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Effects of a new thrombolytic compound LT3001 on acute brain tissue damage after focal embolic stroke in rats

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Abstract

LT3001 is a novel synthetic small molecule with thrombolytic and free radical scavenging activities. In this study, we tested the effects of LT3001 as a potential alternative thrombolytic in focal embolic ischemic stroke rat model. Stroke rats received intravenous injection of 10 mg/kg LT3001 or tPA at 1.5, 3 or 4.5 hours after stroke, respectively, and all outcomes were measured at 24 hours after stroke. We performed multi-parametric MRI, 2,3,5-triphenyltetrazolium chloride (TTC) staining and neurological severity score. Lastly, we assessed the effect of LT3001 on tPA activity in vitro, international normalized ratio (INR), and serum levels of active tPA and plasminogen activator inhibitor-1 (PAI-1). LT3001 treated at 1.5 hours after stroke is neuroprotective by reducing the CBF lesion size, lowering diffusion and T2 lesion size measured by MRI. Non-TTC-stained infarction was also reduced. When treated at 3 hours after stroke, LT3001 had significantly better therapeutic effects regarding reduction of infarct size, swelling rate and hemorrhagic transformation compared to tPA. When treated at 4.5 hours after stroke, tPA, but not LT3001, significantly increased brain swelling and intracerebral hemorrhagic transformation. Lastly, LT3001 did not interfere tPA activity in vitro, or significantly alter INR, serum levels of active tPA and PAI-1 in vivo. Our data suggests that LT3001 is neuroprotective in focal embolic stroke rat model. It might have thrombolytic property, not interfere tPA/PAI-1 activity, and cause less risk of hemorrhagic transformation compared to conventional tPA. Taken together, LT3001 might be developed as a novel reagent for treating thrombotic ischemic stroke.

Key Words

Embolic stroke, Thrombolytic reperfusion therapy, Brain infarction, Hemorrhagic transformation

Introduction

Ischemic stroke is a devastating thrombotic cerebrovascular event. Despite numerous basic and clinical research efforts that have been made in developing an effective therapeutic for ischemic stroke, intravenous administration of recombinant tissue plasminogen activator (rt-PA) remains the only FDA-approved and effective pharmacological intervention for acute ischemic stroke [1, 2], which is intended to reopen occluded vessels by lysis of the thrombus, thereby improving clinical outcome through restoration of regional cerebral blood flow (CBF) and salvage of ischemic brain tissues [3]. However, one major limitation of tPA thrombolysis is the poor reperfusion rate, which results in only partially or fully successful recanalization in about 50% of patients [4, 5]. Accumulating experimental data suggests that exogenous tPA may also have additional actions within the brain [4], such as direct vasoactivity [6, 7], enhanced excitotoxicity [8] and activation of extracellular proteases [8-12]. These non-thrombolytic actions of tPA may exacerbate edema, increase ischemic neurotoxicity, damage the blood brain barrier, increase the risk of cerebral hemorrhage and ultimately compromise its usefulness as a thrombolytic agent [6, 13, 14]. Thereby, seeking an alternative or making more effective and safer thrombolytic reperfusion therapy for managing ischemic stroke is considered clinically significant.

The concept of saving ischemic penumbra is still central to the neuroprotection after ischemic stroke [2, 15]. Very excitingly, emerging application of mechanical thrombectomy (MT) has also resulted in a significant advance in the acute management of ischemic stroke [16]. It has been proposed that successful microvascular reperfusion restoring or maintaining a certain level of cerebral blood flow is essential to the neuroprotection in any type of acute ischemic stroke, and a prerequisite for ischemic brain tissue survival [15]. Thus, pharmacologically maximizing the rate of brain reperfusion, which might overcome the tPA-related poor thrombolytic perfusion efficacy, is

an important target in the current stroke neuroprotection research [17, 18].

Nevertheless, both tPA thrombolytic and mechanical thrombectomy used as the reperfusion therapy may also worsen brain tissue damage, a phenomenon called reperfusion injury including BBB integrity disruption, brain edema, and hemorrhagic transformation [17, 18]. Experimental studies have suggested that increased generation of reactive oxygen species (ROS) plays a key role in reperfusion injury [17, 19], such as oxidative stress suppression or ROS scavenging in combination with reperfusion therapy can decrease reperfusion injury and improve stroke outcome [20]. Recently, the combination of tPA plus antioxidant has been enrolled into a clinical trial [21]. Clearly, a strategy for optimizing thrombolytic therapy requires rebalancing between the potential benefits of reperfusion and the detrimental effects of reperfusion-associated edema and hemorrhagic transformation [10, 22].

Taken together, approaches aimed at attaining both effective reperfusion and lower reperfusion injury might improve the efficacy and safety of stroke reperfusion therapy. Based on two abovementioned principles, a novel small synthetic molecule LT3001 was designed, which is structurally modified from a small thrombolytic peptide [23] that has a triple active molecular structure for thrombolysis, anti-thrombosis and free radical scavenging. In current study, we for the first time evaluated the therapeutic effects of LT3001 in embolic clot and mechanical filament-induced focal ischemic stroke rat models. The thrombolytic reperfusion efficacy of LT3001 was compared with conventional tPA treatment. Acute brain tissue damage including ischemic brain infarction, brain swelling rate, and intracerebral hemorrhagic transformation were assessed and compared.

Methods

Animals

Male Wistar rats weighing from 280-320g (Charles River Laboratories, Wilmington, MA) were used in this study. LT3001 was provided from Lumosa Therapeutics, Taiwan (licensed by the USA patent-US2016/0083423A1). The therapeutic effects of LT3001 were tested in two types of focal ischemic stroke model, focal embolic MCAO and permanent intraluminal MCAO. All experiments were performed following the protocols approved by Massachusetts General Hospital Institutional Animal Care and Use Committee in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Focal cerebral ischemia models and experimental groups

Embolic focal stroke model in rats: Rats were subjected to focal embolic stroke as previously described [24, 25]. This model closely simulates the clinical situation in ischemic stroke patients and has been substantially applied in stroke research testing thrombolytics [26]. Regional cerebral blood flow (rCBF) was continuously monitored by the laser Doppler flowmetry (LDF) before and after embolization. Animals underwent same procedure without clot injection were set as sham controls. At 1.5, or 3, or 4.5 hours after stroke, rats were treated with either saline, or conventional rat-based dose of tPA (Actilyse, Boehringer Ingelheim International GmbH, 10 mg/kg, 10% bolus followed by i.v. infusion the remnant for 30 minutes), or LT3001 (10 mg/kg, i.v. infusion for 15 minutes). The relatively high dose of tPA was chosen based on the data showing approximately 10-fold difference in fibrin-specific enzyme activity between human and rodent [27]. The dose selection of LT3001 was based on our previous study demonstrating LT3001 at 10 mg/kg was effective in restoring cerebral blood flow after $FeCl_3$ induced brain vessel occlusion in adult mice (data not shown). Randomization, blinding and statistical powering in this translational study were

all performed in accordance with STAIR and RIGOR guidelines [28, 29]. Inclusion criteria were set as follows: rCBF is stable and lower than 30% of pre-ischemic baseline after clot injection, no significant autorestoration of rCBF at the end of the monitor process after surgical procedure. Animals dead within 24 hours with subarachnoid hemorrhage were excluded from the study as we previously described[30].

Permanent intraluminal MCAO model in rats: A method involving mechanical vascular occlusion was used to induce focal ischemia in rats via a standard intraluminal approach as previously described [31]. In brief, an incision was created in the ventral aspect of the neck region to expose internal, external, and common carotid arteries. A 4-0 nylon monofilament suture coated with silicon tip was inserted through the external carotid artery into the origin of middle cerebral artery to induce permanent focal ischemia. Successful occlusion was determined by a sharp decrease (at least 70% reduction) in baseline rCBF. At 3 hours after stroke onset, animals were intravenously treated with LT3001 (10mg/kg, i.v. infusion for 15 minutes) or saline.

MRI imaging and data analysis

MRI scans were performed on a 4.7 Tesla small-bore scanner (Bruker Biospec, Erlangen Germany) with a dual RF coil setup, including a 70 mm volume transmitter coil and an actively-decoupled 20 mm surface receiver coil. During MRI, the animals were placed on a plastic cradle with the head fixed with a tooth bar and ear bars to minimize motion. Multi-slice MRI (5 slices, slice thickness/gap=1.8/0.2 mm, field of view=20x20 mm^2, image matrix=48x48) was acquired with echo-planar imaging (EPI) (receiver bandwidth = 225 kHz), with the central slice positioned at 2 mm posterior to bregma. Diffusion MRI was obtained using single-shot isotropic diffusion-weighted MRI with two b-values of 250 and 1,000 s/mm^2 (repetition time (TR)/echo time (TE) = 3250/54 ms, 16 averages, scan time=2 min).[32] T_2-weighted spin-echo images were obtained with

two TE of 30 and 100 ms (TR = 3,250 ms, 16 averages; scan time=2 min).[33] Perfusion imaging was acquired with amplitude-modulated continuous arterial spin labeling (ASL) MRI (TR/Saturation time/TE = 6500/3250/14.8 ms, 32 averages, scan time = 7 min).

Data were processed in MATLAB (MathWorks, Natick, MA). Apparent diffusion coefficient (ADC) maps were calculated as $DC = \frac{\ln(I(b1)/I(b2))}{b2-b1}$, where $I(b_{1,2})$ are diffusion-weighted signals obtained at two diffusion b values (250 and 1,000 s/mm^2). T2 map was calculated as $T_2 = \frac{TE2-TE1}{\ln(I(TE1)/I(TE2))}$, where $I(TE_{1,2})$ are T2-weighted signals obtained at two echo times (TE= 30 and 100 ms). Cerebral blood flow (CBF) map was obtained from ASL MRI[34] and derived as $CBF = \frac{\lambda(I_{ref}-I_{tag})\cdot e^{w/T_{1a}}}{2\alpha\cdot I_{ref}\cdot T_{1w}}$, where I_{tag} is the label image, I_{ref} is the reference image, λ is the brain-blood partition coefficient for water, α is the degree of inversion with transient time correction, w is the post-labeling delay, T_{1w} is the apparent longitudinal relaxation time measured from inversion recovery sequence and T1a is the arterial blood longitudinal relaxation time.[35] The size of ischemic lesions in perfusion-CBF, diffusion-ADC, and T2 maps were automatically defined using a K-means clustering-based algorithm.[36] Briefly, a 1D vector of all the pixels within the brain was formed and clustering algorithm used an iterative refinement technique to partition the data points in the vector into two clusters (lesion vs. normal tissue) based on their intensities.

Assessments of neurological function deficits

The modified neurological severity score (mNSS) was evaluated before giving treatment and at 24 hours after stroke by following a standard method as previously described [37]. Briefly, this scoring system consists a composite of motor, sensory, reflex and balance measurements. One point is gained for failure to accomplish the task or showing a measured reflex. Normal score is recorded as

0 whereas maximal deficit score is 18. Score 1 to 6 indicates mild injury, 7 to 12 indicates moderate injury and 13 to 18 indicates severe injury.

Measurements of brain infarction, swelling rate, and intracerebral hemorrhagic transformation

To examine acute brain tissue outcomes, rats were euthanized at 24 hours after stroke. Intracerebral hemorrhagic transformation (HT) was semi-quantified as the HT index on eight coronal brain slices (2 mm thick) of each rat as previously described [38]. Briefly, a blinded investigator measured the macroscopic HT using a four-point scale and the average score of totally eight brain slices of each animal was reported. Brain slices were then stained with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, USA) to quantify infarct size and swelling rate using computer-assisted image analysis as we previously described [39, 40]. Rats died within 24 hours after stoke were counted for mortality rate.

Plasmin activity assay in vitro

The effect of LT3001 on the plasmin activity was tested in an in vitro fluorogenic assay by following a standard method as previously described[41]. Briefly, various concentrations of LT3001, tPA, N-terminal lysine plasminogen (2.5 mg/mL) and, a fluorogenic plasmin substrate, D-Val-Leu-Lys-AMC (200 nmol/L) were mixed to a final volume of 100μL followed by immediately adding to wells of 96-well culture plate for incubation at 37°C for 30 mins. Subsequently, plasmin activity was read on a fluorescent plate reader at excitation 360 nm and emission 460 nm. The plate readings were expressed as relative fluorescent units for each well, and the final result was represented as fold of plasmin activity generated by saline alone.

Measurements of serum active tissue type plasminogen activator (tPA) and active plasminogen activator inhibitor (PAI-1) levels

At 24 hours after stroke, whole blood of each anesthetized rat was collected into EDTA-coated blood collection tubes. Serum was isolated and stored at -80°C before use. Rat tPA ELISA Kit and Rat PAI-1 ELISA Kit (Abcam, USA) were used to detect serum levels of active tissue plasminogen activator (tPA) and active plasma plasminogen activator inhibitor-1 (PAI-1), respectively, according to the manufacturer's instruction.

INR measurement

The potential effect of tPA or LT3001 on coagulation was indicated by the measurement of International Normalized Ratio (INR), which was read by the INR coagumeter (Roche, Germany) using a small amount of blood collected from the tail cut as previously descried [42].

Statistical analysis

All data are expressed as mean ± SEM. For parametric measurements, we used one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc comparisons. For nonparametric ordinal data, we used nonparametric Kruskal-Wallis followed by post hoc Mann-Whitney tests. A P-value of less than 0.05 was considered statistically significant.

Results

LT3001 increases ischemic brain tissue perfusion when treated at 1.5 hours after focal embolic stroke in rats

Firstly, we tested the thrombolytic efficacy of LT3001 in a standard focal embolic stroke rat model. Rats were treated with intravenous infusion of LT3001 (10 mg/kg) or rat dose of conventional tPA (10 mg/kg) at 1.5 hours after embolization. We measured the lesion size in CBF (perfusion), ADC (diffusion) and T2 maps by MRI before and 1 hour after drug administration, as well as 24 hours after stroke onset, respectively. In acute phase (before drug administration) after stroke, no significant difference in lesion size in CBF map (perfusion-deficient) between saline, tPA and LT3001 groups was observed (Fig. 1a). However, at 1 hour after drug administration, both conventional tPA group (23.5% reduction) and LT3001 group (15.6% reduction) group have shown significantly smaller CBF lesion size (perfusion-deficient) compared to saline control group (Fig. 1b). At 24 hours after stroke, this pro-perfusion effect was maintained in both treated groups, the perfusion-deficient lesion size had 25% reduction in tPA group and 25.5% reduction in LT3001 group (Fig. 1c).

LT3001 reduces brain infarction, swelling rate and neurological deficits when treated at 1.5 hours after focal embolic stroke in rats

Importantly, despite we did not detect significant difference in ischemic lesion size in ADC map between three groups at either acute phase or 1 hour after drug administration, ADC ischemic lesion size was significantly reduced at 24 hours after stroke in both treated groups compared to saline control group (Fig. 2a-c). Consistently, T2 ischemic lesion size analyzed at 24 hours after stroke in both tPA and LT3001 groups was also reduced (47.2% and 33.8%, respectively), but a significant difference was only observed between tPA group and saline control group. We also performed TTC

staining on brain slices at 24 hours after stroke, infarction volume (% of the hemisphere) was significantly reduced by tPA (31.2 ± 5.4%, 41.7% reduction) and LT3001 (32.8 ± 6.3%, 38.7% reduction) compared to saline control group (53.6 ± 2.9%) (Fig. 2e). Additionally, brain swelling rate (% of the hemisphere) was non-significantly decreased in tPA group (32.1% reduction) as well as LT3001 group (28% reduction) (Fig. 2f). Furthermore, both tPA and LT3001 significantly lowered the mNSS score compared to the pre-treatment baseline or post-stroke saline control group (Fig. 2g).

LT3001 reduces 24-hour brain infarction, swelling rate, intracerebral HT and neurologic deficits when treated at 3 hours after focal embolic stroke in rats

Next, we tested and compared effects of intravenous infusion of LT3001 (10 mg/kg) or rat dose of conventional tPA (10 mg/kg) at 3 hours after embolization. At 1 day after stroke, both tPA and LT3001 reduced brain infarction volumes compared to saline controls. However, the reduction in infarction volume by tPA treatment (infarct size: 39.8 ± 3.5%) was non-significant compared to saline control group (infarct size: 48.3 ± 1.5%). But surprisingly, we observed that LT3001 induced a significant decrease in infarction volume (infarct size: 28.1 ± 3.0%) compared to other two groups (41.8% reduction vs saline and 29.4% reduction vs tPA) (Fig. 3a). We also found the brain swelling rate was exacerbated in tPA treated group (17.4% increase) compared to saline control group, but was not increased in LT3001 group (Fig. 3b). Furthermore, neurologic deficit examined by the mNSS score were significantly lowered in both tPA group and LT3001 group (22.3% and 33.8% reduction compared to the saline group, respectively), and LT3001 also significantly lowered the mNSS score compared to the pre-treatment level. However, there was no significant difference in mNSS score between tPA and LT3001 treatment group (Fig. 3c). Additionally, we semi-quantified intracerebral hemorrhage on fresh brain slices before TTC staining at 1 day after stroke, which was presented as hemorrhagic transformation (HT) index. There was a significantly higher HT index

(63.5% increase) in the tPA group (1.57 ± 0.22) than saline group (0.96 ± 0.13). Interestingly, the HT index was significantly lower in LT3001 group (0.85 ± 0.12) compared to tPA group (45.9% reduction) (Fig. 3d). Moreover, mortalities within 24 hours after stroke were 29% (5/17) in the saline group, 25% (4/16) in tPA group and 25% (4/16) in the LT3001 group, however, no significant differences between three groups were detected. These results suggest that LT3001 (10 mg/kg) treated at 3 hours after focal embolic stroke in rats are still neuroprotective, and might have better therapeutic performance than conventional tPA, although its therapeutic effects and underlying mechanisms warrant further validation and investigation.

To ask whether LT3001 has reperfusion-independent neuroprotective effect, we also tested the effect of intravenous infusion of LT3001 (10 mg/kg) given at 3 hours after permanent MCAO in rats. Brain infarction and swelling rate were measured at 24 hours after stroke. Our result showed that there were no significant differences in brain infarction and swelling rate between LT3001 treated and saline control groups (Supplement Fig. 1).

LT3001 does not increase 24-hour brain swelling rate and intracerebral HT when treated at 4.5 hours after focal embolic stroke in rats

In a separate experiment, we given treatment in an extended the treatment time window (4.5 hours after stroke) using the same focal embolic stroke rat model. However, there were no significant differences in 24-hour brain infarct size (Fig. 4a), mNSS score (Fig. 4c), and mortality rate (30.7% in saline group, 35.7% in tPA group and 30.7% in LT3001 group) between saline, 10 mg/kg tPA, and 10 mg/kg LT3001 group. Notably, significantly increased brain swelling rate as well as HT index by tPA treatment were not observed in LT3001 group (Fig. 4b, 4c). These results suggest that LT3001 significantly lowered the risk of intracerebral hemorrhage compared to conventional tPA treatment when given at 4.5 hours after stroke.

LT3001 does not alter endogenous tPA and PAI-1 activity, and INR levels at 24 hours after focal embolic stroke in rats

We performed the cell-free tPA-converting plasmin activity in vitro assay to ask whether LT3001 interfere with endogenous thrombolysis, and found that LT3001 has no capability of converting plasminogen to plasmin, and does not directly alter tPA-converted plasmin generation or activity (Fig. 5a). Furthermore, we measured serum active tPA level and PAI-1 activity at 1 day after stroke in the 3-hour treatment experiment. In saline control group, there was a significantly elevated tPA activity (194% increase) but PAI-1 activity remains unchanged compared to sham controls. In the tPA treatment group, serum tPA activity was significantly decreased (48% reduction), but PAI-1 activity was significantly increased (138.8% increase) compared to the saline group. However, in the LT3001 group, there were no significant alterations in serum tPA and PAI-1 activities compared to saline group at 24 hours after stroke (Fig. 5b, 5c). We did not detect any significance in INR measurements between each group or time points (Fig. 5d). These results suggest that LT3001 might not interfere with circulating factors affecting thrombolytic activity, such as tPA and PAI-1 measured in this study, although its thrombolytic activity and underlying molecular mechanisms warrant further investigation.

Discussion

In this animal study, we performed a series of experiments to evaluate potential therapeutic effects of LT3001 (intravenous administration, 10 mg/kg) in a clinically relevant and well-established focal embolic rat model. In the first set of experiment, we asked whether LT3001 has thrombolytic activity and neuroprotective effects when treated at 1.5 hours after stroke onset. By measuring the ischemic lesion size in CBF, ADC and T2 MRI maps, respectively, we demonstrated that LT3001 exerts thrombolytic efficacy comparable to the conventional tPA therapy in focal embolic stroke rat model. Reduced CBF lesion size can be detected as early as 1 hour after treatment and maintained up to 24 hours post-stroke in both tPA and LT3001 group. ADC lesion size reduction was significant at 24 hours post-stroke in both treated groups. No significant difference being observed in reduction of T2 lesion size in tPA and LT3001 group indicates the sample size expansion may be required. We also performed 24-hour TTC brain infarction examination, the results showed both LT3001 and tPA treated at 1.5 hours after stroke significantly reduced brain infarction, which is consistent with our ADC findings. Importantly, these experimental data, based on a relatively reproducible rat model of focal embolic stroke with similar ischemic severity in all groups before receiving different treatments, demonstrated that the possible mechanism underlying LT3001-induced infarction reduction might be, at least in part, contributed by its thrombolytic reperfusion activity.

In the following experiment, we asked whether LT3001 is still neuroprotective when initiated at 3 hours after stroke. 24-hour brain infarction assay showed that conventional tPA only slightly reduced the brain infarction. However, LT3001 significantly reduced brain infarction compared to other two groups. Furthermore, a significant worsening of brain swelling and intracerebral hemorrhagic transformation we observed in tPA group compared to saline controls were eliminated

in LT3001 group. Interestingly, in a filament-induced permanent focal stroke rat model, we found LT3001 had no effects on brain infarction and brain swelling when treated at 3 hours after stroke onset. Additionally, in a separate set of experiment using same focal embolic stroke rat model, there were no significant differences between three groups in brain infarct size, brain swelling rate, modified neurological severity score, and mortality when treated at 4.5 hours after stroke, However, a significantly increased HT index in tPA treatment group was not observed in LT3001 group. These results suggest that, when compared with conventional tPA treatment, LT3001 might have comparable or better therapeutic efficacy and less risk of hemorrhagic transformation in focal embolic stroke rat model, although its therapeutic effects and underlying molecular mechanisms warrant further validation and investigation. Most importantly, our experimental results suggest that LT3001 might be further developed as a novel and effective neuroprotectant for treating acute ischemic stroke.

One important mechanistic and safety concern is that whether LT3001 can directly interfere tPA activity or LT3001 may alter endogenous thrombolysis in stroke rats. To address these questions, we firstly performed tPA-converting plasmin generation assay in vitro. Our results demonstrate that plasminogen is not cleaved by LT3001 alone for plasmin generation, and LT3001 also does not interfere tPA-converted plasmin generation and activity. Secondly, we examined serum level of tPA and PAI-1 activity at 24 hours after stroke in rats. As expected, ischemic stroke *per se* triggered the elevation of endogenous tPA activity, however, tPA treated stroke rats showed a significant decrease in serum tPA activity with a significantly higher serum PAI-1 activity compared to saline group. This observation is consistent with our previous report explaining that, at 24 hours after stroke, the elevated PAI-1 activity might be contributed by tPA directly stimulated PAI-1 secretion from the vascular wall and platelet in the circulation, and lower tPA activity might be due to the shorter half-life of tPA that is neutralized by elevated PAI-1[43]. Lastly, we did not find significant

differences in INR measurements between each group or time point. These results suggest that thrombolytic role of LT3001 might be independent of tPA and PAI-1 activity. Indeed, thrombolytic mechanisms of LT3001 warrant further investigation.

However, we are aware of several limitations in current study. First, our experimental data indicate a thrombolytic property of LT3001, however, the detailed molecular mechanisms are still unknown, which warrants further investigations. Second, ROS scavenging might be another chemical property of LT3001, which was not examined in this study and needs to be defined in the future investigation. However, very interestingly, if the ROS scavenging function can be furtherly validated in animal studies, together with its thrombolytic activity and the lower risk of hemorrhagic transformation, LT3001 can potentially be a novel candidate for thrombolytic stroke therapy since the tPA/PAI-1 independent thrombolytic activity of LT3001 might promote total thrombolytic efficacy, while the ROS scavenging property of LT3001 might attenuate reperfusion injury. Third, the therapeutic time window may differ between the current focal embolic stroke rat model and real stroke patients. For example, a key criterion for initiating reperfusion therapy is the size of penumbra, which is highly variable and should be meticulously examined by CT perfusion or MRI perfusion imaging test in stroke patients [44, 45]. Forth, based on our current experimental data showing beneficial effect of LT3001 in focal embolic stroke rat model, the precise molecular mechanisms underlying its neuroprotective effects, other than thrombolytic and speculated ROS scavenging functions, remain to be fully elucidated. One more limitation is that although LT3001 did not show neuroprotection when treated at 3 hours after filament induced permanent MCAO in rats, we cannot simply conclude that LT3001 has no direct neuroprotective effects on ischemic brain tissue beside its thrombolytic reperfusion effect without conducting further investigations. For example, there are several possibilities may explain this result: the dose (10 mg/kg) for intravenous infusion of LT3001 might not be optimal, salvageable ischemic penumbra may be

significantly decreased at 3 hours after permanent MCAO, and neuroprotective role of LT3001 might be mainly reperfusion-dependent. Lastly, this study was proposed as a proof-of-concept investigation, all translational aspects, determination of optimal dose regimen and long-term outcomes, and testing and comparing both male and female animals, should be further evaluated in a well-controlled preclinical translational setting.

Summary

In summary, in this pilot pharmacological investigation, we found that LT3001 administration is neuroprotective in focal embolic stroke rat model. Its neuroprotective mechanisms might be partially related to its thrombolytic activity, which is tPA/PAI-1 activity independent. LT3001 showed certain thrombolytic efficacy but less risk of hemorrhagic transformation compared to the conventional tPA, although its therapeutic effects and precise underlying molecular mechanisms warrant further validation and investigation. LT3001 might be developed as a new and effective reagent for treating thrombotic ischemic stroke. However, further preclinical investigations are required to define underlying thrombolytic pharmacology and evaluate its translational potential.

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Compliance with Ethical Standards

All experiments were performed following protocols approved by Massachusetts General Hospital Institutional Animal Care and Use Committee in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Conflict of Interest

The authors declare no competing interests. LT3001 was provided from the Lumosa Therapeutics Co. Ltd, Taiwan (licensed by the USA patent-US2016/0083423A1).

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Figure Legends

Fig 1. LT3001 increases ischemic brain tissue perfusion when treated at 1.5 hours after focal embolic stroke in rats. a Representative images and quantification of ischemic lesions in cerebral blood flow (CBF) map immediately after stroke onset, **b** 1 hour post-treatment and **c** 24 hours post-stroke. N=6 per group. *P＜0.05 versus saline group.

Fig 2. LT3001 reduces 24-hour brain infarction, swelling rate and neurological deficits when treated at 1.5 hours after focal embolic stroke in rats. a Representative images and quantification of ischemic lesions in apparent diffusion coefficient (ADC) map immediately after stroke onset (before giving the treatment), **b** 1 hour post-treatment and **c** 24 hours post-stroke. **d** Representative images and quantification of ischemic lesions in T2 map at 24 hours post-stroke. Rat brains were harvested for further analysis after the 24-hour imaging test. **e** Representative images of TTC stained brain sections and the quantification of brain infarction volume at 24 hours post-stroke. **f** Quantification of brain swelling rate. **g** Modified neurologic severity score. N=6 per group. *P＜0.05 versus saline group; $P＜0.05 versus pre-treatment level.

Fig 3. LT3001 reduces 24-hour brain infarction, swelling rate, intracerebral hemorrhagic transformation and neurological deficits when treated at 3 hours after focal embolic stroke in rats. a Representative images of TTC stained brain sections and quantification of brain infarction volume. **b** Quantification of brain swelling rate. **c** Modified neurologic severity score. **d** Representative images of hemorrhagic transformation on brain sections and quantification of hemorrhagic transformation index. Yellow arrowheads indicate intracerebral hemorrhage. N=12 per

group. *P＜0.05 versus saline group; #P＜0.05 versus tPA group; $P＜0.05 versus pre-treatment level.

Fig 4. LT3001 does not increase 24-hour brain swelling rate and intracerebral hemorrhagic transformation when treated at 4.5 hours after focal embolic stroke in rats. a Representative images of TTC stained brain sections and quantification of brain infarction volume. **b** Quantification of brain swelling rate. **c** Modified neurologic severity score. **d** Representative images of hemorrhagic transformation on brain sections and quantification of hemorrhagic transformation index. Yellow arrowheads indicate intracerebral hemorrhage. N=9 per group. *P＜0.05 versus saline group.

Fig 5. LT3001 does not alter endogenous tPA thrombolytic activity and coagulation. a Plasmin activity assay in vitro. **b** Serum level of active tPA at 24 hours after stroke when treated at 3 hours after stroke onset. **c** Serum level of active PAI-1 at 24 hours after stroke when treated at 3 hours after stroke onset. **d** Measurement of international normalized ratio (INR) at different time points. N=4 or 6 per group. *P＜0.05 versus saline group; #P＜0.05 versus tPA group; &P＜0.05 versus sham group.

Supplemental Fig 1. LT3001 does not change 24-hour brain infarction and swelling rate when treated at 3 hours after intraluminal permanent MCAO in rats. a Representative images of TTC stained brain sections and quantification of brain infarction volume. **b** Quantification of brain swelling rate. N=10 per group.

Fig 1

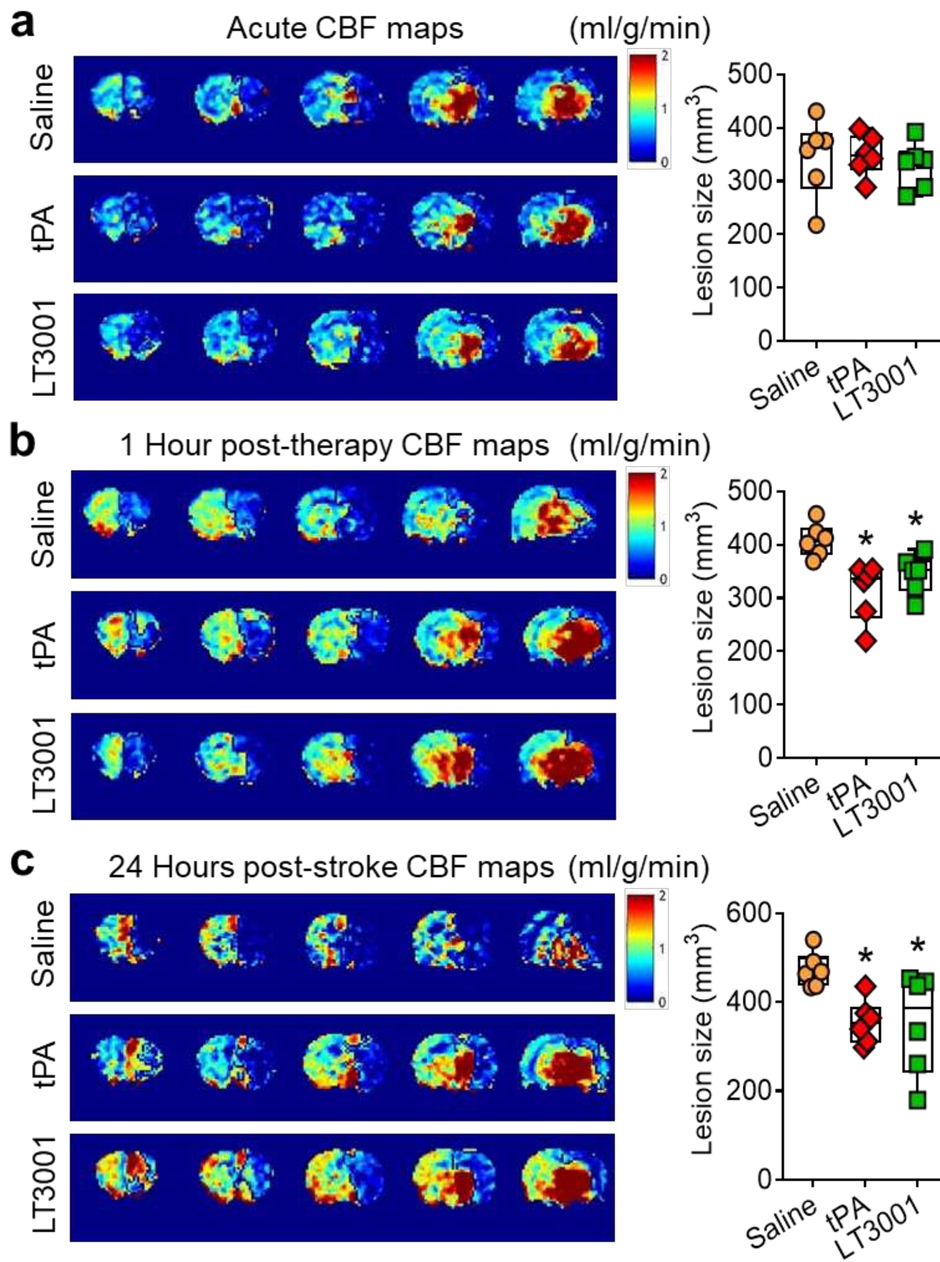


Fig 2

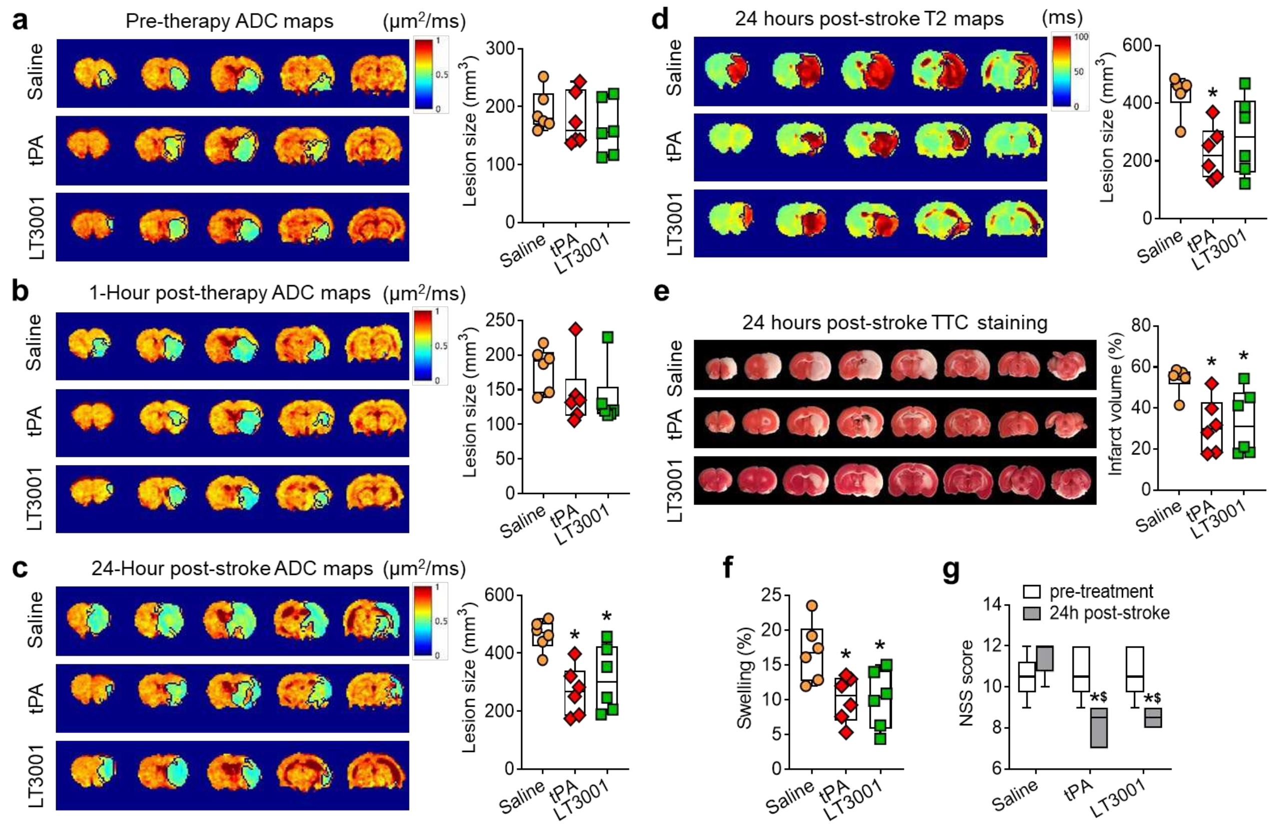
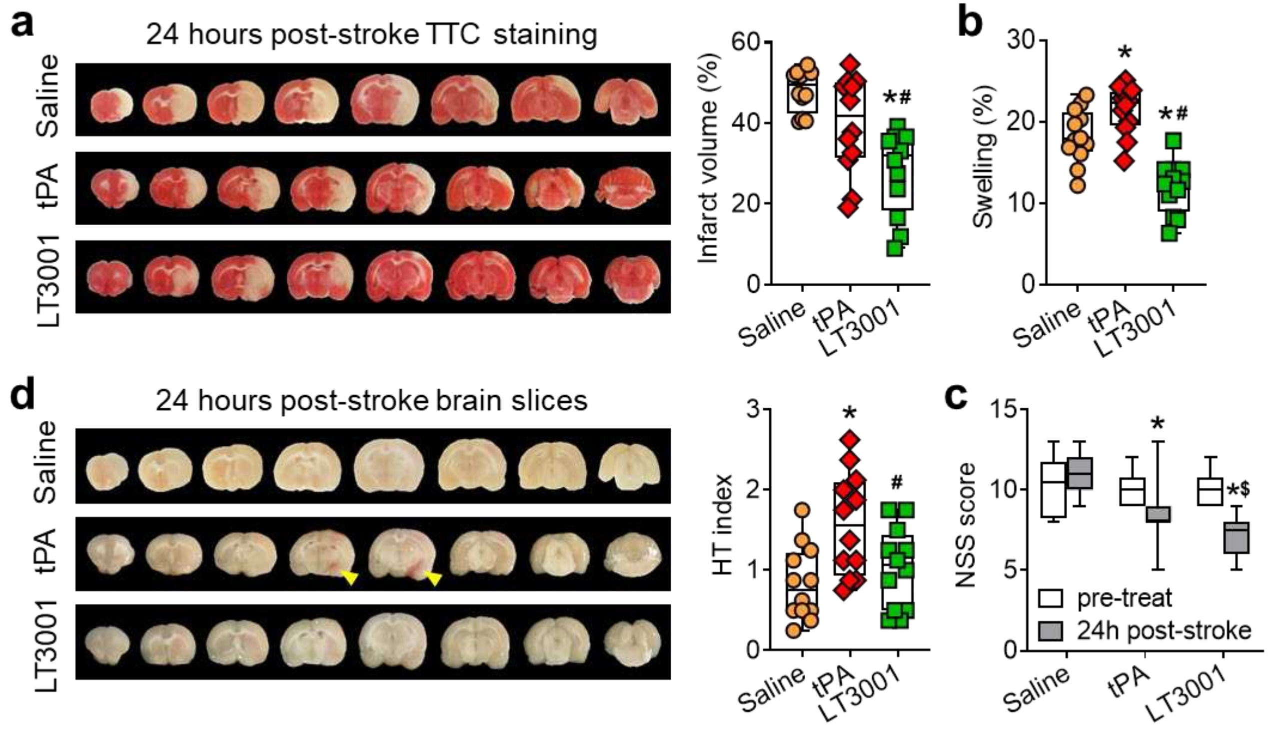


Fig 3



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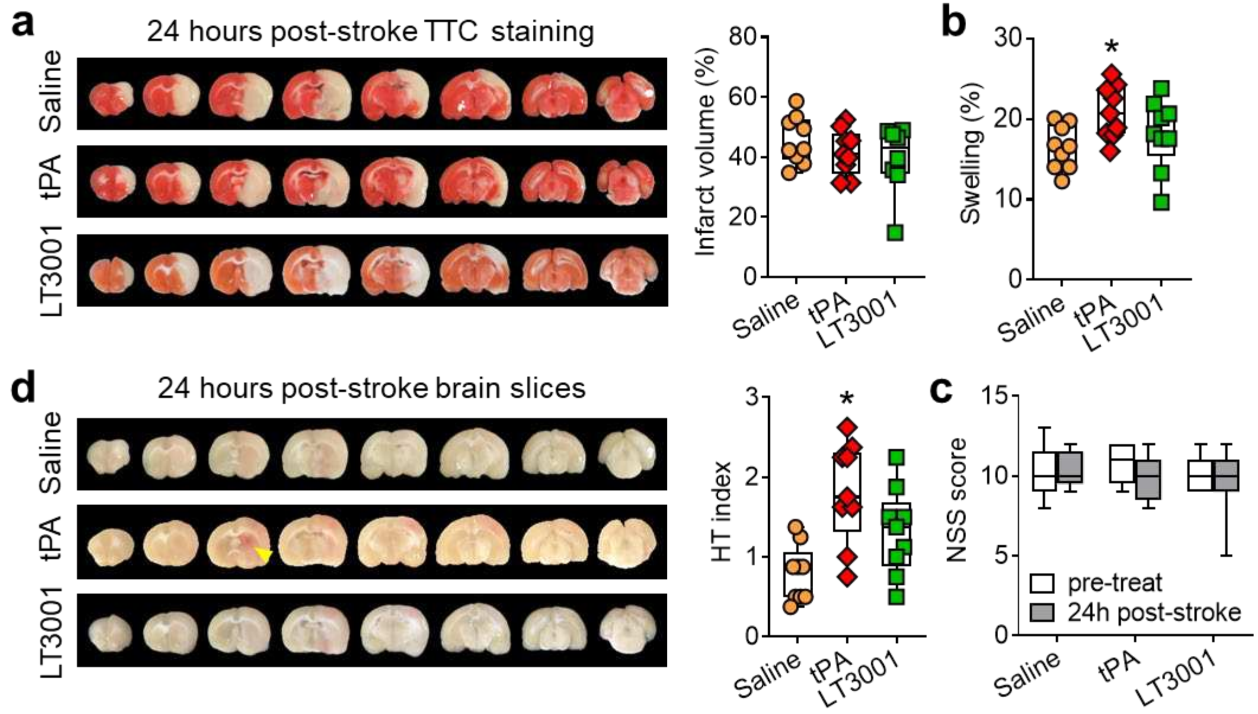
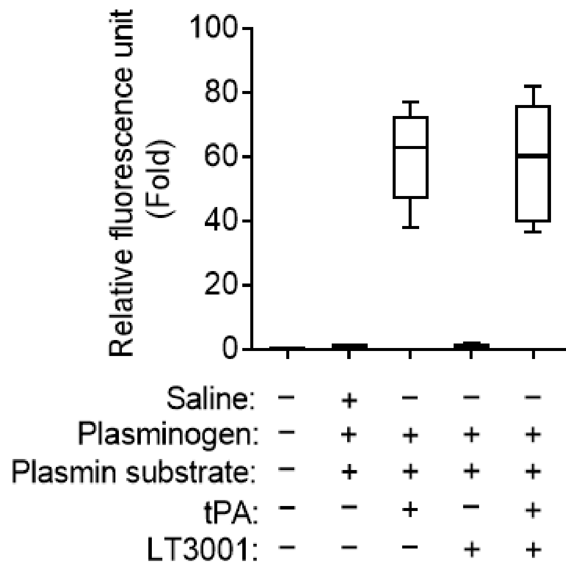
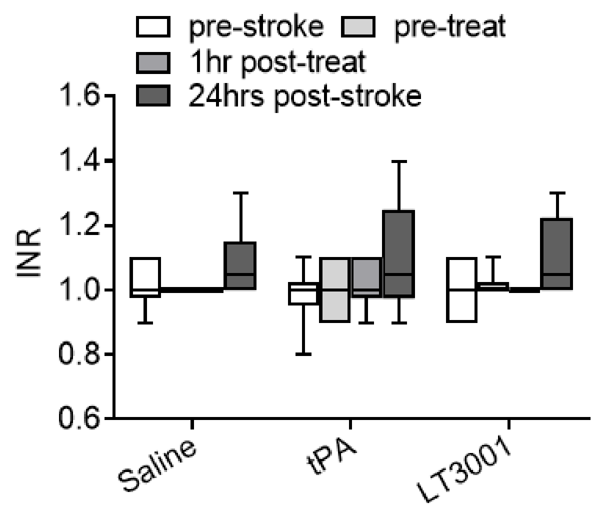
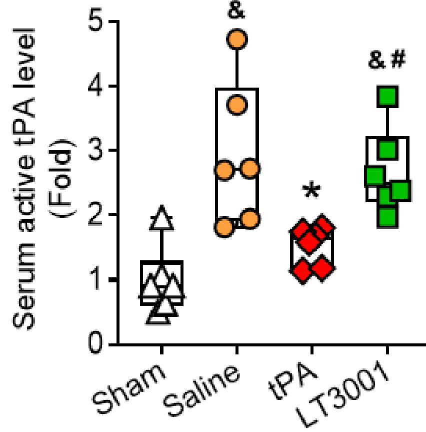
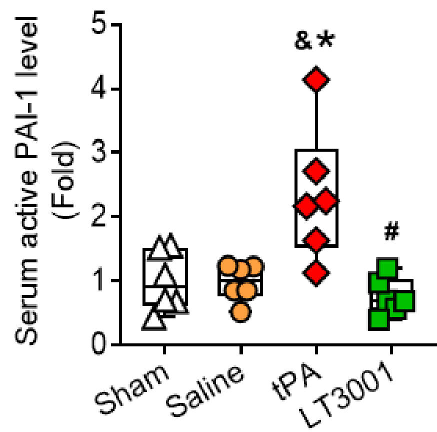


Fig 5









Supplemental Fig 1