Asiatic Acid Attenuates Infarct Volume, Mitochondrial Dysfunction, and Matrix Metalloproteinase-9 Induction After Focal Cerebral Ischemia

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Background and Purpose—Asiatic acid (AA) has been shown to attenuate cerebral infarction in a mouse model of focal ischemia and shows promise as a neuroprotective stroke therapy. To facilitate translation of these findings to clinical studies, we determined pharmacokinetics, a dose–response relationship, the therapeutic time window, and efficacy using multiple stroke models. We also explored potential mechanisms of action.

Methods—Escalating doses of intravenous AA were administered and serum concentrations were measured at multiple time points for the pharmacokinetic studies. Subsequently, a dose–response relationship was determined followed by administration at different intervals after the onset of ischemia to establish a therapeutic time window for neuroprotection. Outcome measurements included both histological and behavioral. Mitochondrial function and matrix metalloproteinase activity in controls and treated rats were also determined.

Results—The pharmacokinetic studies showed that AA (75 mg/kg) has a half-life of 2.0 hours. AA significantly decreased infarct volume and improved neurological outcome even when administration was at time points up to 12 hours after the onset of ischemia. Infarct volume was also significantly decreased in female rats and spontaneously hypertensive rats. AA attenuated mitochondrial dysfunction and reduced matrix metalloproteinase-9 induction.

Conclusions—Our study shows AA is effective against multiple models of focal ischemia, has a long therapeutic time window, and is also effective in females and hypertensive animals. AA may mediate neuroprotection by protecting mitochondria and inhibiting matrix metalloproteinase-9 induction and activation. Taken together these data suggest that AA is an excellent candidate for development as a stroke therapy. (Stroke. 2012;43:1632-1638.)

Key Words: asiatic acid ■ matrix metalloproteinase-9 ■ mitochondria ■ neuroprotective ■ stroke

Stroke is a leading cause of mortality and the largest single cause of adult disability worldwide.1 Treatment with tissue-type plasminogen activator, the only approved pharmacological therapy, is limited by its narrow time window and the risk of hemorrhagic complications. A plethora of neuroprotectants have failed to translate to clinically effective therapies and a desperate need exists for new therapies.2

Asiatic acid (AA) is a triterpene isolated from Centella asiatica, which has been widely used as an antioxidant and anti-inflammatory agent.3 AA has also been shown to display robust neuroprotective properties both in vitro and in vivo and shows promise as a novel therapeutic agent for acute stroke therapy.4-7

The Stroke Therapy Academic Industry Roundtable (STAIR)8 has published recommendations for preclinical testing of candidate stroke therapies. These recommendations include determining: a dose–response relationship, a therapeutic time window, and efficacy in multiple models including permanent and transient ischemia, female animals, aged animals, and animals with comorbidities. Efficacy in both behavioral and histological time points was also recommended.

Previously, we reported that AA is neuroprotective in a mouse model of permanent focal ischemia and protects the integrity of the blood–brain barrier (BBB) after ischemia.7 In accord with STAIR guidelines, we have now tested AA in...
multiple stroke models using both histological and functional outcomes and established a dose–response relationship and a therapeutic time window. We also investigated the mechanism of action and provide evidence of reduced mitochondrial damage and matrix metalloprotease inhibition.

Materials and Methods

Animals
Adult male Sprague-Dawley (SD), female SD rats, and male spontaneously hypertensive rats (SHR) weighing 250 to 300 g were used. All experiments were performed in accord with the National Institutes of Health Policy and Animal Welfare Act under the approval by Institutional Animal Care and Use Committee at Michigan State University.

Drugs
AA was dissolved to a concentration of 50 mg/mL in ubisol-Aqua (Zymes LLC, Hasbrouck Heights, NJ). The lateral tail vein was used for the administration of AA or ubisol-Aqua (vehicle).

Blinding and Randomization
Treatment groups were allocated in a randomized fashion using a Researcher Randomizer (www.randomizer.org/). Investigators were blind to the allocation of treatment when doing surgeries and doing outcome evaluations.

Justification for the Ischemia Models Used in These Studies
There is no ideal stroke model that completely reflects clinical stroke in humans. We used both permanent (pMCAO) and transient (tMCAO) middle cerebral artery occlusion models using a filament to occlude the middle cerebral artery through the carotid (see the online-only Data Supplement). The pMCAO models the clinical situation of occlusion of the cerebral vessel without recanalization. The tMCAO models the recanalization of a previously occluded cerebral vessel either spontaneously or with treatment.9 Because stroke can involve both situations, we tested both models.

Experimental Groups
Detailed methods of blood sampling and pharmacokinetics studies, surgery and induction of ischemia, neurological score, behavioral testing, calculation of infarct volume using triphenyltetrazolium chloride and cresyl violet staining, brain mitochondrial isolation, respiration measurements, in vivo matrix metalloprotease (MMP) level measurements, and statistical analyses are available in the online-only Data Supplement.

Experiment 1: Determination of Pharmacokinetics and Safety/Tolerability of AA
We sought to determine pharmacokinetics and safety/tolerability of AA. Male SD rats (9–10 weeks) were randomly divided into 4 groups (n=7 rats/group) and given a single intravenous bolus injection of vehicle or 10, 25, and 75 mg/kg AA. Blood samples were drawn before administration and at 15 minutes, 1 hour, 3 hours, 6 hours, 12 hours, and 24 hours postadministration. The concentration of AA in serum at each time point was measured using high-performance liquid chromatography/mass spectroscopy. Rats were allowed to survive for 14 days. For safety and tolerability evaluations, the daily assessment occurred of the following: (1) food consumption; (2) body weight; (3) activity; and (4) mortality. All animals underwent blood analysis for chemistry and hematology. In a separate set of experiments, AA or vehicle was administered to rats (n=4 per group) and blood pressure was monitored for 24 hours.

Experiment 2: Determination of a Dose–Response Relationship
We sought to determine the dose–response relationship and the therapeutic time window in healthy young males in both pMCAO and tMCAO models.

Male SD rats were randomly assigned to treatment with AA (50–75 mg/kg) or vehicle at 30 minutes (AA 50 mg/kg, n=21; AA 75 mg/kg, n=23; vehicle, n=25) before ischemia or 6 (AA 75 mg/kg, n=20; vehicle, n=20), 9 (AA 75 mg/kg, n=18; vehicle, n=17), and 12 (AA 75 mg/kg, n=19; vehicle, n=18) hours after ischemia onset in the pMCAO model.

Similarly, rats were randomly assigned treatment with AA or vehicle at 6 (AA 75 mg/kg, n=18; vehicle, n=17), 9 (AA 75 mg/kg, n=18; vehicle, n=20), or 12 (AA 75 mg/kg, n=19; vehicle, n=21) hours after ischemia onset in the tMCAO model. Infarct volumes were evaluated at 24 hours after middle cerebral artery occlusion (MCAO).

Experiment 3: Determination of the Influence of Gender on Outcomes
Clinical stroke affects both genders. We sought to determine the influence of gender on outcomes. Female SD rats (9–10 weeks) were subjected to pMCAO and treated with AA (75 mg/kg, n=11) or vehicle (n=12) at 6 hours after ischemia onset.

Experiment 4: Determination of the Influence of Hypertension on Outcomes
Clinical stroke affects patients with comorbidities like hypertension. We sought to determine the efficacy of AA in the presence of hypertension. SHRs were subjected to tMCAO. Only tMCAO was used because SHRs have very high mortality rates with pMCAO.10 SHRs were subjected to 1 hour tMCAO and treated with AA (75 mg/kg, n=12) or vehicle (n=13) at 6 hours after ischemia onset. Infarct volumes were evaluated at 24 hours after MCAO.

Experiment 5: Determination of Long-Term Histological and Behavioral Outcomes
We sought to determine long-term histological and behavioral outcomes. Male SD rats were randomly divided into 3 groups. (Only the transient model was used because like other investigators, we also observed high long-term mortality.) The sham group had surgery but was not subjected to tMCAO (n=6). The other 2 groups were subjected to tMCAO and blindly treated with AA (75 mg/kg, n=23) or vehicle (n=18) at 6 hours after ischemia onset. The neurological scores, behavioral tests, and infarct volumes were evaluated during and at the end of 2 weeks post-MCAO.

Experiment 6: Exploration of Possible Mechanisms of Action
We explored possible mechanism of action by examining the effects of AA on brain mitochondrial bioenergetics and MMP induction and activity. Both these mechanisms play a role in ischemic injury.11–13 Male SD rats were randomly divided into 2 groups and subjected to tMCAO and treated with AA (75 mg/kg, n=8) or vehicle (n=8) at 6 hours after ischemia onset. The brain tissues were isolated at 24 hours after MCAO for mitochondrial isolation and Western blots. (Details of the tissue isolation are provided in the only-only Data Supplement).

Results

Safety, Tolerability, and Pharmacokinetics
After a single intravenous injection of 10, 25, and 75 mg/kg, serum concentrations of AA increased to 2.95, 6.16, and 8.62 μmol/L, respectively, at 15 minutes. The subsequent fall in serum concentrations within the first 1 hour was rapid (Figure 1). The T1/2 of 75 mg/kg AA was 2.00±0.18 hours. The maximal concentration value and area under the curve
value of 75 mg/kg AA was 9.70±0.82 mg/L and 27.67±2.91 mg·hr/L, respectively (online-only Data Supplement Table VI). AA was well tolerated up to a dose of 75 mg/kg. Higher doses could not be tested because of toxicity associated with the solvent. Food consumption, activity, weight, mean arterial pressure, heart rate, and mortality are presented in online-only Data Supplement Figures I through V.

AA Attenuates Infarct Volume After Ischemic Stroke

Dose–Response Relationship and Therapeutic Time Window

AA administration (50 or 75 mg/kg, 30 minutes before MCAO) had a dose-dependent protective effect, attenuating infarct volume against pMCAO at 24 hours by 37.0% and 52.5%, respectively (Figure 2A). To determine the therapeutic time window, rats were administered AA (75 mg/kg) at 6, 9, or 12 hours after onset of ischemia. As illustrated in Figure 2B, treatment with AA significantly decreased infarct volume even 12 hours after pMCAO. In tMCAO, infarct volume was significantly reduced with AA treatment at 6 or 9 hours, 53.1% and 52.3%, respectively (Figure 2C).

Efficacy in Female Rats and Hypertensive Rats

Additional studies were performed in female animals and hypertensive animals. As illustrated in Figure 3A, 6 hours posttreatment with AA (75 mg/kg) in female rats decreased infarct volume by 56.4%. Similarly, AA (75 mg/kg) 6 hours postischemia in SHR significantly reduced infarct volume by 25.6% (Figure 3B).

Determination of Long-Term Efficacy of Histological and Functional Outcomes

To test whether the neuroprotective effect of AA is sustained, AA (75 mg/kg) was administered 6 hours after onset of ischemia. Neurological function and cerebral infarct volume were determined at 14 days. AA reduced infarct volume by 36.3% (Figure 4A). AA treatment also significantly improved functional outcome and decreased motor, sensory, and reflex impairment 1, 3, 7, and 14 days after ischemia (Figure 4B). Additional functional testing using the adhesive removal and Rotorod tests were done before (baseline) and 1, 3, 7, and 14 days after ischemia (Figure 4C–D). In the Rotorod test, a strong trend toward better performance of the group injected with AA was observed at postoperative Day 14 (repeated-measures analysis of variance, P=0.056). Significant improvement for the adhesive removal test was observed at postoperative Day 14 (P=0.027; Figure 4D).

AA Influences Mitochondrial Health and MMPs

Influence of AA on Mitochondrial Respiration Functions

Brain mitochondrial bioenergetics were characterized by measuring oxygen consumption rates of different substrates and calculating the respiratory control ratio (RCR; Figure 5). The measured rates of oxygen consumption of different mitochondrial substrates and the derived RCR serve as important indicators of mitochondrial respiratory capacity and functional homeostasis.11,12 Adenosine 5'-triphosphate phosphorylation (State III) rates were significantly decreased in mitochondria sampled from the ipsilateral hemisphere after
MCAO (Figure 5A). AA treatment restored State III. The RCR (State III respiration divided by State IV respiration) is an index of how coupled the electron transport chain is to adenosine 5'-triphosphate production. Figure 5B shows alterations in RCR values after ischemia injury in both the ipsilateral (injured) hemisphere and contralateral (uninjured) hemisphere. After MCAO, RCR was significantly attenuated in the ischemic cortex sample by 69.8%. AA treatment decreased the attenuation of RCR by 42.8%.

Influence of AA on MMPs
The MMPs are induced in the brain after ischemia and are involved in disruption of the BBB among other deleterious processes. To determine whether AA treatment could inhibit MMP activity in the brain after ischemia, the levels of MMP-2 and MMP-9 protein in the brain were determined by Western blotting. Ischemic rats showed an increase in MMP-9 levels in the ipsilateral hemisphere, whereas no changes in MMP-2 were detected (Figure 6A–B). AA treatment significantly inhibited MMP-9 induction (Figure 6C).

To determine whether AA inhibits MMP activity, an in vitro assay using recombinant MMP-9 was performed. The MMP-9 was incubated with various concentrations (0.1, 0.5, and 1 mmol/L) of AA, and the effect on enzyme activity was determined by the ability to degrade fluoroescently labeled gelatin. AA significantly inhibited MMP-9 (Figure 6D) in a concentration-dependent manner. Because MMPs are zinc-dependent endopeptidases, AA inhibitory activity in excess zinc was examined to determine whether AA has a metal-chelating effect on zinc. Our results suggest that AA does not inhibit MMP activity by zinc chelation.

Discussion
We have previously shown in a mouse model of pMCAO that AA is neuroprotective, has antioxidant activity, and protects BBB integrity after ischemia. The current study, using rigorous blinding and randomization methodology, has established a dose–response relationship and confirmed efficacy in 4 other MCAO stroke models: pMCAO/tMCAO in male rats, pMCAO in female rats, and tMCAO in SHRs. We also show that AA has a long and clinically relevant therapeutic time window. Furthermore, we have identified ischemia-activated deleterious mechanisms that are favorably influenced by AA.

Several of our findings are particularly noteworthy. First, AA exhibited significant neuroprotection, even when administered 12 hours after the onset of ischemia, which could translate to a clinically long time window for treatment and will allow the treatment of many more patients. Second, because almost half of all strokes affect females and hypertension is the biggest risk factor for stroke, efficacy in female rats and the SHR model strengthens the case for further preclinical development. Third, our findings that AA improves both histological and functional outcomes 14 days after ischemia suggest sustained benefit.

Our pharmacokinetic data suggest that AA has a half-life of 2.0 hours. This relatively short half-life raises the possibility that maintaining serum levels of AA for longer periods of time by using multiple dosing or bolus plus infusion regimens may afford greater efficacy. Future studies will explore multiple dosing strategies to determine if there is greater efficacy using those regimens. It is also possible that multiple dosing regimens may allow smaller doses to be administered.

AA favorably influences a number of deleterious mechanisms that are activated after stroke. We have previously shown that AA is a powerful antioxidant and also that it maintains the integrity of the BBB after ischemia. We have now extended our mechanistic studies to include the influence of AA on mitochondrial redox activity and MMP induction and enzymatic activity and show that AA also favorably influences these pathways. The postischemic brain exhibits prominent changes in redox activity of mitochondrial respiratory chain components. Mitochondria isolated from ischemic brains exhibited decreases in State III respiratory rates of approximately 70% with nicotinamide-adenine
In the current study, we confirm previous studies that showed that reperfusion after cerebral ischemia resulted in mitochondrial damage.18–20 Our data show that AA inhibited ischemia induced reductions of RCR, the index of mitochondrial respiratory function indicating that AA protects mitochondria during ischemia.

MMPs degrade the extracellular matrix and are involved in several brain diseases including stroke.21,22 Enhanced MMP-9 expression and activity have been demonstrated in the ischemic brain.23,24 MMP inhibitors and MMP-9 gene deletion attenuated BBB disruption and brain tissue infarction after ischemia.25,26 Clinical studies have also shown a correlation between plasma MMP-9 levels and the rate of hemorrhagic transformation in human stroke.27 In the present study, we confirmed that MMP-9 expression was increased after ischemia and showed that AA treatment attenuated the increase in MMP-9. It is, however, possible that the attenuation in

**Figure 4.** Improvement of long-term histological and neurological functional outcomes after tMCAO. AA (75 mg/kg) or vehicle were administered at 6 hours after ischemia onset. Infarct volume was determined at 14 days after 2 hours tMCAO by Nissl staining (A). AA treatment significantly reduced infarct volume and improved functional outcome. B, Neurological scores. C, Rotorod test. D, Adhesive tape removal test at the right. AA decreased motor, sensory, and reflex functional damage after 2 hours tMCAO determined at 1, 3, 7, and 14 days after ischemia by neurological deficit scoring (0–18). The adhesive removal and Rotorod tests were done both before (baseline) and 1, 3, 7, and 14 days after ischemia. There was a strong trend toward better performance of the AA-treated animals (white circle) in both Rotorod and adhesive tape removal tests. Scale bar, 1 cm. All values are means±SEM and analyzed by 1-way analysis of variance. *P<0.05, **P<0.01, ***P<0.001 versus vehicle group. tMCAO indicates transient middle cerebral artery occlusion; AA, asiatic acid.

**Figure 5.** Mitochondrial bioenergetics after tMCAO. A, Representative traces from nonsynaptic mitochondrial oxygen consumption measurements in the presence of oxidative substrates (pyruvate and malate), ADP, oligomycin, CCCP, and succinate. B, The respiratory control ratios (RCR), which is State III respiration divided by State IV respiration. Data were expressed as mean±SEM. **P<0.05, ***P<0.01 versus contra group; #P<0.05, ##P<0.01 versus vehicle group. Contra indicates contralateral hemisphere; ipsil, ipsilateral hemisphere; tMCAO, transient middle cerebral artery occlusion; ADP, adenosine 5′diphosphate; CCCP, carbonylcyanide m-chlorophenylhydrazone.
MMP-9 induction may be due to the smaller infarct volume rather than the direct effect of AA on MMP induction. Our in vitro data on the other hand show that AA inhibits MMP enzyme activity. The mechanism through which AA inhibits MMPs is not known; our study suggests that this inhibition is not mediated by AA chelating zinc, an important cofactor for MMPs. Inhibition of MMP-9 induction and/or activity may underlie the maintenance of BBB integrity that we previously reported with AA after focal ischemia.7

Many promising preclinical drugs have failed in clinical testing. Reasons for failure include inadequate preclinical testing and new guidelines (STAIR)8 have been published to address this issue. Although we present considerable data showing the efficacy, safety, and pharmacokinetics of AA, more studies are still needed to fully meet the STAIR guidelines and before AA can be tested in humans. Future studies will test multiple dosing regimens including bolus with infusion, compatibility with tissue-type plasminogen activator, and efficacy in other comorbidity models and aged animals. Pre-Investigational New Drug Food and Drug Administration mandated safety and toxicity studies would also be needed.

In conclusion, we have shown that AA is neuroprotective in multiple stroke models, it has a long therapeutic time window, and favorably influences both early and late histological and functional outcomes. We also provide evidence that AA protects mitochondria and attenuates MMP-9 induction and activity. Taken together these data suggest that AA is an excellent candidate for further development as a stroke therapy.

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Figure 6. Effects of AA on MMP-2 and MMP-9. A, Western blot showing MMP-9 expression in the brain is enhanced after ischemia in the ipsilateral hemisphere in rats receiving vehicle (Lane 2), but this effect is attenuated by AA (Lane 4). MMP-2 levels were unchanged (β-actin illustrates protein loading in different lanes for MMP-9 and MMP-2). B, MMP-2. C, MMP-9. AA inhibited MMP-9 activity in vitro regardless of excess ZnCl2. D, MMP09. Data were expressed as mean±SEM *P<0.05, **P<0.01 versus contra group; #P<0.05, ##P<0.01, ###P<0.001 versus vehicle group. Contra indicates contralateral hemisphere; ipsil, ipsilateral hemisphere; AA, asiatic acid; MMP, matrix metalloproteinase.

Disclosures
None.

References


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