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Synergistic Neuroprotection by a PAF Antagonist Plus a Docosanoid in Experimental Ischemic Stroke: Dose-Response and Therapeutic Window

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Abstract

Objective: We tested the hypothesis that blocking pro-inflammatory platelet-activating factor receptor (PAFR) with LAU-0901 (LAU) plus administering a selected docosanoid, aspirin-triggered neuroprotectin D1 (AT-NPD1), which activates cell-survival pathways after middle cerebral artery occlusion (MCAo), would lead to neurological recovery. Dose-response and therapeutic window were investigated.

Materials and methods: Male SD rats were subjected to 2 hours of MCAo. Behavior testing (days 1–7) and *ex vivo* MRI on day 7 were conducted. In dose-response, rats were treated with LAU (45 and 60 mg/kg; IP), AT-NPD1 (111, 222, 333 μg/kg; IV), LAU+AT-NPD1 (LAU at 3 hours and AT-NPD1 at 3.15 hours) or vehicle. In the therapeutic window, vehicle, LAU (60 mg/kg), AT-NPD1 (222 μg/kg), and LAU +AT-NPD1 were administered at 3, 4, 5, and 6 hours after onset of MCAo.

Results: LAU and AT-NPD1 treatments alone improved behavior by 40–42% and 20–30%, respectively, and LAU+AT-NPD1 by 40% compared to the vehicle group. T2-weighted imaging

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Authors contributions

NGB and LB conceptualized and designed the experiments. MMR, LK, CR, AO, NP, and PMK acquired the data. LB, AO, and MMR analyzed and interpreted the data. MMR and LB performed the statistical analysis. NGB, LB, and MMR drafted the article. LB, NGB, and RO critically revised the article. All authors reviewed the submitted version of the manuscript. LB and NGB approved the final version of the manuscript on behalf of all authors. RO, PMK, NP, and LB provided Administrative/technical/material support. NGB and LB supervised the study.

Declaration of Competing Interest

The authors report no conflicts of interest.

(T2WI) volumes were reduced with all doses of LAU and AT-NPD1 by 73–90% and 67–83% and LAU+AT-NPD1 by 94% compared to vehicle. In the therapeutic window, LAU+AT-NPD1, when administered at 3, 4, 5, and 6 hours, improved behavior by 50, 56, 33, and 26% and reduced T2WI volumes by 93, 90, 82, and 84% compared to vehicle.

Conclusions: We have shown here for the first time that LAU plus AT-NPD1 treatment affords high-grade neuroprotection in MCAo, equaling or exceeding that afforded by LAU or AT-NPD1 alone at consider-ably moderate doses. It has a broad therapeutic window extending to 6 hours after stroke onset.

Keywords

LAU-0901; AT-NPD1; MRI; Penumbra; MCAo; Behavior

Introduction

Stroke is a primary cause of mortality and neurological dysfunction worldwide, with limited therapeutic options. Thrombolysis or thrombectomy is a common therapeutic strategy, but it is often accompanied by cerebral ischemia/reperfusion (I/R) injury.[1] The occurrence of I/R damage after stroke may trigger various molecular cascades associated with dysregulation of numerous neuro-inflammatory pathways[2] and disruption of neuronal circuits, which aggravate brain damage.[3] Therefore, developing neuroprotection strategies to safeguard the brain from cerebral ischemia and I/R is an important goal. Although stroke is a complex disorder with activation of multiple detrimental signaling cascades, almost all neuroprotective strategies to date have attempted a monotherapy against a single target. It has been discussed that no single compound will have maximal efficacy and that a multi-target compound or drug cocktail approach may be needed to promote neuroprotection and recovery of behavioral functions.[4]

Accumulating evidence indicates a central role of inflammation in all aspects of stroke, including its initiation, the progression of injury, and recovery.[5] The patho-physiologic disruption that results from ischemic stroke is complex and includes excitotoxicity, intracellular calcium influx, ion homeostasis, inflammatory cell signaling, cytokine release, and blood-brain barrier hampering.[6] To address these multifaceted pathogenic stroke components, we proposed a combination therapy to block pro-inflammatory events[7,8] while simultaneously promoting neuroprotection and inflammation resolution.[9] Activating inflammatory factors is a significant obstacle in the reperfusion process due to the disturbance of metabolic processes.[10] We questioned whether joint inhibition and inflammation resolution of both processes would be more neuroprotective than either on its own. The following small molecules were investigated: a) LAU-0901, a plate-let-activating factor receptor (PAFR) antagonist that blocks pro-inflammatory signaling, and b) aspirin-trig-gered Neuroprotectin D1 (AT-NPD1), which activates cell-survival pathways and exerts potent anti-inflammatory activity in the brain. Therefore, we assessed the effects of combined LAU-0901 + AT-NPD1 on the motor deficit and the extent of cerebral (cortical and subcortical grey matter) infarction produced by middle cerebral artery occlusion (MCAo) in the rat and compared results to appropriate single agent and vehicle controls.

Platelet-activating factor (PAF) is a bioactive phospho-lipid that accumulates during I/R and is involved in the activation of platelets, neutrophils, and pro-inflammatory signaling. It has been suggested that PAF enhances brain I/R damage.[11,12] In recent experimental studies of focal cerebral ischemia, we have shown that LAU-0901 (2,4,6-trimethyl-1, 4-dihydro-pyridine-3, 5-dicarboxylic acid), a highly potent and selective PAFR antagonist, is markedly neuroprotective in moderate doses in acute focal cerebral ischemia in rats and mice, both improving behavioral function as well as local cerebral blood flow and reducing the extent of histological damage with a therapeutic window of at least 2 h.[13,14] Thus, we used these doses of LAU-0901 (45 and 60 mg/kg) in our studies.

Endogenous mechanisms in the resolution of acute inflammation are of interest since excessive inflammation underlies many pathologies. We reported the discovery of a novel aspirin-triggered docosahexaenoic acid (DHA) metabolite, a potent anti-inflammatory pro-resolving molecule, namely AT-NPD1 (10R, 17R dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid).[15] We demon-strated that AT-NPD1 treatment reduced polymorphonuclear (PMN) recruitment in murine peritonitis with decreased transendothelial PMN migration and enhanced efferocytosis of apoptotic human PMN by macrophages.[15] We tested AT-NPD1 in a model of MCAo in rats and found that AT-NPD1 provided sustained neurobehavioral recovery and reduced brain infarction and brain edema when administered at 3 h after MCAo.[16]

We recently showed that a combinatory treatment with LAU-0901 and AT-NPD1[17] was more effective than the single therapy when administered at 3 h after stroke. Yet, we did not assess the dose-response and therapeutic window. The present study used magnetic resonance imaging in combination with behavioral tests to expand our under-standing of this novel therapeutic approach.

Material and methods

Animals and ethical approval

All animal procedures complied with the National Institutes of Health Guidelines for animal research and were approved by the Institutional Animal Care and Use Committee of Louisiana State University Health New Orleans. Maximum efforts were taken to minimize the number and suffering of animals. Eighty-eight male Sprague-Dawley rats (8–10 weeks old) were purchased from Charles River Laboratories (Wilmington, Massachu-setts, USA). Experimental rats were anesthetized with 3% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen, orally intubated, and mechanically ventilated. The catheters were inserted into the right femoral artery for blood sampling and the femoral vein to administer drugs. Serial analyses of arterial blood gases, plasma glucose, hematocrit, and arterial blood pressure were conducted before and during the surgical procedure. Rectal (CMA/150 Temperature Controller, CMA/Microdialysis AB, Stockholm, Sweden) and cranial (temporalis muscle; Omega Engineering, Stamford, Connecticut, USA) temperatures were maintained at 36°C to 37.5°C before, during, and after MCAo. Rectal temperature and body weight were monitored daily during the 7-day survival period.

MCAo/Reperfusion model

Rats were subjected to 2 h of right MCAo by an intraluminal filament, as previously described.[18] A nylon filament coated with poly-L-lysine was advanced through the external carotid artery to the MCA until mild resistance was felt. Animals were allowed to awaken from anesthesia and were tested on a standardized neurobehavioral battery at 60 min to confirm the presence of a high-grade neurological deficit.[18] After 2 h of MCAo, rats were reanesthetized with the same anesthetic combination, the suture was withdrawn, and reperfusion was allowed for 7 days.

Dose-response study

LAU-0901 was dissolved in 45% Cyclodextrin and administered intraperitoneally (IP) at 3 h after stroke onset. AT-NPD1 was dissolved in 0.9% saline and administered intravenously (IV) at 15 min after LAU-0901 delivery. Vehicle (45% Cyclodextrin and 0.9% Saline) was administered at 3 h and 3.15 h. Rats were randomly assigned to following groups: LAU-0901 (45 and 60 mg/kg, IP), AT-NPD1 (111, 222, 333 μg/kg, IV), LAU-0901 (60 mg/kg, IP) + AT-NPD1 (333 μg/kg, IV) or vehicle (n=5–8 per group). LAU-0901 was administered first at 3 h and AT-NPD1 at 3.15 h.

Therapeutic window study

The dose-response study showed that LAU-0901 (60 mg/kg) and AT-NPD1 (222 μg/kg) were highly neuro-protective; thus, these doses were applied. LAU-0901 (60 mg/kg, IP) and AT-NPD1 (222 μg/kg, IV) were administered at 3 h and 3.15 h, respectively, after stroke onset. LAU-0901+AT-NPD1 was administered at 3, 4, 5, and 6 h after onset of MCAo (n=5–9 per group). LAU-0901 was administered first and AT-NPD1 15 min after LAU-0901 delivery. Vehicle (45% Cyclodextrin and 0.9% Saline) was administered at 3 h and 3.15 h, respectively.

In both studies, behavior testing was conducted on days 1, 2, 3, and 7 followed by *ex vivo* MRI on day 7. All treatments were administered by researchers blinded to the treatment groups.

Behavioral tests

As we previously described, a standardized battery of behavioral tests was used to assess the neurological deficits after MCAo.[18] The battery incorporated two tests: 1) the postural reflex test, to examine upper body posture while the animal is suspended by the tail, and 2) the fore-limb placing test, to assess the forelimb placing responses to visual, tactile, and proprioceptive stimuli.[18] The behavioral function was graded on a 12-point scale (0=normal and 12=maximal deficit).[18] The severity of stroke injury was assessed by behavioral examination of each rat 60 min after the onset of MCAo. Rats that did not demonstrate high-grade contralateral deficit (score of 10–12) were excluded from further study. All tests were conducted by an investigator who was blinded to the treatment groups at 60 min (during MCAo) and then on days 1, 2, 3, and 7 after MCAo.

Magnetic resonance imaging (MRI) and analysis of total lesion, core, and penumbra

On day 7, a high-resolution *ex vivo* MRI was performed on 4% paraformaldehyde-fixed brains using an 11.7T Bruker Advance 8.9 cm horizontal bore instrument equipped with an 89 mm (ID) receiver coil (Bruker Bio-spin, Billerica, Massachusetts, USA). T2-weighted imaging (T2WI), diffusion-weighted images (DWI), and T2 relaxation maps were computed as previously described.[19] Hierarchical Region Splitting (HRS) automatically identified core and penumbra volumes (total lesion = core + penumbra) from T2 relaxation maps.[19]

Our core and penumbral tissue determination using HRS (implemented in Matlab) were validated previously using perfusion-weighted imaging (PWI)/DWI subtractions at each brain level.[19] The penumbra from HRS was defined using T2 values (ms) between normal-appearing brain tissue and ischemic core. Data from each modality were summarized per group.

Statistical analysis

All values are reported as means ± SEM. Two-tailed Student's t-test was used for two-group comparisons. For multiple-group comparisons, repeated-measures analysis of variance (ANOVA) followed by Bonferroni tests. A value of $p < 0.05$ was regarded as statistically significant.

Results

Physiological variables and mortality

All animals in this study showed similar values for rectal and cranial temperatures, MABP, arterial blood gases, and plasma glucose with no significant differences between groups. No adverse behavioral side effects were observed after LAU-0901, AT-NPD1, or LAU-0901 + AT-NPD1 administration. Dose-response study: No rats died in any treated groups, except three in the vehicle group on days 2, 3, and 7. Therapeutic window study: a total of three rats died (two rats in the vehicle group on days 1 and 7 and one in LAU-0901+AT-NPD1–3h on day 2). Autopsies revealed a large ipsilateral hemispheric infarct and extensive brain edema in all animals.

Dose-response

The experimental design is presented in Fig. 1A. All rats had a total neurological score of 0 before ischemia and developed a high-grade behavioral deficit (10–11; maximum possible, 12) when examined at 60 min of MCAo (Fig. 1B); thus, no animals required exclusion based on an inadequate degree of cerebral ischemia during the behavioral assessment. Vehicle-treated rats continued to exhibit severe behavioral impairments throughout the 7-day survival period. All rats treated with different doses of LAU-0901, AT-NPD1, and LAU-0901+AT-NPD1 significantly improved the total neurological score compared to the vehicle on days 1, 2, 3, and 7 (Fig. 1B).

The neuroprotective effect was enhanced using the LAU-0901+AT-NPD1 compared to AT-NPD1 (111 and 222), which resulted in improved behavioral scores of 20% and 31% on day 7 (Fig. 1B).

Fig. 2A–C depicts the significant improvement of tactile (dorsal and lateral) and proprioceptive placing at different times in all treated rats compared to the vehicle group.

Representative T2WI and core/penumbra images are presented in Fig. 3A. Large lesions and T2 hyperinten-sities were observed in the ischemic core and penumbra of vehicle-treated rats. In contrast, all treatments had smaller lesion volumes, mainly visible in the ischemic core and partially in the penumbra. Total, ischemic core, and penumbra lesion volumes computed using T2WI on day 7 were significantly reduced by all treatments com- pared to the vehicle group (Fig. 3B). Rats treated with combinatory treatment showed smaller lesions than LAU-0901 and AT-NPD1 treatments alone, but this was not statistically significant (Fig. 3B).

Time window

The experimental design is presented in Fig. 4A. Treatment with LAU-0901 and AT-NPD1 alone, when administered at 3 h, improved total neurological score on day 7 by 43% and 30% (Fig. 4B). In contrast, the neuroprotective effect was enhanced using LAU-0901+AT-NPD1, which improved behavior by 50% when administered at 3 h, 56% at 4 h, 33% at 5 h, and 26% at 6 h (Fig. 4B). The combinatory treatment administered at 4 h was significantly better than AT-NPD1 alone (Fig. 4B). Representative T2WI and core/penumbra images are presented in Fig. 5.

Large lesions and T2 hyperintensities were observed in saline-treated rats' ischemic core and penumbra. Rats treated with LAU-0901 and AT-NPD1 alone had smaller lesions with partial cortex and subcortical involvement. In contrast, lesions were reduced by combinatory treatment administered at 3, 4, 5, and 6 h and were primarily restricted to subcortical areas (Fig. 5). Ischemic core, penumbra, and total lesion volumes were reduced by all treatments when administered up to 6 h after onset of MCAo (Fig. 6). There were no statistical differences between all treatment groups.

Discussion

In the first part of this study, we have shown that LAU-0901 and AT-NPD1 substantially improve behavioral function and reduce the volume of cerebral infarction when administered promptly at moderate doses, which are potentially amenable to clinical applications. In the second portion of the study, we demonstrated a broad therapeutic window of neuroprotective efficacy with moderate doses of LAU-0901 and AT-NPD1. Treatment initiated even 6 hours after stroke onset of ischemia was highly effective. The present study was prompted by our earlier finding demonstrating the neuroprotective efficacy of LAU-0901 and AT-NPD1 in focal cerebral ischemia. We discovered that these lipid mediators promote neuronal cell survival with important anti-inflammatory activity.

Inflammation is currently considered a prime target for developing new stroke therapies.[20] Several pre-clinical and clinical proof-of-concept studies have suggested the effectiveness of pharmacological interventions that target inflammation post-stroke. The biological activity of LAU-0901 and AT-NPD1 due to specific activation or modulation of signaling pathways was investigated. We questioned whether joint inhibition and inflammation resolution of

both processes would be more neuroprotective than either on its own. Emerging evidence suggests that platelets contribute crucially to inflammation and immune responses. PAF is a potent phospholipid regulator of inflammation that exerts its effect via binding specific PAFR. PAF is pivotal in central and peripheral immune systems and can mediate platelet aggregation and leukocyte functions.[21,22] It has comprehensive biological functions, such as promoting the synthesis of cytokines and activating platelet aggregation systems.[22] PAFR is a G protein-coupled seven-transmembrane receptor activating multiple intracellular signaling pathways and exists in microglia, neurons, and astrocytes in CNS.[23] Once released, activation of PAFR results in acute inflammation, which can contribute to the clinical manifestation of I/R injury.[24] Many researchers investigated the related underlying mechanisms because inflammation is influenced by the delicate balance between PAF/PAFR activation and pyroptosis. It has been demonstrated that 1) PAFR deficiency promoted neuroprotection in global cerebral ischemia in PAFR$^{-/-}$ mice,[25] 2) knockout of PAFR decreased the expressions of IL-1β, TNF, and IL-6 in the hippocampus, prevented neuroinflammation and brain dysfunction after traumatic brain injury in mice,[26] and 3) PAFR-deletion induced reduction of ROS after myocardial I/R injury in mice.[27]

While basal levels of PAF are virtually undetectable in resting tissues, PAF is rapidly synthesized in the brain during ischemia, mediates the release of the neurotransmitter glutamate in the hippocampus, and selectively activates a network of early response gene expression, including the rapid induction of COX-2 and TNFα.[7] During ischemia, PAF synthesis and degradation rates no longer maintain a modulated PAF pool size; PAF accumulates and becomes a pro-inflammatory messenger and a mediator of neurotoxicity.[7] The level of PAFR was increased following one hour of MCAo in mice and OGD/R injury, indicating that PAFR tangled in ischemic brain injury.[22]

Our earlier findings demonstrated the neuroprotective efficacy of LAU-0901, a highly potent and selective PAFR antagonist in acute focal cerebral ischemia in rats and mice.[13,14] LAU-0901 therapy significantly reduced cortical and subcortical infarct volumes, improved behavioral scores, attenuated microglia infiltration, and promoted astrocytic and neuronal survival when administered promptly after 2 h of MCAo in rats.[28] We demonstrated that mice treated with LAU-0901 after one hour of MCAo increased local cerebral blood flow by 77% of baseline at 6h compared to an LCBF of 41% relative to baseline observed in the vehicle-treated rats.[13] In our long-term survival study, 60 mg/kg of LAU-0901, administered 2 h after onset of stroke in rats, not only yielded short-term improvements by day 7 and resulted in beneficial effects on the behavioral outcome that persisted up to 4 weeks after MCAo.[14] In addition, LAU-0901-treated animals, resulted in a 20% increase in preserved brain tissue at 30 days, a remarkable reduction in the incidence of extensive cystic necrotic lesions, and markedly increased GFAP and Nissl-positive cell count.[14]

Fatty acids and lipid mediators derived from fatty acids can potentially influence the immune system.[29] Lipid mediators are produced through conserved biosynthetic pathways involving specific enzymes, which function on lipid precursors released from cell membranes.[29] There are several families of lipid mediators, which can be divided into pro-inflammatory lipid mediators and the more specialized pro-resolving lipid mediators (SPMs). Pro-inflammatory lipid mediators include prostaglandins and leukotrienes, while

SPM has lipoxins, resolvins, maresins, and protectins.[30] SPMs derived from DHA and eico-sapentaenoic acid (EPA), including families of resolvins and protectins, have played a significant role in resolving inflammation.[31] Complete mechanisms of action for SPMs have not been identified, but data indicate G-protein-coupled receptors on leukocytes bind SPMs to reduce infiltration and promote tissue regeneration.[31] Thus, endogenous SPMs, particularly those derived from omega-3 fatty acids, may represent a valuable target in shifting the balance of neuroinflammatory processes from inflammation-driving to inflammation-resolving conditions in the damaged central nervous system.[32]

Recently, we reported the discovery of novel AT-NPD1, a potent anti-inflammatory pro-resolving molecule.[15] The new aspirin-triggered product demonstrated decisive regulatory actions with leukocytes in vivo, reduced human polymorphonuclear (PMN) transendothelial migration, and enhanced efferocytosis.[15] As proposed for other potent polyunsaturated fatty acid-derived local mediators, the aspirin-triggered protectin pathway likely involves the conversion of the hydroperoxide 17R-HpDHA to an epoxide intermediate.[15] This aspirin-triggered pathway was first identified in the resolution phase of inflammatory exudates and brain tissues of mice treated with aspirin, and its basic structure was proposed.[33,34] We demonstrated that novel docosanoids inhibited brain I/R-mediated leukocyte infiltration and pro-inflammatory gene expression.[33] We identified a novel biosynthetic pathway that leads to the formation of an AT-NPD1 mediator when aspirin plus DHA are administered after stroke.[16] Then, we performed the total chemical synthesis of this molecule and tested it in the set- ting of 2 h MCAo in rats. We have shown that synthetic AT-NPD1, delivered at 3 h after stroke onset, improves behavioral scores, reduces brain infarction and brain edema, improves tissue matrix (ADC), and protects white matter.[16]

The current study established efficacy and dose-response relation for the neuroprotective effect of LAU-0901 and AT-NPD1 in a rat model of focal cerebral ischemia. The most remarkable result was found in rats treated with medium and high doses of LAU-0901 and AT-NPD1. All LAU-0901 and AT-NPD1 doses used in this study were highly neuroprotective. LAU-0901 (60 mg/kg) and AT-NPD1 (222 and 333 μg/kg) treatments alone were the most effective in improving behavioral scores and reducing brain lesions on day seven after MCAo. The neuroprotective effect was enhanced using the LAU-0901 +AT-NPD1, which improved behavior throughout the 7-day survival period and dramatically reduced total, core, and penumbra volumes by 93, 94, and 92% compared to the vehicle group. Due to the challenges of treating patients on time, an extension of the therapeutic window is necessary to allow for broader appeal to clinicians treating ischemic stroke victims. We asked if LAU-0901 plus AT-NPD1 could prolong the therapeutic window in a setting of focal cerebral ischemia. We selected a dose in the middle of the effective dose range of AT-NPD1 (222 μg/kg) and LAU-0901 (60 mg/kg) for our therapeutic window study, which proved effective even with a delay of treatment up to 6 h. LAU-0901 plus AT-NPD1, when administered at 3, 4, 5, and 6 h, improved behavior by 50, 56, 33, and 26% compared to the vehicle. The effect of combinatory treatment was demonstrated clearly in the cortical (penumbral region) and subcortical (ischemic core) of the infarct and reduced total T2WI volumes at 3 h by 93%, 4 h by 91%, at 5 h by 82%, and 6 h by 84% compared to the vehicle. The exact mechanisms of LAU-0901 plus AT-NPD1 remain obscure. We can speculate that

joint inhibition and inflammation resolution by LAU-0901 and AT-NPD1 provides additive neuroprotection in experimental stroke.

The beneficial effect of LAU-0901 and AT-NPD1 has been shown in a well-controlled animal model of MCAo.[18] Intraluminal suture occlusion of the MCA has become increasingly popular as a focal ischemia model because of its simplicity and minimally invasive. We employed a poly-L-lysine-coated suture and have found that this technique produces consistent subcortical and cortical lesions (coefficient of variation of infarct volume, 9%).[18] This infarction closely mimics, in extent and severity, the large hemispheric infarcts resulting from proximal MCA and internal carotid artery occlusion in patients. Essential to the consistency of the model is the close monitoring of physiological variables, including body and cranial temperature. Cranial temperature control is critical, as it is an essential determinant of the extent of brain injury. We demonstrated that LAU-0901 and AT-NPD1 did not directly affect body and brain temperature, arterial blood pressure, and blood gases and did not produce any adverse side effects.

Conclusion

We have shown here for the first time that LAU-0901 plus AT-NPD1 treatment affords high-grade neuroprotection in MCAo, equaling or exceeding that afforded by LAU or AT-NPD1 alone, at considerably moderate doses. It has a broad therapeutic window extending to 6 h after stroke onset. This 6-hour time frame is clinically relevant in that it is logistically challenging to institute therapy in many patients with acute ischemic stroke at early times. Taken together, this finding suggests that this combinatory treatment offers great promise in the treatment of patients with acute ischemic stroke.

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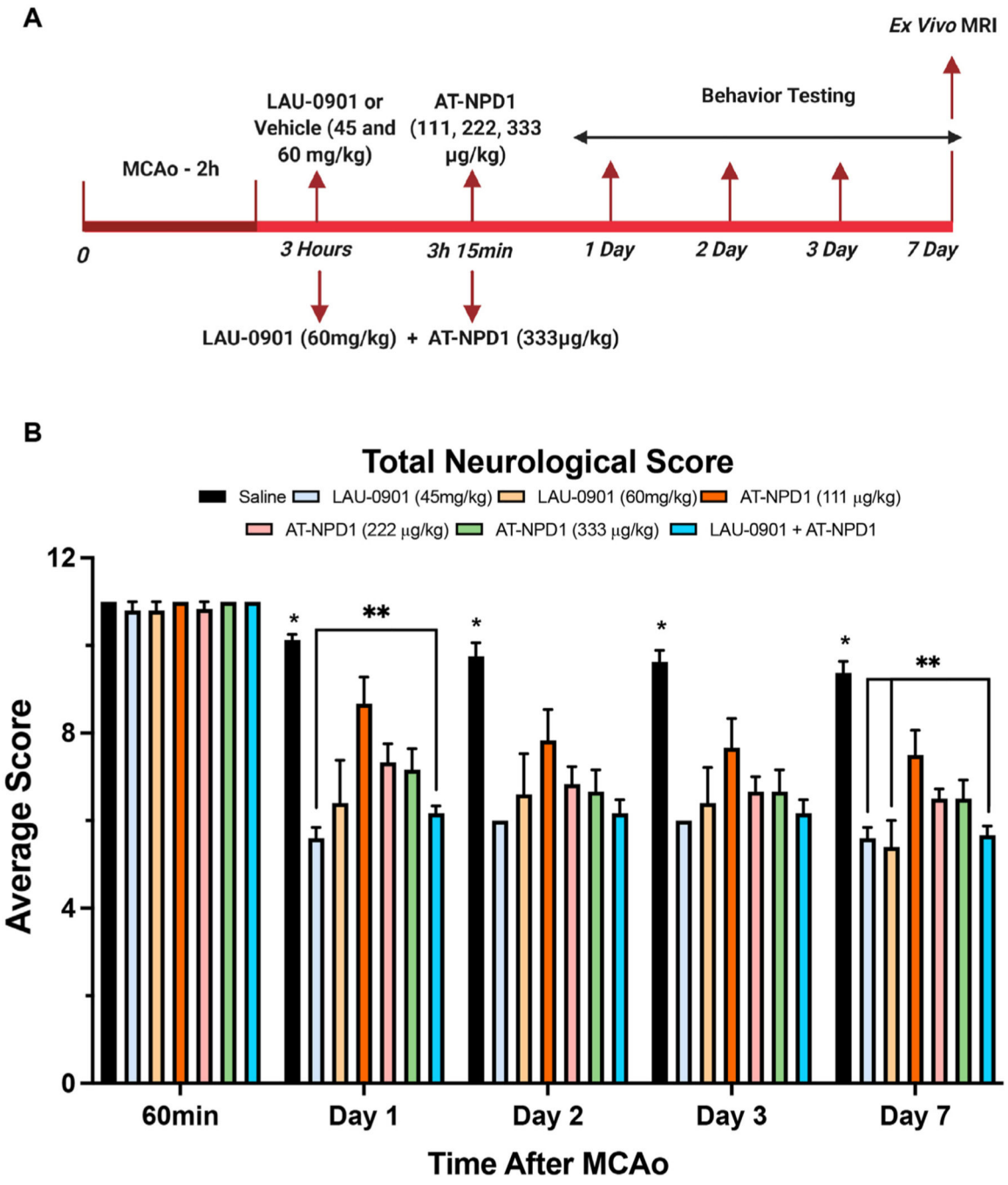

Fig. 1.

Dose-Response study: LAU-0901, AT-NPD1, and their combination improved functional recovery after MCAo. (A) Experimental design. (B) Total neurologic score (normal=0, maximal deficit=12) in rats during MCAo (60 min) and various times after MCAo. All doses of LAU-0901 and AT-NPD1 and combinatory treatment significantly improved the total neurological score on days 1, 2, 3, and 7 compared to the vehicle group. Values shown are means ± SE (n=5–8 per group). *P < 0.05, vehicle vs all treatments; ** LAU-0901+AT-

NPD1 vs AT-NPD1 (111 and 222 μg/kg) (Repeated-measures ANOVA followed by Bonferroni tests).

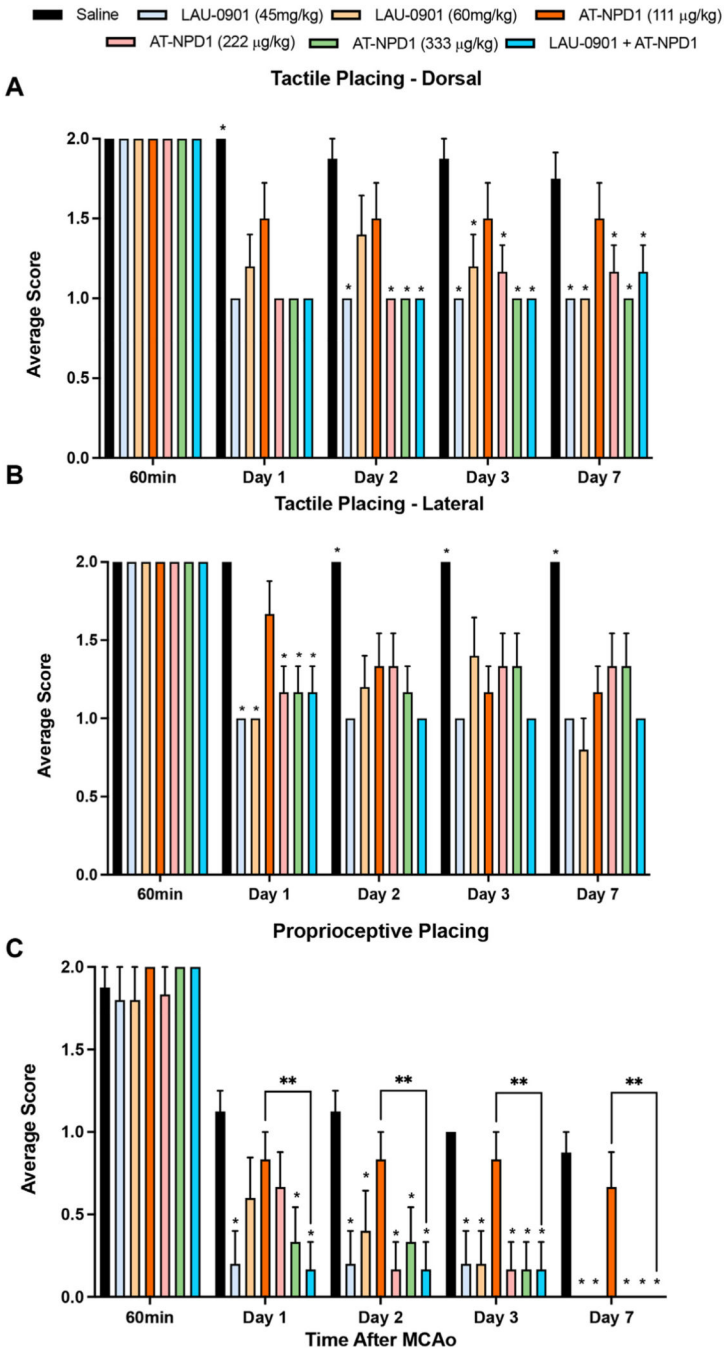

Fig. 2.

(A-C) Time-course of recovery of tactile (dorsal and lateral) and proprioceptive forelimb placing reactions (normal=0, maximal deficit=2). Values shown are means ± SE (n=5–8 per group). *P < 0.05, vehicle vs. all treatments; ** LAU-0901+AT-NPD1 vs. AT-NPD1 (111 μg/kg) (Repeated-measures ANOVA followed by Bonferroni tests).

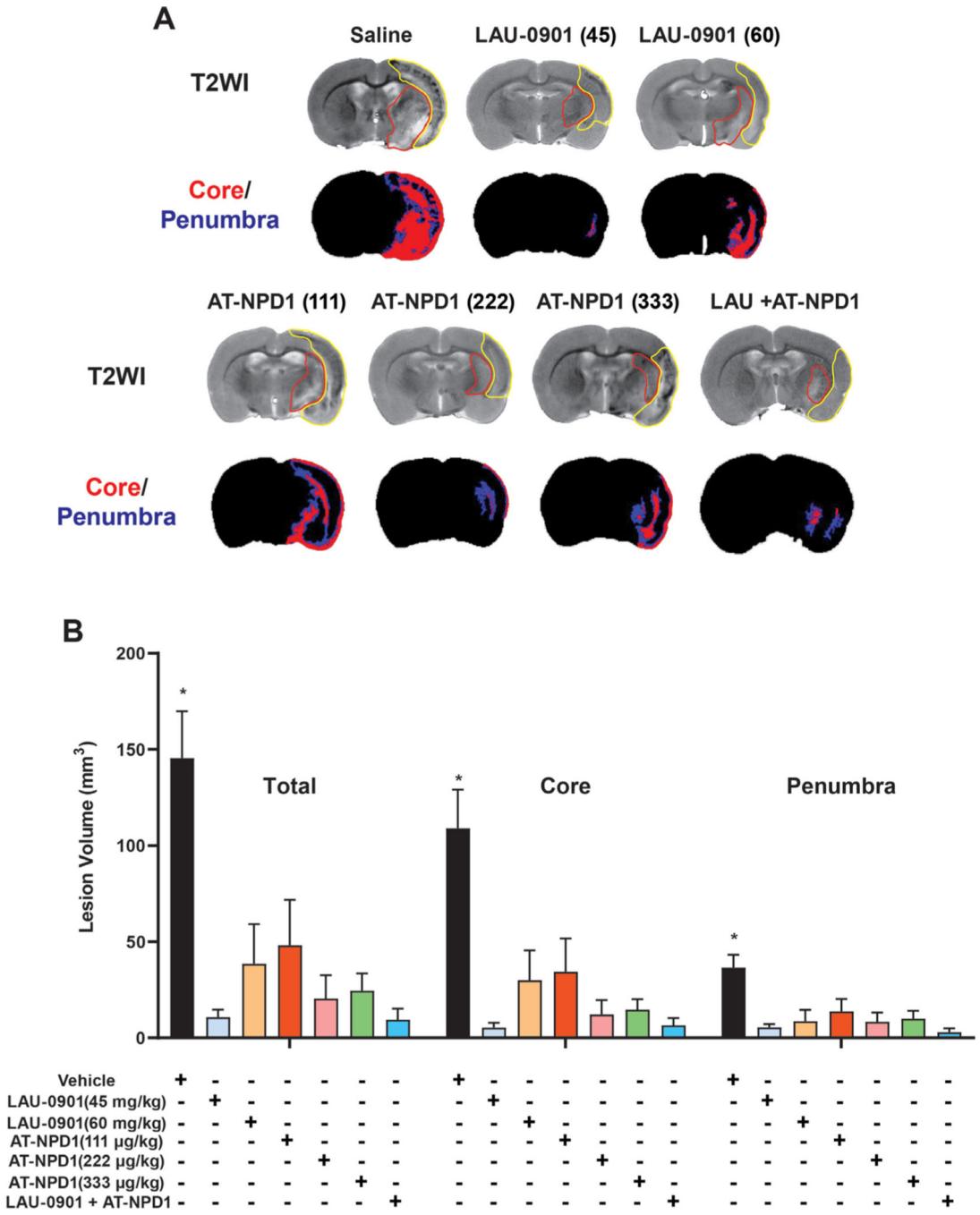

Fig. 3.

LAU-0901, AT-NPD1, and their combination attenuated MRI lesion volumes and ischemic core and penumbra after stroke. (A) Representative T2-weighted images (T2WI) and core/penumbra from each group on day 7. Core (red) and penumbral (blue) tissues were automatically extracted from the entire brain using the MRI method hierarchical region splitting for penumbra identification. T2 hyperintensities were observed in vehicle-treated rats' ischemic core and penumbra, consistent with edema formation. In contrast, all-treated animals had smaller lesion sizes. (B) All treatments significantly reduced total lesion

volumes, computed from T2WI on day 7 compared to the vehicle group. Values shown are means ± SE (n=5–8 per group). *P < 0.05, vehicle vs. all treatments (Repeated-measures ANOVA followed by Bonferroni tests).

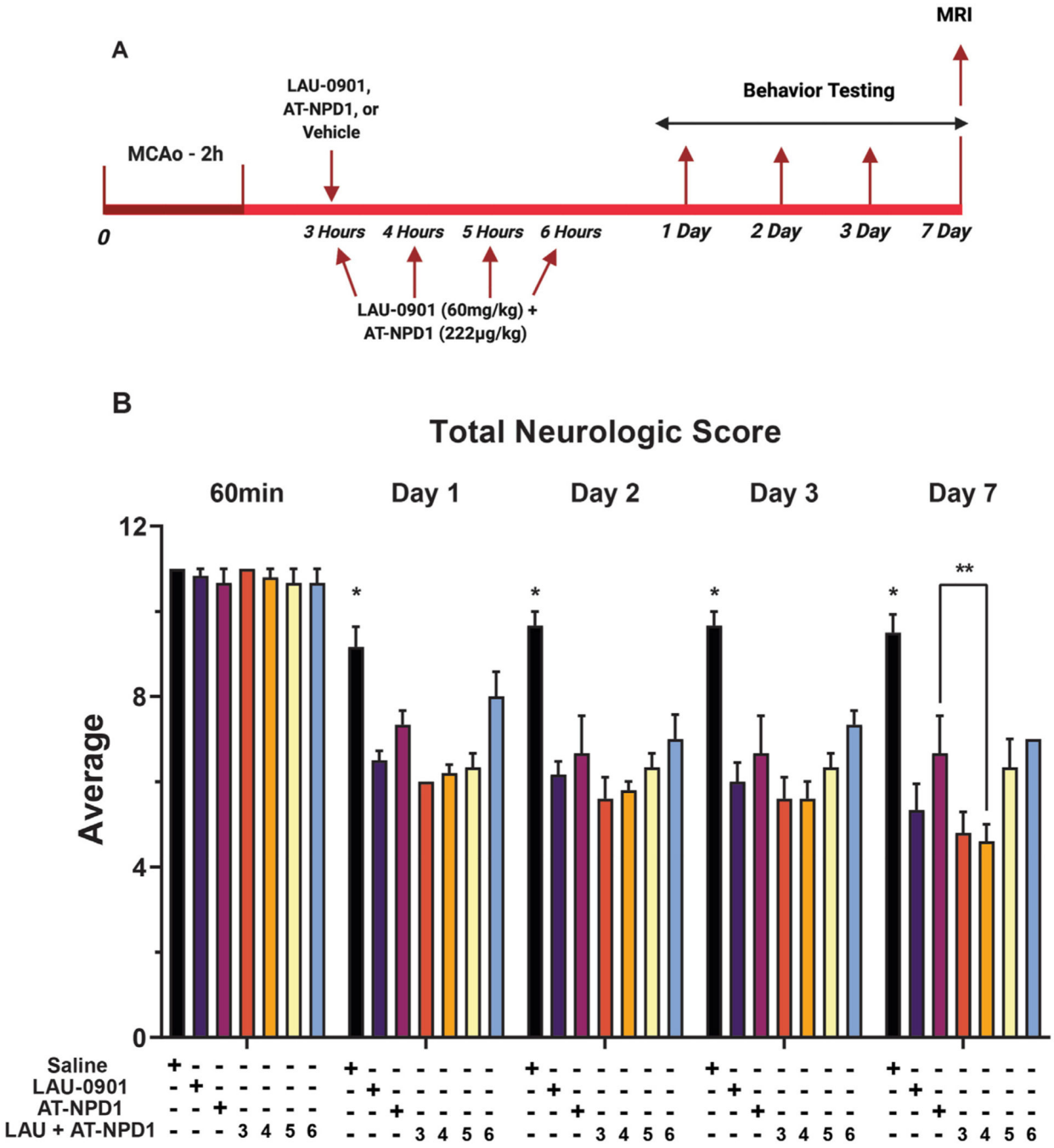

Fig. 4.

Therapeutic window study. LAU-0901, AT-NPD1, and their combination improve neurological scores when administered up to 6 h following MCAo. (A) Experimental design. (B) Total neurological score (normal=0, maximal deficit=12) in all groups. Values shown are means ± SE (n=5–9 per group). *P < 0.05, vehicle vs. all treatments ** LAU-0901+AT-NPD1–4h vs. AT-NPD1 (Repeated-measures ANOVA followed by Bonferroni tests).

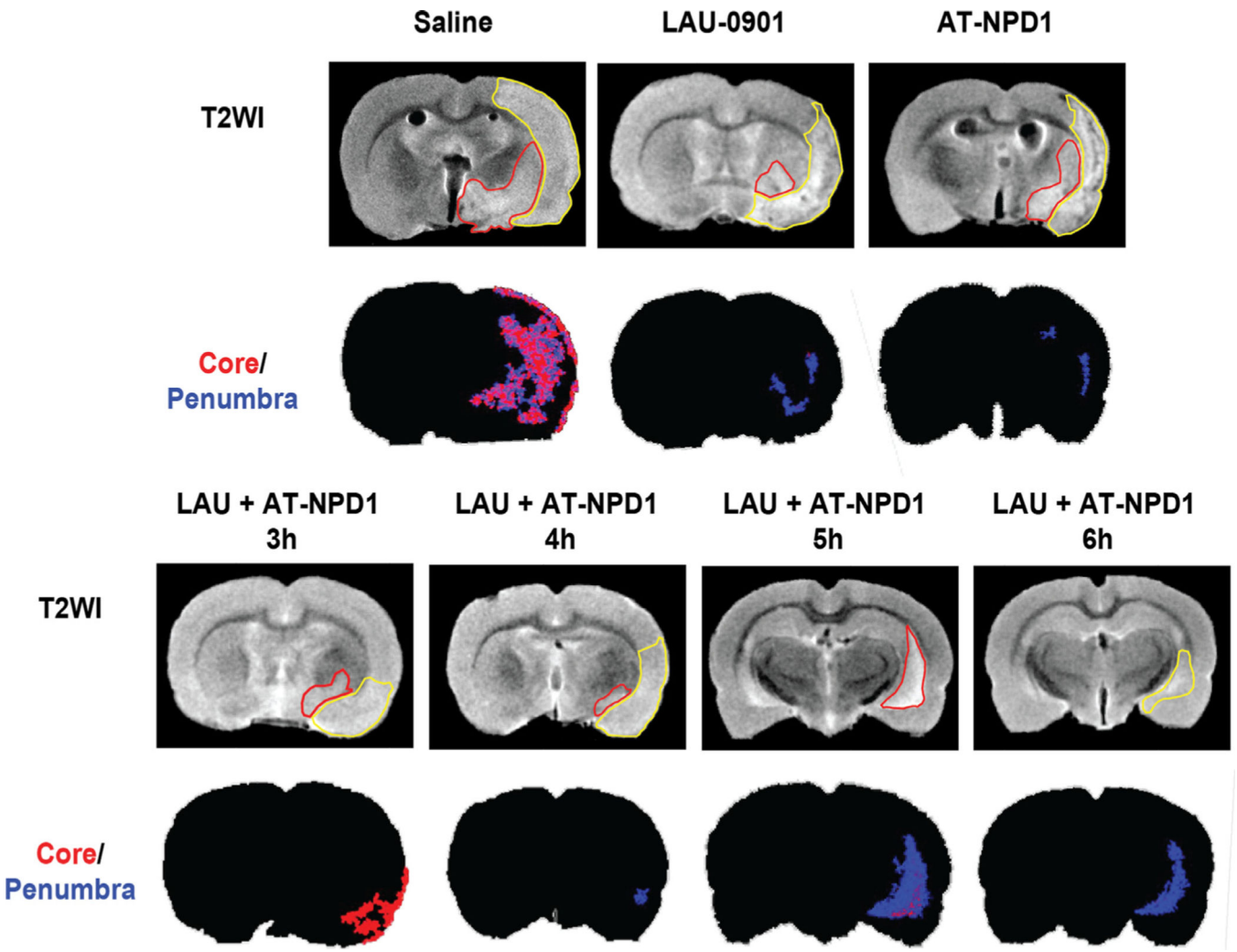

Fig. 5.

LAU-0901, AT-NPD1, and their combination reduce MRI lesions and protect the ischemic core and penumbra. (A) Representative T2WI and core/penumbra from the vehicle and all treated groups. Core (red) and penumbral (blue) tissues were automatically extracted from the entire brain. T2 hyperintensities were observed in the ischemic core and penumbra of vehicle-treated rats, consistent with edema formation. In contrast, all-treated animals had smaller lesion sizes, primarily located in subcortical areas.

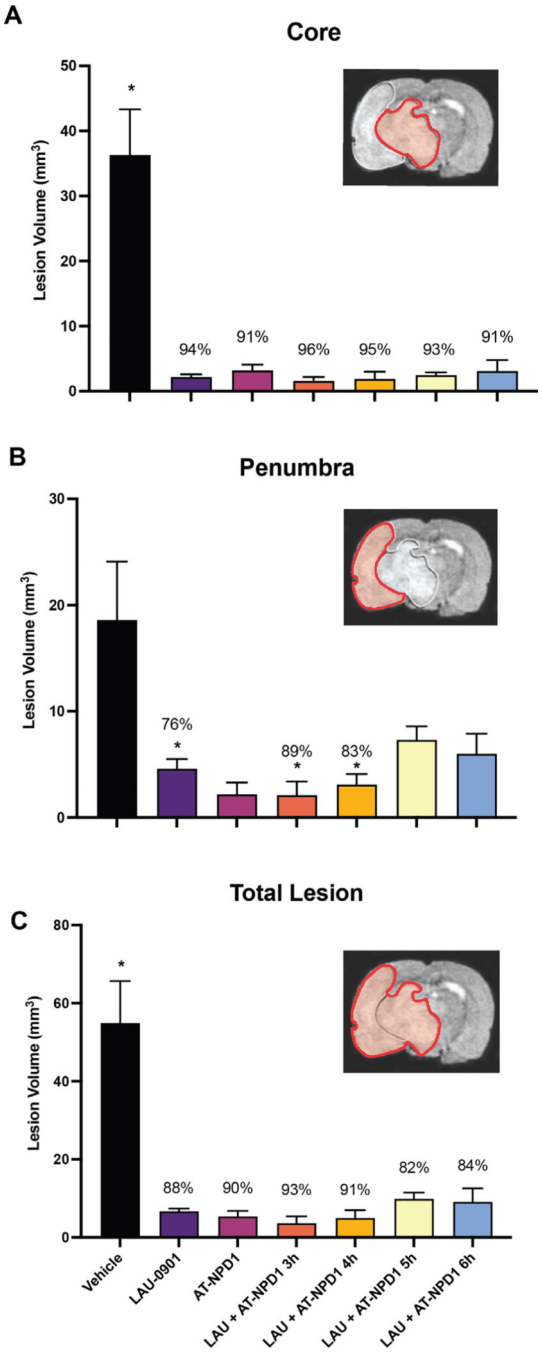

Fig. 6.

(A-C) Cortical, subcortical, and total lesion volumes were computed from T2WI maps. All treatments dramatically reduced lesion volumes. Values shown are means ± SE (n=5–9 per group). *P < 0.05, vehicle vs. all treatments (Repeated-measures ANOVA followed by Bonferroni tests).