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Articles

HU-211, a Novel Noncompetitive *N*-Methyl-D-Aspartate Antagonist, Improves Neurological Deficit and Reduces Infarct Volume After Reversible Focal Cerebral Ischemia in the Rat

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Abstract

Background and Purpose HU-211 is a nonpsychotropic cannabinoid analogue that has been shown to act as a functional N-methyl-D-aspartate receptor blocker. We investigated the neuroprotective efficacy of HU-211 in a model of reversible middle cerebral artery occlusion (MCAo) in rats.

Methods Male Wistar rats were anesthetized with halothane and subjected to 90 minutes of temporary MCAo by retrograde insertion of an intraluminal nylon suture, coated with poly-L-lysine, through the external carotid artery into the internal carotid artery and MCA. The drug (HU-211 in cosolvent, 4 mg/kg IV) or vehicle was administered in a blinded fashion 70 minutes after onset of MCAo. Behavioral tests were evaluated during occlusion (60 minutes) and for a 3-day period after MCAo. Three days after MCAo, brains were perfusion-fixed, and infarct volumes were determined.

Results HU-211 significantly improved the neurological score compared with vehicle during the 3 days after MCAo. Treatment with HU-211 also significantly reduced both infarct volume (mean±SEM, 66.6±12.5 versus 149.8±36.3 mm^3) and brain swelling (2.61±1.33% versus 6.66±1.24%) compared with vehicle-treated rats (n=17 in each group).

Conclusions These results demonstrate the neuroprotective ability of HU-211 in focal cerebral ischemia as judged by neurological score, infarct size, and brain swelling. Reversible MCAo with the use of a poly-L-lysine-coated intraluminal suture proved to be a reliable and effective modification of this technique, yielding consistent results.

Key Words:
cerebral ischemia, focal
N-methyl-D-aspartate
neuroprotection
rats

In evaluating the role of NMDA receptors in focal cerebral ischemia, several recent studies have made use of models of temporary MCAo[1 2] to produce a lesion that simulates focal stroke in humans. During cerebral ischemia, high extracellular concentrations of glutamate associated with activation of glutamate receptors have been implicated in neuronal damage.[3 4 5] Cerebroprotection with NMDA antagonists has also been well documented after focal cerebral ischemia.[6 7 8]

HU-211 [the synthetic cannabinoid (+)-(3S,4S)-7-hydroxy-Δ^6-tetrahydrocannabinol 1,1-dimethylheptyl] acts functionally as an NMDA receptor antagonist.[9 10] HU-211 exhibits pharmacological, autonomic, and behavioral effects typically caused by NMDA receptor antagonists. In vitro studies of brain membrane preparations and neuronal tissue culture have shown that this compound is a functional noncompetitive antagonist of the NMDA receptor, preventing the influx of calcium through the NMDA receptor-linked channel, and

rescues neurons from NMDA toxicity in culture.[11] [12] This new synthetic cannabinoid can protect against selective hippocampal neuronal damage induced by global cerebral ischemia in rats and gerbils.[13] [14] [15] The anti-ischemic efficacy of HU-211 has also been detected in head injury in the rat.[16] [17] We thus decided to investigate the effect of HU-211 on the histopathological and neurobehavioral outcome of focal cerebral ischemia in the rat.

An additional aim of the present study was to evaluate a modified method of intraluminal suture occlusion of the MCA on brain damage in rats subjected to a temporary focal ischemic insult. We initially adopted the method of Zea Longa et al[2] to study focal ischemia in the rat but found that the technique produced inconsistent infarct volume. We decided to coat the sutures used for intraluminal MCA occlusion with poly-L-lysine, a polycationic polymerized amino acid, to induce the intraluminal adhesion cascade. Poly-L-lysine has been used to coat glass slides in the preparation of tissue sections for immunocytochemical staining.[18] The polycationic nature of this molecule allows an interaction with the anionic sites of tissue, promoting adhesion. As the polycationic polylysine molecules adsorb strongly to various solid surfaces, leaving cationic sites that combine with the anionic sites on cell surfaces,[19] we suspected that this might encourage adherence of the suture to the vascular endothelium. Successful occlusion of the MCA was judged by the presence of neurological deficits and histopathology. We found that the technique was reliable and produced consistent results.

Materials and Methods

Studies were performed with 43 adult male Wistar rats (weight, 270 to 320 g) obtained from Charles River Laboratories, Inc, Wilmington, Mass.

Surgical Preparation

Animals were fasted overnight but were allowed free access to water. Atropine sulfate (0.5 mg/kg IP) was injected 10 minutes before anesthesia. Anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen. Rats were orally intubated and mechanically ventilated. During ventilation, the animals were paralyzed with pancuronium bromide (0.6 mg/kg IV). Temperature probes were inserted into the rectum and the left temporalis muscle, and heating lamps were used to maintain rectal and cranial temperatures at 37°C to 38°C (Mon-a-therm 7000; Mallinckrodt Inc). Polyethylene catheters were introduced into the right femoral artery and vein for blood pressure recording, blood sampling, and drug infusion. Rectal temperature and body weight were monitored before MCAo and periodically for 3 days after MCAo. MABP was measured by an indwelling femoral arterial catheter connected to a precalibrated Statham pressure transducer (model P23XL, Viggo-Spectramed, Inc) and was recorded continuously (model RS3400, Gould, Inc). Serial measurements were made of arterial blood gases and pH (model ABL 330, Radiometer America, Inc) and plasma glucose (model 2300 Stat, Yellow Springs Instrument Co, Inc).

MCAo was induced as described by Zea Longa et al.[2] Under an operating microscope, the right CCA was exposed through a midline neck incision and was carefully dissected free from surrounding nerves and fascia from its bifurcation to the base of the skull. The occipital artery branches of the ECA were then isolated, and these branches were dissected and coagulated. The ECA was dissected further distally and coagulated along with the terminal lingual and maxillary artery branches, which were then divided. The ICA was isolated and carefully separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated close to its origin with a 5-0 nylon suture. Next, a 5-0 silk suture was tied loosely around the mobilized ECA stump, and a 4-cm length of 3-0 monofilament nylon suture (Harvard Apparatus) was inserted via the proximal ECA into the ICA and thence into the circle of Willis, effectively occluding the MCA. The silk suture around the ECA stump was tightened around the intraluminal nylon suture to prevent bleeding.

Before use, the tip of the suture was blunted by heating near a flame. A 20-mm distal segment of the suture was then coated with poly-L-lysine solution (0.1% [wt/vol], in deionized water, Sigma) and dried in a 60°C oven for 1 hour. (The diameter of the suture was not changed by the coating process.) The suture was inserted 18 to 20 mm from the bifurcation of the CCA, according to the animal's body weight. After the intraluminal suture was placed, the neck incision was closed with a silk suture.

The animals were then awakened from anesthesia and returned to their cages. Rats that did not demonstrate a right upper extremity paresis during this recovery period were excluded from further study. After 70 minutes of MCAo, rats were reanesthetized with the same anesthetic combination. HU-211 (Pharmos Corp) was dissolved in cosolvent (Chemophor EL/ethanol, 50 mg/mL) and diluted (1:20) in saline before injection. We administered 4 mg/kg IV of HU-211 or drug vehicle. This dose was chosen on the basis of a previous dose-response study,[15] in which the 4-mg/kg dose proved to confer optimal protection in a global ischemia model. Temperature probes were reinserted, and the intraluminal suture was carefully removed 90 minutes after the onset of MCAo. The CCA and ICA were then inspected to ensure the return of good pulsations. The neck incision was closed with silk suture, and the animals were allowed to survive for 3 days with free access to food and water.

Behavioral Testing

Behavioral tests were performed in all 43 rats before MCAo, during occlusion (at 60 minutes), and daily during the 72-hour observation period by an investigator (L.B.) who was blinded to the experimental groups. The battery consisted of two tests that have been used previously to evaluate various aspects of neurological function: (1) the postural reflex test, developed by Bederson at al[20] to examine upper body posture while the animal is suspended by the tail, and (2) the forelimb placing test, developed by De Ryck et al[21] to examine sensorimotor integration in forelimb placing responses to visual, tactile, and proprioceptive stimuli. Neurological function was graded on a scale of 0 to 12 (normal score, 0; maximal score, 12). Rats with convulsions or sustained disturbances of consciousness were excluded from the study; most of these cases proved to have subarachnoid hemorrhage secondary to suture-induced rupture of the ICA. Four animals were excluded for the above reasons.

Infarct Assessment

Animals were allowed to survive for 3 days. Brains were then perfusion-fixed as previously described[22] with a mixture of 40% formaldehyde, glacial acetic acid, and methanol (FAM, 1:1:8 by volume), and brain blocks were embedded in paraffin. Ten-micrometer-thick sections were cut in the coronal plane and stained with hematoxylin and eosin. To quantitate infarct volume and depict infarct frequency distribution, coronal sections were viewed microscopically at low power, and the areas of infarction at nine coronal levels throughout the brain were traced and measured with the aid of a camera lucida microscope attachment.[23] [24] These drawings of infarcted zones were then video-digitized and saved as digital images. A value of 1 was assigned to each pixel inside of an infarcted region, and the remaining pixels were assigned a value of 0. We then mapped corresponding sections into a preselected "template" section derived from one of the animals studied. This mapping procedure was based on a well-validated image matching/registration algorithm termed "disparity analysis."[25] Pixel-based summation of these mapped digital histological drawings resulted in frequency maps showing, for each image pixel, the number of animals with infarction (Fig 1⇓). Computational procedures were performed on a MicroVAX 3600 computer (32 megabytes of RAM), and image display and analysis were carried out on a VAX Station 3200 (8-bit color plane, 16 megabytes of RAM) (Digital Equipment Corp). The volume of infarction was calculated by numerical integration, by an investigator who was blinded to the experimental groups. The degree of associated brain swelling was determined as the difference in brain volume between the two hemispheres.[26] [27]

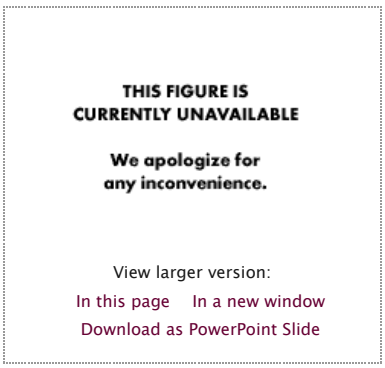

Figure 1.

Frequency distribution of cerebral infarction and results of Fisher's exact test at two different coronal levels (left column, bregma −1.3; right column, bregma −1.8). Top and middle rows depict vehicle- (A, D) and HU-211-treated (B, E) groups, respectively (n=17 in each group); colors represent

numbers of animals having infarction at that pixel (eg, red corresponds to 17 animals, yellow to 15 animals, etc). Bottom row (C, F) depicts the results of Fisher's exact tests performed on every pixel at these two coronal levels; the color bar is calibrated as (1−P), where P represents the level of statistical significance; red corresponds to P=.01, and light blue corresponds to P=.05.

Statistical Analysis

Physiological variables and infarct areas and volumes were analyzed by repeated-measures ANOVA and by Student's t test; P<.05 was regarded as significant. Values are presented as mean±SEM.

Results

Rectal and cranial (temporalis muscle) temperatures, MABP, plasma glucose, and blood gases in the 43 animals of this study showed no significant differences between groups (Table⇓). The neurological scores at 24, 48, and 72 hours after MCAo were significantly better in the HU-211 group than in vehicle-treated animals (2.59±0.29 versus 5.18±0.6; 1.81±0.28 versus 4.9±0.76; and 1.71±0.28 versus 4.07±0.47, respectively; Fig 2⇓). Contralateral forelimb placing deficits were clearly present at 60 minutes after the onset of MCAo in all rats. Fig 3⇓ demonstrates significant improvement of visual and tactile placing reactions after the recovery period in HU-211–treated rats compared with the vehicle-treated group (P<.05).

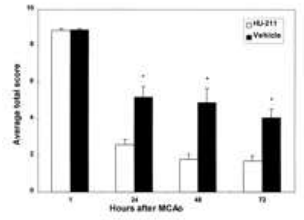

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Figure 2.

Bar graph shows total neurological score (normal score=0) at various times after MCAo (mean±SEM) in HU-211– (n=19) and vehicle-treated (n=24) rats. *P<.05, HU-211 vs vehicle (one-way ANOVA).

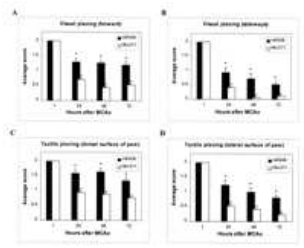

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Figure 3.

Time course of recovery of visual and tactile contralateral forelimb placing reactions after MCAo in rats. Bar graphs show improvement of visual (A and B) and tactile (C and D) placing reactions in HU-211– (n=16 to 18) and vehicle-treated (n=15 to 17) rats. *P<.05 (one-way ANOVA). Normal score=0; maximal score=2 (mean±SEM).

Table 1.

Physiological Variables Before and During MCA Occlusion in Rats

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Survival after MCAo tended to be improved in the HU-211 group (only 2 of 19 rats died during the 72-hour period) compared with the vehicle-treated group (in which 7 of 24 animals died), but this difference did not reach statistical significance (Fisher's exact test). Autopsy in these rats typically revealed a large ipsilateral

hemispheric infarct and extensive brain edema.

Histological examination of the brains of both groups after 72-hour survival showed a remarkably constant pattern of ischemic brain damage, characterized by a mixture of infarction and selective ischemic neuronal changes.[28] All animals of the vehicle-treated group (n=17) had infarcts localized to the lateral segment and, to a varying extent, the medial segment of the caudate nucleus. The infarcted zones were well demarcated and included pancellular necrosis as well as dense areas of eosinophilic, shrunken neurons along the edges of the infarct. In addition, vehicle-treated rats had ischemic cell change in the cortex, and five had infarction in the deeper cortical layers. In HU-211-treated rats (n=17), infarcts affecting the lateral caudoputamen were smaller, and no cortical changes were observed. Infarct volume was significantly reduced by the administration of HU-211 compared with vehicle (66.6±12.5 versus 149.8± 36.3 mm^3, respectively; Fig 4A⇓). Fig 4B⇓ illustrates the rostrocaudal distribution of infarct areas in the two groups standardized according to the atlas of König and Klippel.[29] Infarct volume was significantly reduced by HU-211 at coronal levels 5 (coordinate=6.06; level of fimbria), 6 (coordinate=5.15; level of rostral hippocampus), and 8 (coordinate=2.18; level of aqueduct). Treatment with HU-211 also significantly reduced brain swelling compared with vehicle-treated rats (2.61±1.33 versus 6.66±1.24%, respectively; Fig 4C⇓).

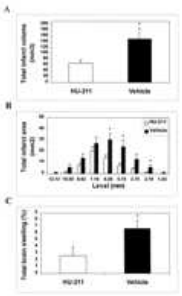

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Figure 4.

Bar graphs show effect of HU-211 on total infarct volume and brain swelling (mean±SEM) 3 days after reversible MCAo in HU-211- and vehicle-treated rats (n=17 in each group). A, Total infarct volume in the two groups. *P<.05, HU-211 vs vehicle (one-way ANOVA). B, Rostrocaudal distribution of the area of total cerebral hemispheric infarction at nine coronal levels in HU-211- and vehicle-treated rats. *P<.05, HU-211 vs vehicle (one-way ANOVA). C, Total brain swelling in HU-211- and vehicle-treated rats. *P<.05, HU-211 vs vehicle (one-way ANOVA).

Discussion

We have demonstrated that the administration of HU-211 reduced infarct volume, diminished brain swelling, and improved neurological deficits resulting from reversible MCAo. In previous studies, HU-211, a synthetic nonpsychotropic cannabinoid, has been shown to rescue neurons in culture from excitotoxic insults.[30] These data suggest that the neuroprotective activity of HU-211 may be directly associated with the NMDA receptor channel. Indeed, a previous study has established that HU-211 functions as a noncompetitive NMDA antagonist.[9] The reduction in tissue damage may also result from the free radical-scavenging activity of HU-211,[31] which may be especially important during the early reperfusion period.

In models of global ischemia, we have previously shown that HU-211 protects against hippocampal neuron damage when administered 15 minutes before ischemia[13] and is equally effective when given 30 and 60 minutes after ischemia.[15] In a dose-response study of global ischemia, we found that a 4-mg/kg dose of HU-211 was significantly neuroprotective.[15] Thus, we used this dose in the present study of focal ischemia. HU-211 also protects blood-brain barrier integrity when injected in a dose of 4 mg/kg 30 minutes after photochemically induced cortical infarction in rats.[32] In a model of closed head injury in rats,[17] it was found that a single dose of HU-211 given 1 hour after closed head injury improved the clinical outcome during a 30-day survival period. Repetitive doses of HU-211 iniected during the posttraumatic period had similar effects. HU-211 can also block $^{45}Ca^{2+}$ uptake through the NMDA-receptor/ion channel in primary cell cultures of rat forebrain.[12] In another study (V. Nadler et al, unpublished data), HU-211 indeed

attenuated the accumulation of ^{45}Ca in the injured brain during the first 3 days after closed head injury.

The early activation of NMDA receptors, which results in the accumulation of Ca^{2+}, triggers a number of mechanisms affecting cellular functions. NMDA and more recently non-NMDA antagonists have been shown to be potent neuroprotective agents in focal ischemia models.[33] [34] However, these compounds also have behavioral, physiological, and neuropathological effects that limit their clinical utility. HU-211, unlike MK-801, has no effect on blood pressure and heart rate at doses as high as 20 mg/kg IV, and neuroprotective doses do not produce these side effects.[35] These considerations formed the rationale for the present investigation of the 4-mg/kg dose of HU-211 in focal ischemia.

Animal models of focal cerebral ischemia that use MCAo reproduce the pattern of ischemic brain damage observed in many human ischemic stroke patients.[36] [37] Techniques for inducing transient MCAo by an intraluminal filament have been used extensively in experimental models of ischemic stroke.[2] [38] [39] These methods have the advantage of not requiring craniotomy with its associated operative trauma, and they permit reperfusion of the occluded MCA. Koizumi et al[1] used a silicone-coated 4-0 nylon surgical thread (diameter, 0.25 to 0.30 mm), while Zea Longa et al[2] used a 4-0 uncoated nylon thread whose tip was blunted by heating near a flame. Both groups showed that reperfusion occurred when the threads were removed. As discussed by Laing et al,[40] the method of Zea Longa et al[2] has relatively low reproducibility, with a success rate of only 56% in comparison to 93% in the model of Koizumi et al.[1] In another study in which a silicon-coated thread was used, approximately 30% of experimental animals were excluded.[41] Unsuccessful outcomes consisted of animals without neurological deficits and rats in which subarachnoid hemorrhage caused by rupture of the intracranial ICA had occurred. In the study of Nagasawa and Kogure,[38] in which the MCA was occluded with a silicone rubber cylinder attached to a nylon surgical thread, 38 of 41 rats died within 48 hours after MCAo; the overall mortality rate was 92.7%.

In our own preliminary experiments in which uncoated sutures were used, the success rate was also rather low. Thus, we used the method of Zea Longa et al[2] while modifying the technique of suture preparation: We blunted the tip of the thread and then coated it with poly-L-lysine. Poly-L-lysine has previously been shown to promote the adherence of cells and proteins to glass and plastic surfaces.[19] This substance spreads as a monolayer and is thought to change the negative charge of a suture surface to a positive one, which is then available to attract the anionic sites of endothelial cells, causing the suture to adhere to the endothelial surface,[18] so that flow around the suture is prevented.

Successful occlusion of the MCA was achieved in 43 of 47 animals in this series (92%) as judged by neurological deficits and histopathology. In this ischemic model, the lateral segment of the caudate nucleus was most frequently damaged at 72 hours after MCAo. In five vehicle-treated animals, infarction extended from the caudoputamen to the overlying cortex. MCAo-induced infarcts are typically composed of two zones.[42] In the zone of cortical infarction, part of the affected tissue (corresponding to the ischemic penumbra) possesses sufficient collateral blood supply to enable at least some neurons potentially to recover under the influence of agents such as NMDA antagonists or non-NMDA antagonists.[34] [43] [44] In contrast, the striatum tends to be more severely ischemic as it is supplied by end arteries.[45] In the present study of temporary MCAo, the neuroprotective effect of HU-211 was apparent in both the cortex and striatum of treated rats, and total infarct volume was significantly reduced (45%) at 3 days after ischemia.

Evidence for acute brain edema was seen after MCAo as revealed by apparent swelling of the affected hemisphere. We have adopted a correction formula as previously reported,[26] [27] which is based on the difference in brain volume between the two hemispheres, to calculate the percentage of swelling and the indirect total infarct volume. Treatment by HU-211 significantly reduced total brain swelling (39%) at 72 hours after MCAo.

Observation of neurological deficits is generally important not only in clinical cases of stroke but also in experimental cerebral ischemia models. Focal ischemia induced a neurological deficit characterized by sensorimotor dysfunction, which has been noted by previous workers.[20] [46] [47] While Wahl et al[48] reported a lack of correlation between neurological deficit and the extent of the infarct, others have shown that the neurological deficit correlated significantly with the size of the

infarcted area.[20 49 50] In our study we observed that neurological grade was significantly correlated to infarct volume (linear regression analysis, P<.005). There were no adverse behavioral side effects observed with HU-211 administration.

Brain temperature during the ischemic insult and during the early hours after cerebral reperfusion is an important factor affecting histopathological outcome.[36 51 52] Small variations in brain temperature during and after ischemia markedly influence the extent of ischemic neuronal pathology.[53] Since brain temperature in rats, particularly during and after ischemia, cannot be reliably predicted from a knowledge of rectal temperature alone, it must be independently monitored.[54 55] The protective effect of HU-211 in this study could not be explained by differences in body or brain temperatures, arterial pressure, or arterial blood gases because these variables were carefully controlled and did not differ among groups.

In summary, we have shown that HU-211 is a powerful neuroprotective agent in an in vivo model of temporary focal cerebral ischemia. The model of MCA suture-occlusion, as modified in our laboratory, proved to be a useful means of studying focal cerebral ischemia.

Selected Abbreviations and Acronyms

CCA =common carotid artery
ECA =external carotid artery
ICA =internal carotid artery
MABP =mean arterial blood pressure
MCA =middle cerebral artery
MCAo =middle cerebral artery occlusion
NMDA=N-methyl-D-aspartate

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Footnotes

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