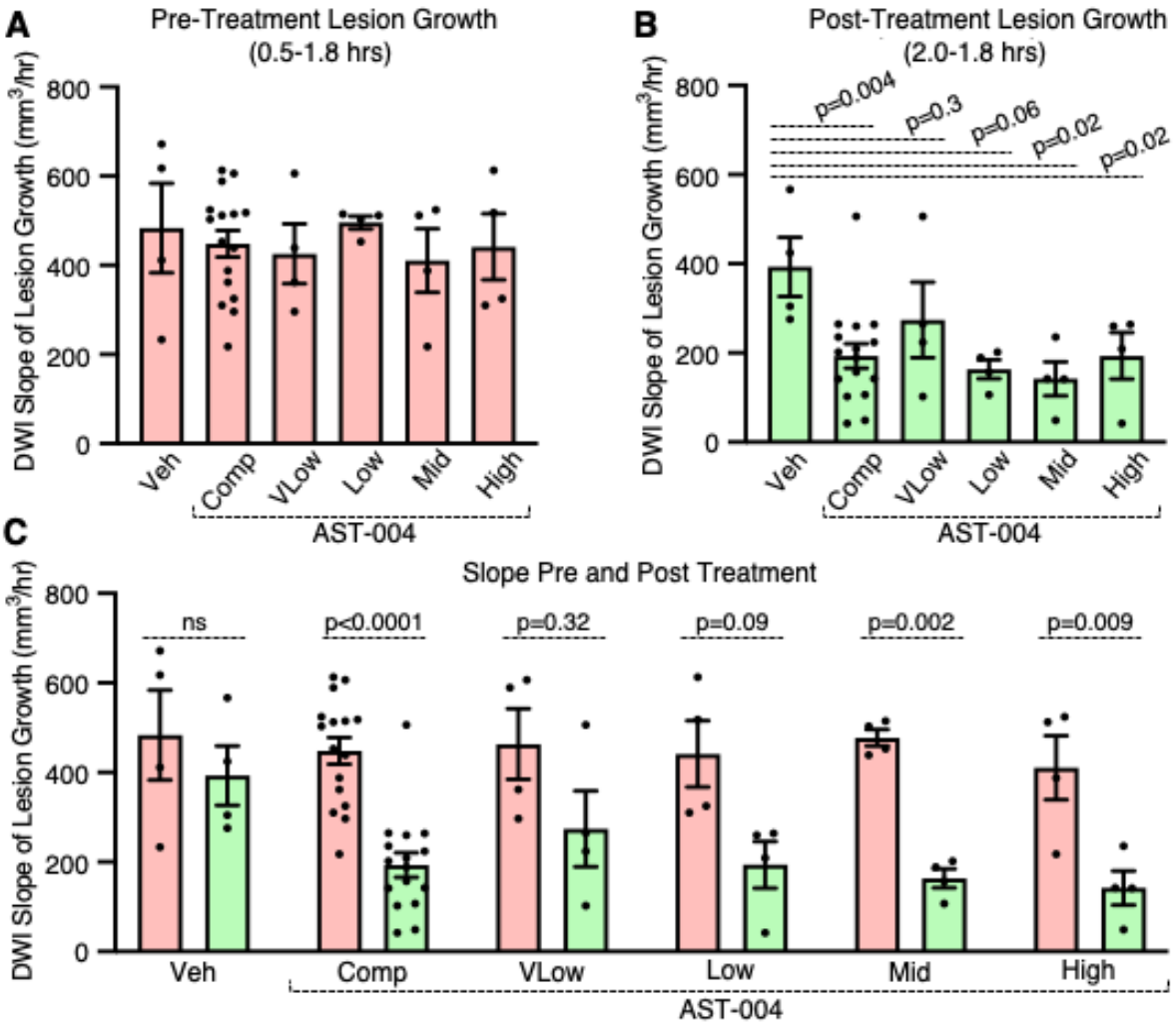
A
Pre-Treatment Lesion Growth
(0.5-1.8 hrs)
DWI Slope of Lesion Growth (mm3/hr)
800
600
400
200
0
Veh
Comp
VLow
Low
Mid
High
AST-004
B
Post-Treatment Lesion Growth
(2.0-1.8 hrs)
p=0.004
p=0.3
p=0.06
p=0.02
p=0.02
C
Slope Pre and Post Treatment
ns
p<0.0001
p=0.32
p=0.09
p=0.002
p=0.009

Figure 1

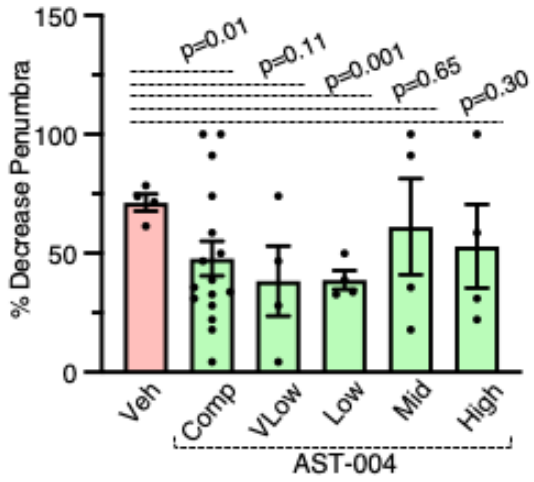

Figure 2.

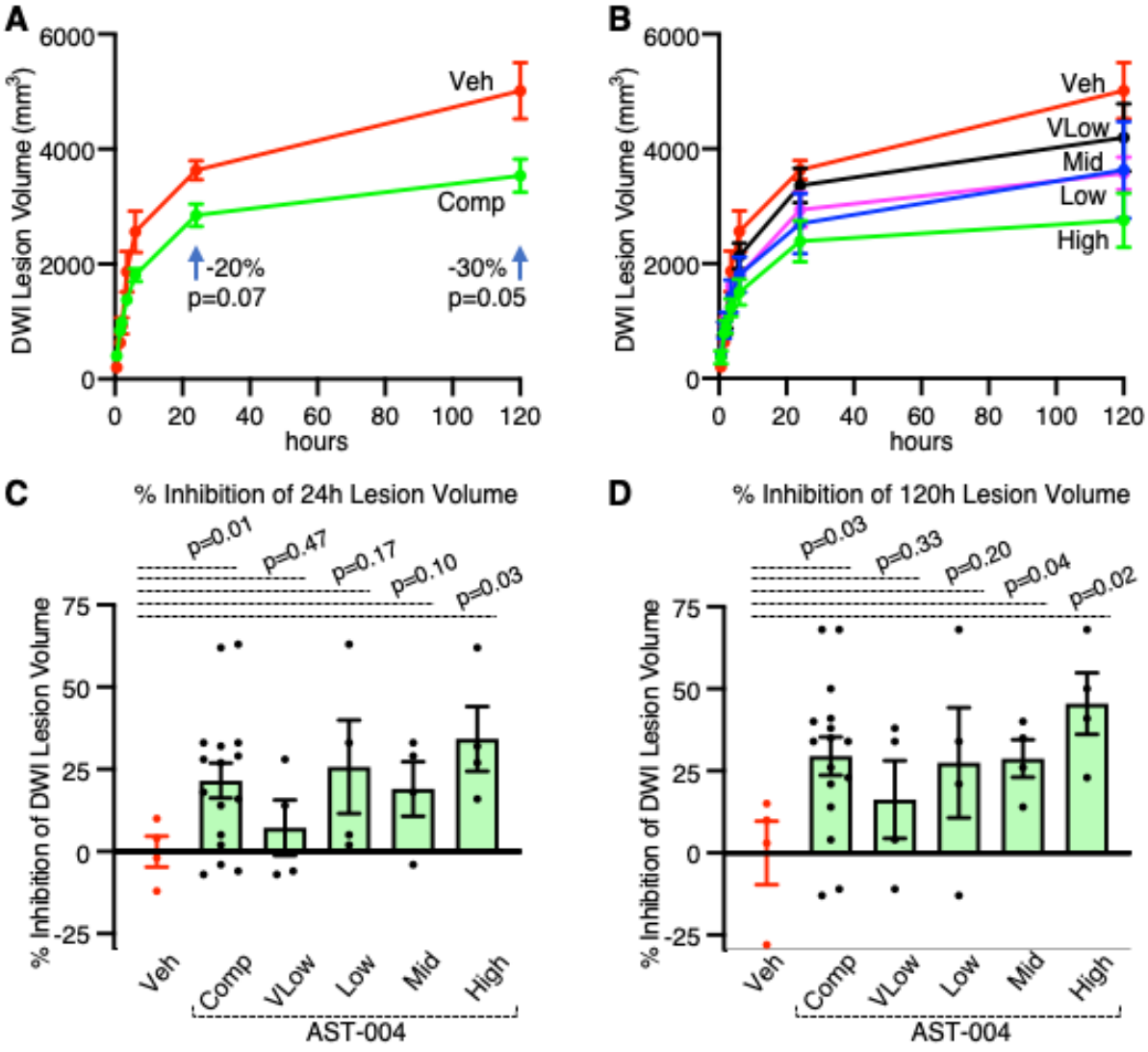
A
DWI Lesion Volume (mm3)
6000
4000
2000
0
Veh
Comp
-20%
p=0.07
-30%
p=0.05
0
20
40
60
80
100
120
hours
B
DWI Lesion Volume (mm3)
Veh
VLow
Mid
Low
High
hours
C
% Inhibition of 24h Lesion Volume
p=0.01
p=0.47
p=0.17
p=0.10
p=0.03
% Inhibition of DWI Lesion Volume
75
50
25
0
-25
Veh
Comp
VLow
Low
Mid
High
AST-004
D
% Inhibition of 120h Lesion Volume
p=0.03
p=0.33
p=0.20
p=0.04
p=0.02
% Inhibition of DWI Lesion Volume
AST-004

Figure 3

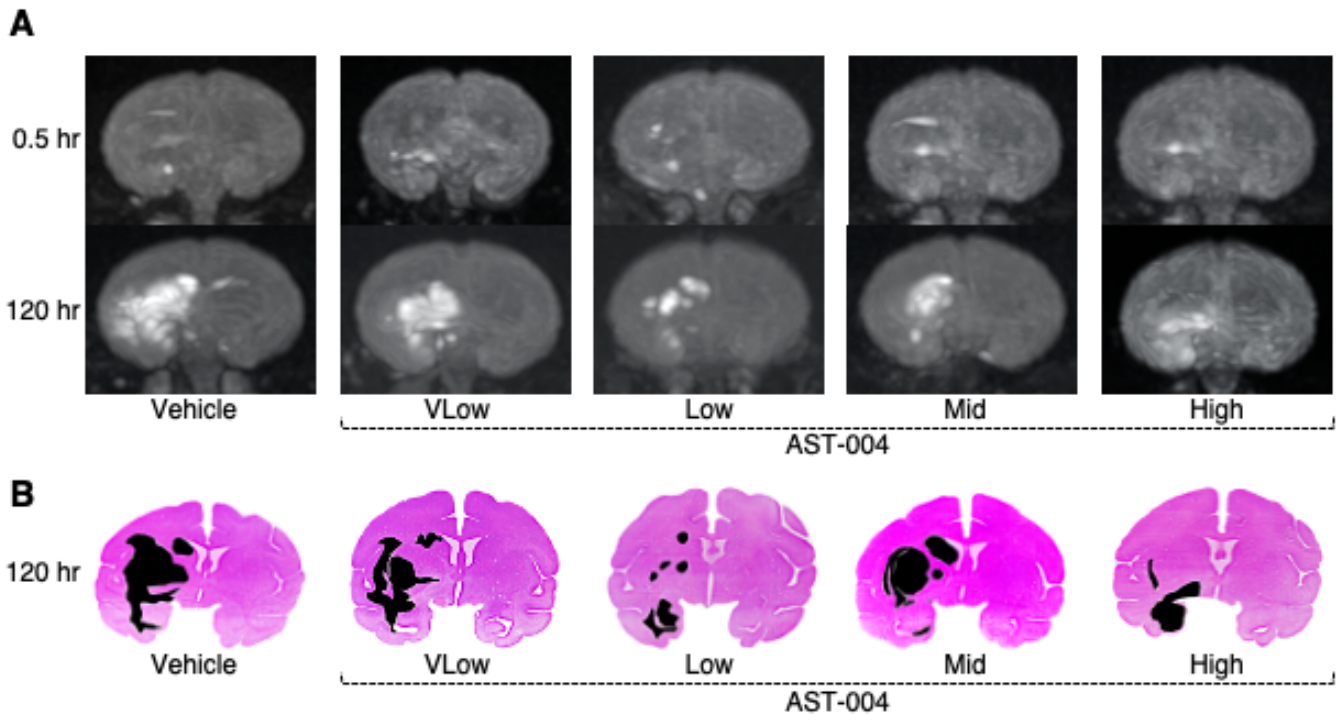
A
0.5 hr
120 hr
Vehicle
VLow
Low
Mid
High
AST-004
B
120 hr
Vehicle
VLow
Low
Mid
High
AST-004

Figure 4.

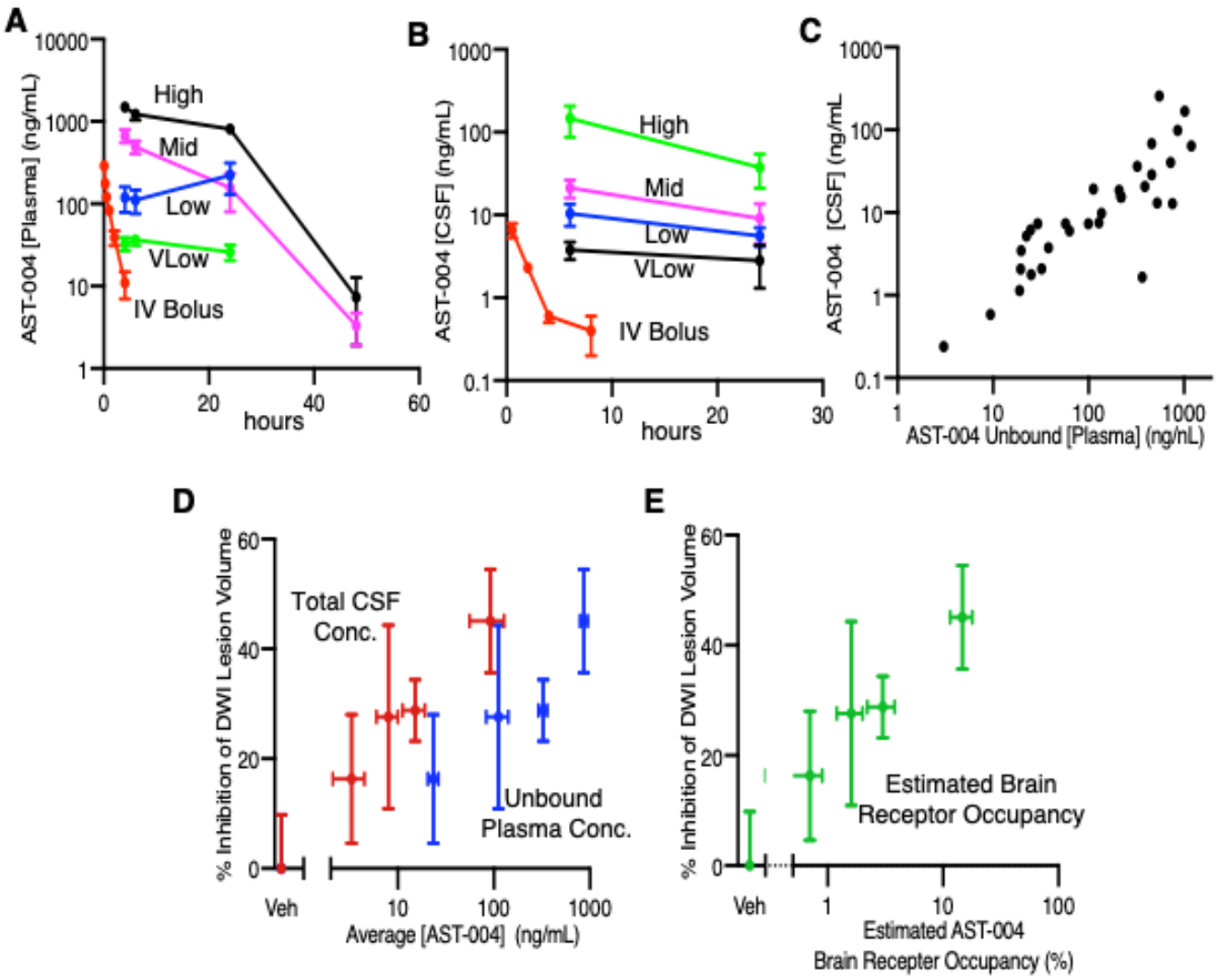
A
AST-004 [Plasma] (ng/mL)
10000
1000
100
10
1
High
Mid
Low
VLow
IV Bolus
0
20
40
60
hours
B
AST-004 [CSF] (ng/mL)
1000
100
10
1
0.1
High
Mid
Low
VLow
IV Bolus
0
10
20
30
hours
C
AST-004 [CSF] (ng/mL
1000
100
10
1
0.1
1
10
100
1000
AST-004 Unbound [Plasma] (ng/nL)
D
% Inhibition of DWI Lesion Volume
60
40
20
0
Total CSF
Conc.
Unbound
Plasma Conc.
Veh
10
100
1000
Average [AST-004] (ng/mL)
E
% Inhibition of DWI Lesion Volume
60
40
20
0
Estimated Brain
Receptor Occupancy
Veh
1
10
100
Estimated AST-004
Brain Recepter Occupancy (%)

Figure 5.