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## Protection from Cerebral Ischemia by Inhibition of TGF $\beta$ -activated kinase

Benjamin J White, BS<sup>1</sup>, Sami Tarabishy, BS<sup>2</sup>, Venugopal Reddy Venna, PhD<sup>2</sup>, Bharti Manwani, MBBS<sup>2</sup>, Sharon Benashki, MS<sup>2</sup>, Louise D. McCullough, MD, PhD<sup>2,3,\*</sup>, and Jun Li, MSc., PhD<sup>2</sup>

<sup>1</sup>University of Connecticut School of Medicine, Farmington, Connecticut, USA

<sup>2</sup>Department of Neuroscience, University of Connecticut Health Center, Farmington, Connecticut, USA

<sup>3</sup>Departments of Neurology and Neuroscience, University of Connecticut Health Center, Farmington, Connecticut, USA

### Abstract

**Objective**—Transforming growth factor- $\beta$ -activated kinase (TAK1) is a member of the mitogen-activated protein kinase family that plays important roles in apoptosis and inflammatory signaling, both of which are critical components of stroke pathology. TAK1 has recently been identified as a major upstream kinase that phosphorylates and activates adenosine monophosphate-activated protein kinase (AMPK), a major mediator of neuronal injury after experimental cerebral ischemia. We studied the functional role of TAK1 and its mechanistic link with AMPK after stroke.

**Methods**—Male mice were subjected to transient middle cerebral artery occlusion (MCAO). The TAK1 inhibitor 5Z-7-oxozeanol was injected either intracerebroventricularly or intraperitoneally at various doses and infarct size and functional outcome after long term survival was assessed. Mice with deletion of the AMPK  $\alpha$ 2 isoform were utilized to assess the contribution of downstream AMPK signaling to stroke outcomes. Levels of pTAK1, pAMPK, and other TAK1 targets including the pro-apoptotic molecule c-Jun-N-terminal kinase (JNK)/c-Jun and the pro-inflammatory protein cyclooxygenase-2 were also examined.

**Results**—TAK1 is critical in stroke pathology. Delayed treatment with a TAK1 inhibitor reduced infarct size and improved behavioral outcome even when given several hours after stroke onset. This protective effect may be independent of AMPK activation but was associated with a reduction in JNK and c-Jun signaling.

**Conclusions**—Enhanced TAK1 signaling, via activation of JNK, contributes to cell death in ischemic stroke. TAK1 inhibition is a novel therapeutic approach for stroke as it is neuroprotective with systemic administration, has a delayed therapeutic window, and demonstrates sustained neuroprotective effects.

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\*Corresponding author: Louise D. McCullough Department of Neuroscience, University of Connecticut Center, 263 Farmington Avenue, Farmington, CT 06030, USA telephone: 860-679-2271; fax: 860-679-1181 lmcullough@uchc.edu.

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## Keywords

TAK1; AMPK; stroke; JNK

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## INTRODUCTION

Transforming-growth-factor-beta-activated kinase-1 (TAK1), also known as mitogen-activated protein kinase kinase kinase-7 (MAPKKK7), is a mitogen-activated protein kinase that can be activated by TGF- $\beta$ , tumor necrosis factor-alpha (TNF- $\alpha$ ) and other cytokines including interleukin-1 (IL-1) (Ninomiya-Tsuji, et al., 1999, Sakurai, et al., 2003, Yamaguchi, et al., 1995). Activation of TAK1 requires association with TAK1-binding protein 1 (TAB1) and phosphorylation at multiple sites including threonine-184 and threonine-187 (Sakurai, et al., 2000, Shibuya, et al., 1996). The function of TAK1 has been studied in a number of cell lines with conflicting results. Several studies have demonstrated an anti-apoptotic response to TAK1 activation (Omori, et al., 2008, Thiefes, et al., 2005), however others have suggested that TAK1 is pro-apoptotic via activation of downstream JNK signaling (Sorrentino, et al., 2008). TAK1 is also emerging as a possible contributing factor to pro-inflammatory signaling through induction of the transcription factor AP-1 with subsequent expression of inflammatory genes including *COX-2* (Kumar, et al., 2009, Ninomiya-Tsuji, et al., 2003, Zhang, et al., 2010). Inflammation and apoptosis both contribute to ischemic neuronal cell death (Sims and Muyderman, 2010, Tuttolomondo, et al., 2009), and due to the delayed activation of these pathways after injury, may represent an attractive target for the development of neuroprotective agents.

TAK1 has been recently identified as one of the three upstream kinases that phosphorylates and activates adenosine monophosphate-activated protein kinase (AMPK), a key metabolic enzyme and sensor of cellular metabolic state (Li, et al., 2010). AMPK's activity is primarily regulated by the cellular AMP:ATP ratio (Omori, et al., 2006) but AMPK can also be activated by other cellular stressors including ischemia and hypoxia. AMPK is activated by phosphorylation at Threonine 172 via an upstream kinase triggering a cascade of events that reduces the activity of anabolic enzymes and increases catabolic pathways, thereby maintaining ATP levels. Although activation of AMPK appears to be a protective adaptive response to stress in peripheral tissues, in the brain, ischemia-induced AMPK activation exacerbates injury by enhancing metabolic failure and inducing lactic acidosis (Li, et al., 2010, Li and McCullough, 2010, Li, et al., 2007). Deletion of the catalytic isoform of AMPK responsible for phosphorylation and activation of AMPK is neuroprotective, as is pharmacological inhibition of AMPK. We hypothesized that inhibition of TAK1, due to its function as an upstream activator of AMPK (Li and McCullough, 2010), would lead to a reduction in stroke-induced AMPK phosphorylation and subsequent neuroprotection. TAK1 was recently shown to be activated in rodents after neonatal hypoxic-ischemic injury (Nijboer, et al., 2009), in a rodent model of middle cerebral artery occlusion (MCAO), and *in vitro* after oxygen glucose deprivation (Neubert, et al., 2011) but the signaling pathways induced by TAK and actions on AMPK signaling are unknown. In the present study, the function of TAK1 was studied in a rodent model of ischemic stroke using the highly specific small molecule TAK1 inhibitor 5Z-7-oxozeaenol (Ninomiya-Tsuji, et al., 2003, Sicard, et al., 2009) and putative downstream targets of TAK1 were investigated.

## MATERIALS AND METHODS

### Stroke model

C57BL/6 mice were purchased from Charles River Laboratories (Willimantic, Connecticut, USA). Experiments were performed according to NIH guidelines for the care and use of

animals in research and under protocols approved by the University of Connecticut Health Center Animal Care and Use Committee. Prior to experiments, animals were randomized into vehicle and drug groups. All treatments were performed by a blinded investigator. Focal transient cerebral ischemia was induced in male mice (20 to 25g) by 90 minutes of right middle cerebral artery occlusion (MCAO) under isoflurane anesthesia followed by reperfusion as described previously (Kilic, et al., 2002, Li, et al., 2007). In a separate cohort, physiological variables such as mean arterial blood pressure and cortical cerebral perfusion data were obtained as previously described (Li, et al., 2010). In this cohort, LDF was measured continuously from a baseline value pre-stroke (100%) through the ischemic period and for 30 minutes after reperfusion was initiated. The LDF data was calculated by averaging the LDF values taken at 15 minutes intervals, then provided as the percentage of the baseline value (the LDF value before the suture was inserted).

### Drug treatment

The TAK1 inhibitor 5Z-7-oxozeaenol (Neubert, et al., 2011) (Tocris Bioscience) was dissolved in DMSO. 5Z-7-oxozeaenol was injected intracerebroventricularly (0.32  $\mu$ g or 1.6  $\mu$ g in 2  $\mu$ L of DMSO). Additional cohorts were treated with intraperitoneal doses (0.5 mg/kg in 10% DMSO in PBS; pH = 7.4 or 5.0 mg/kg in DMSO/PBS). Control mice were injected with vehicle. Animals were randomly assigned to treatment cohorts. Separate cohorts were injected with 5 mg/kg IP of 5Z-7-oxozeaenol with delayed dosing, either 2 hours or 3.5 hours after onset of ischemia.

### Functional outcome measurements

Please refer to supplemental materials.

### Tissue staining and Infarct Quantification

24 hours after stroke, mice were euthanized and brains removed and cut into 5 2-mm coronal slices which were stained with a 1.5% 2,3,5-triphenyltetrazolium (TTC) solution at 37°C for 10 minutes. The stained slices were fixed with 4% formalin, images were digitalized, and the infarct volumes (corrected for edema) were analyzed using SigmaScan Pro5 as previously described (Li, et al., 2010). A separate cohort was subjected to the same procedure 7 days following stroke.

### Western blotting

Brains were obtained 4 hours after stroke onset. Please refer to supplemental materials for methods (Li, et al., 2010, Li and McCullough, 2010, Li, et al., 2007).

### Statistical analyses

Data from individual experiments are presented as mean  $\pm$  SEM, except for neurological deficit scores, which are presented as mean  $\pm$  interquartile range. Non-parametric data was log-transformed and compared using ANOVA (SPSS 17, SAS Corporation, Cary, NC). Parametric data was compared using ANOVA with Bonferroni correction for multiple comparisons where appropriate. When an effect of an intervention or characteristic was shown by ANOVA, the appropriate groups were compared with Student's t-test or Mann-Whitney U-test with Z approximation, as appropriate.  $p < 0.05$  was considered statistically significant. All behavioral and histological assessments were performed by an investigator blinded to drug treatment.

## RESULTS

### Intracerebroventricular application of 5Z-7-oxozeaenol reduced stroke injury

ICV administration of 0.32 ug of 5Z-7-oxozeaenol resulted in no significant differences in infarct volume (Cortex:  $53.66 \pm 5.72\%$  for vehicle versus  $39.01 \pm 7.15\%$  for drug,  $p = 0.151$ ; Striatum,  $48.89 \pm 7.98\%$  for vehicle versus  $33.41 \pm 7.01$  for drug,  $p = 0.142$ ; in whole-hemisphere,  $44.58 \pm 5.67\%$  for vehicle versus  $32.58 \pm 5.93\%$ ,  $p = 0.149$ ,  $n = 7$  vehicle, 9 drug). However, injection of a higher dose (1.6 ug) significantly reduced cortical (Fig. 1a) ( $59.04 \pm 4.98\%$  for vehicle versus  $35.00 \pm 7.10\%$  for drug,  $p = 0.014$ ) and whole-hemisphere infarct volume ( $52.30 \pm 4.12\%$  for vehicle versus  $31.79 \pm 5.36\%$  for drug,  $p = 0.008$ ,  $n = 6$  vehicle, 7 drug). Although there was a trend towards neuroprotection in the striatum, this did not reach statistical significance ( $54.89 \pm 4.95\%$  for vehicle versus  $39.89 \pm 6.03\%$  for drug, ( $p = 0.066$ )). ICV injection of low dose (0.32 ug) of 5Z-7-oxozeaenol had no effect on stroke-induced behavioral deficits ( $p = 0.081$ ,  $n = 7$  vehicle, 9 drug). However, at the higher dose (1.6 ug), there was a significant improvement in behavioral scores reflecting the neuroprotective effect of the drug (3(0) for vehicle-treated mice versus 2(0) for drug-treated mice,  $p = 0.038$ ,  $n = 6$  vehicle, 7 drug).

### Systemic injection of 5Z-7-oxozeaenol reduced infarct volume

We then evaluated the effect of IP administration of 5Z-7-oxozeaenol. IP injection of 0.5 mg/kg resulted in no significant differences in infarct volume in any brain area (data not shown). However, injection of a higher 5 mg/kg dose showed statistically-significant differences in cortical ( $50.62 \pm 6.27\%$  for vehicle versus  $23.12 \pm 6.72\%$  for drug,  $p = 0.011$ ), striatal ( $44.91 \pm 7.51\%$  for vehicle versus  $26.41 \pm 4.22\%$  for drug,  $p = 0.044$ ), and whole-hemisphere infarct volume (Fig. 1b) ( $45.99 \pm 6.16\%$  for vehicle versus  $20.95 \pm 5.52\%$  for drug,  $p = 0.010$ ,  $n = 7$  vehicle, 8 drug). Mice administered the 5 mg/kg dose showed an improvement in behavioral deficits; however this difference did not reach statistical significance (Table 3) (3(1) for vehicle-treated mice versus 2(0.5) for drug-treated mice,  $p = 0.073$ ,  $n = 7$  vehicle, 8 drug). No differences were seen in any physiological parameters (including pH, pCO<sub>2</sub>, pO<sub>2</sub>, glucose, mean arterial pressure) (Table 1) or local cerebral blood flow as measured by laser Doppler flowmetry (LDF) between drug- and vehicle-treated groups (Table 2).

### Post stroke treatment of 5Z-7-oxozeaenol provided sustained neuroprotection

To determine if this agent could salvage ischemic brain when administered after stroke onset, IP administration of 5mg/kg of 5Z-7-oxozeaenol was delayed until after reperfusion. IP injection of 5.0 mg/kg 2 hours after the onset of MCAO resulted in a significant reduction in cortical ( $52.72 \pm 5.08\%$  for vehicle versus  $22.29 \pm 8.34\%$  for drug,  $p = 0.007$ ), striatal ( $56.63 \pm 7.65\%$  for vehicle versus  $20.64 \pm 3.83\%$  for drug,  $p = 0.001$ ), and whole-hemisphere infarct volume at 24 hours (Fig. 1c) ( $49.18 \pm 4.18\%$  for vehicle versus  $17.81 \pm 5.86\%$  for drug,  $p = 0.001$ ,  $n = 7$  vehicle, 8 drug).

To determine whether the effect of this agent was durable and could lead to chronic neuroprotection, we subjected a separate cohort of mice to delayed treatment (3.5 hours after stroke onset) and determined infarct volumes 7 days later. Drug treatment decreased infarct volumes at 7 days, with significant decreases in cortical (Fig. 2a) ( $66.08 \pm 2.73\%$  for vehicle versus  $43.15 \pm 9.14\%$  for drug,  $p = 0.028$ ) and whole-hemisphere infarct volumes ( $57.16 \pm 2.38\%$  for vehicle versus  $38.35 \pm 8.97\%$  for drug,  $p = 0.049$ ) ( $n = 8$  vehicle, 8 drug).

### TAK1 inhibition improved memory function in wild type (WT) mice subjected to stroke but had no effect on total locomotor activity

Delayed drug treatment also had a profound effect on memory/recognition tasks. Administration of the drug to WT mice 3.5 hours after onset of MCAO led to a statistically-significant increase in the percentage of time that the mice spent exploring a novel object (Bevins, et al., 2006) (Fig. 3) ( $33.15 \pm 5.04\%$  for vehicle versus  $59.85 \pm 3.62\%$  for drug,  $p = 0.001$ ,  $n = 7$  vehicle, 7 drug). This effect was not observed in mice subjected to sham surgery ( $57.51 \pm 4.61\%$  for vehicle versus  $61.94 \pm 2.17\%$  for drug,  $p = 0.359$ ,  $n = 5$  vehicle, 5 drug) which showed the expected preference for a novel vs. a familiar object (Bevins, et al., 2006). Delayed administration of the drug had no effect on average locomotor activity as measured by total beam breaks by the mice 1 day after MCAO (Fig. 4) ( $3881 \pm 604$  for vehicle versus  $3219 \pm 451$  for drug,  $p = 0.366$ ), 3 days after MCAO ( $4676 \pm 275$  for vehicle versus  $3694 \pm 459$  for drug,  $p = 0.07$ ), or 7 days after MCAO ( $4310 \pm 850$  for vehicle versus  $5114 \pm 786$  for drug,  $p = 0.488$ ,  $n = 8$  vehicle, 7 drug). The levels of activity were similar in sham groups when drug treated groups were compared with vehicle treated groups (Fig. 4). No differences were seen in a corner test between drug treated and vehicle treated stroke mice (day 1 vehicle 0.90 ( $n=9$ ) versus drug 0.89 ( $n=9$ ); day 3 vehicle 0.88 ( $n=9$ ) versus drug 0.89 ( $n=9$ ); day 7 vehicle 0.86 ( $n=8$ ) versus drug 0.74 ( $n=7$ ),  $p>0.05$  on each day).

### 5Z-7-oxozeanol reduced the level of activated TAK1 in stroke

Experimental stroke reduced the level of phosphorylated TAK1 (the activated form) when measured 4 hours after ischemia (Figs. 5a and b) ( $p = 0.002$ ,  $n = 5$  sham, 6 stroke). Drug treatment significantly reduced levels of phosphorylated TAK1 compared to vehicle after MCAO (Figs. 5a and b) ( $p = 0.016$ ,  $n = 6$  vehicle, 5 drug), an effect not observed in sham mice ( $p = 0.203$ ,  $n = 5$  vehicle, 5 drug). Total TAK1 levels were not significantly affected by either stroke (Figs. 5c and d) ( $p = 0.128$ ) or drug treatment ( $p = 0.672$ ) ( $n = 5$  vehicle shams, 6 vehicle strokes, 5 drug shams, 5 drug strokes). When pTAK1 levels were normalized to total TAK1, the ratio in the drug treated stroke group was not significantly reduced compared to that of the vehicle treated stroke groups, however a significant effect was still observed when compared to drug treated shams (Figs. 5e and f).

### Effect of 5Z-7-oxozeanol in stroke was independent of AMPK signaling

Treatment with 5Z-7-oxozeanol reduced infarct volume, suggesting that TAK1 activation exacerbates ischemic injury. To explore possible underlying mechanisms, we first examined AMPK, a putative downstream target for TAK1 that plays an important role in stroke pathology. Consistent with previous reports (Li, et al., 2010, Li and McCullough, 2010), levels of pAMPK were elevated after stroke, but drug treatment had no effect on total or pAMPK levels (Figs. 5g and h) ( $p = 0.755$ ,  $n = 7$  vehicle shams, 9 vehicle strokes, 7 drug shams, 9 drug strokes).

To further explore the possible contributions of AMPK to TAK1 inhibition-induced protection, we administered 5Z-7-oxozeanol to AMPK  $\alpha 2$  deficient mice 3.5 hours after the onset of MCAO and performed histological analysis 7 days after stroke. Administration of 5Z-7-oxozeanol led to a significant decrease in cortical (Fig. 2b) ( $54.61 \pm 3.29\%$  for vehicle versus  $37.60 \pm 4.96\%$  for drug,  $p = 0.008$ ) and whole-hemisphere ( $47.81 \pm 3.34\%$  for vehicle versus  $36.96 \pm 3.65\%$  for stroke,  $p = 0.037$ ) infarct volume 7 days after stroke ( $n = 11$  vehicle, 9 drug), suggesting that the neuroprotective effects of 5Z-7-oxozeanol are independent of AMPK.

AMPK KO mice showed similar results to WT in open-field testing, with no difference in locomotor activity 1 day after MCAO ( $1550 \pm 499$  for vehicle versus  $1609 \pm 356$  for drug,  $p$

= 0.92), 3 days after MCAO ( $2117 \pm 318$  for vehicle versus  $3420 \pm 663$  for drug,  $p = 0.083$ ), or 7 days after MCAO ( $4227 \pm 725$  for vehicle versus  $3872 \pm 430$  for drug,  $p = 0.685$ ,  $n = 11$  vehicle, 10 drug).

### Activated JNK levels were reduced by TAK1 inhibition after stroke

As the neuroprotective effects of TAK1 inhibition were not secondary to a reduction in stroke-induced pAMPK signaling, we investigated another major apoptotic pathway activated by TAK1, c-Jun N-terminal kinase (JNK) (Benakis, et al., 2010, Sanna, et al., 2002). Levels of phosphorylated JNK (the activated form of pJNK) were reduced following treatment with 5Z-7-oxozeaenol in stroke animals (Figs. 5i and j,  $p = 0.002$ ,  $n = 5$  vehicle shams, 6 vehicle strokes, 5 drug shams, 5 drug strokes). Total levels of JNK were not significantly affected by stroke ( $p = 0.213$ ) or by 5Z-7-oxozeaenol treatment (Figs. 5k and l) ( $p = 0.132$ , ( $n = 8$  vehicle shams, 10 vehicle strokes, 8 drug shams, 8 drug strokes). Drug treatment increased the ratio of pJNK/total JNK in sham animals ( $p = 0.009$ ). However, the difference of the ratio of pJNK/total JNK in stroke animals between drug and vehicle treated mice was not significant (Figs. 5m and n) ( $p = 0.121$ ). c-Jun, a component of the transcription factor complex AP-1 and a downstream target for phosphorylation by JNK, was also reduced with 5Z-7-oxozeaenol treatment (Figs. 5o and p) ( $p = 0.006$ ,  $n = 2$  vehicle sham, 3 vehicle stroke, 2 drug sham, 3 drug stroke).

### COX-2 levels were increased by stroke but not effected by TAK1 inhibition

As TAK1 has recently been shown to have pro-inflammatory effects (Neubert, et al., 2011), (Kumar, et al., 2009), (Zeng, et al., 2010, Zhang, et al., 2010), levels of COX-2 were also evaluated. Levels of COX-2 were increased following stroke as has been reported previously (Candelario-Jalil and Fiebich, 2008) (Figs. 5q and r) ( $p = 0.014$ ,  $n = 2$  sham, 3 stroke). However, in stroke animals, TAK1 inhibition had no effect on COX-2 levels ( $p = 0.569$ ,  $n = 3$  drug, 3 vehicle).

## DISCUSSION

TAK1 has recently been implicated as a contributor to cell death in several *in vitro* and *in vivo* models (Sicard, et al., 2009, Tang, et al., 2008). This study reveals several novel and important findings related to the role of TAK1 signaling in cerebral ischemia. First, levels of pTAK were significantly lower in the ischemic brain compared to sham. Second, direct intracerebroventricular delivery of 5Z-7-oxozeaenol, a TAK1 inhibitor, reduced the severity of experimental stroke as measured by both behavioral deficit scores and infarct volumes indicating a CNS-specific effect of this inhibitor. Third, systemic injection of 5Z-7-oxozeaenol also provided similar beneficial effects suggesting TAK1 inhibition is a potentially translatable therapy for stroke. Fourth, delayed treatment also reduced infarct size as 5Z-7-oxozeaenol, even when given 3.5 hours after stroke, provided sustained neuroprotection assessed 7 days following stroke, an effect that was also reflected in reduced neurological deficits as measured by the novel object recognition test. Fifth, treatment with 5Z-7-oxozeaenol specifically reduced pTAK1 levels after stroke, confirming drug specificity for this molecular pathway. TAK1 inhibition did not reduce stroke-induced AMPK activation, suggesting that TAK1's function as an upstream kinase of AMPK is not the mechanism by which TAK inhibition reduces ischemic injury. This was confirmed by demonstrating equivalent neuroprotective efficacy of TAK inhibition in both WT and AMPK  $\alpha 2$  KO mice. Finally, treatment with 5Z-7-oxozeaenol selectively reduced levels of activated JNK, a known downstream target of TAK1, a finding that may shed light into the mechanism of neuroprotection of TAK1 inhibition.

5Z-7-oxozeaenol is a highly-selective inhibitor of TAK1 activity that has been used in several models to evaluate the role of TAK1 in apoptosis (Ninomiya-Tsuji, et al., 2003, Sicard, et al., 2009). 5Z-7-oxozeaenol selectively inhibits the catalytic activity of TAK1 but has minimal effects on other MAP kinases or JNK (Ninomiya-Tsuji, et al., 2003). As such, it is a powerful tool for examining TAK1 signaling. Most previous studies that have utilized 5Z-7-oxozeaenol have been performed in *in vitro* cell culture systems, in isolated organs, or utilized local administration of this agent. Our results indicate that TAK1 inhibition (as measured by significant decreases in p-TAK) can be obtained with either intracerebroventricular or systemic administration of 5Z-7-oxozeaenol. Interestingly, drug treatment did not affect levels of activated TAK1 in sham mice. However, after stroke, levels of activated TAK1 were significantly lower in drug-treated mice than in vehicle-treated mice. Ischemia may allow passage of the drug into the brain secondary to blood-brain barrier dysfunction, allowing it to exert its effects on TAK1. In contrast to our findings, previous work in a neonatal model of hypoxic-ischemic injury found higher levels of pTAK1 in the brain 3 hours after injury (Nijboer, et al., 2009). In that model, neonatal rats were subjected to 120 minutes of hypoxia, followed by 3 hours of permanent MCA occlusion. The differences in levels of TAK1 expression between these studies could be secondary to species, developmental and model difference. Protein phosphatase 6 (Brogie, et al., 2010, Kajino, et al., 2006) or protein phosphatase 2A (Kim, et al., 2008) have been shown to inactivate TAK1 in cell culture models, however their roles in regulating TAK1 levels in the stroke model remain to be investigated. As TAK1 inhibition by 5Z-7-oxozeaenol is neuroprotective, the observed decrease seen in vehicle-treated mice in pTAK1 in the reperfusion period may reflect an attempt at endogenous neuroprotection. We have previously found that inhibition of AMPK is protective after experimental stroke (Li, et al., 2010, Li, et al., 2007). The mechanisms by which acute AMPK activation enhances injury are unclear, but exacerbated lactate accumulation, enhanced autophagy, and increased glucose uptake due to unregulated glucose transporters in the reperfusion phase likely contribute to stroke damage (Li, et al., 2010). There are three known kinases (TAK1, LKB1 (Hawley, et al., 1995, Sakamoto, et al., 2005) and CaMKK $\beta$  (Suter, et al., 2006)) that can activate AMPK by phosphorylation. The response of pAMPK to the manipulation of these upstream kinases has not been examined in the ischemic brain. We initially hypothesized that TAK1 was an important upstream regulator of AMPK in the ischemic brain (Li and McCullough, 2010) due to its effects on apoptosis and inflammatory signaling. We postulated that inhibition of TAK1 would lead to neuroprotection via a reduction in pAMPK levels. However, no decrease in stroke-induced pAMPK levels was seen after TAK1 inhibition, despite robust effects of the drug on pTAK1 levels. This suggests that stroke-induced AMPK phosphorylation is triggered by LKB1 or CaMKK $\beta$  rather than TAK1. This was further confirmed by studies utilizing mice deficient in AMPK  $\alpha$ 2, the isoform responsible for the detrimental effect of AMPK activation (Li, et al., 2007) in which TAK inhibition led to robust neuroprotection despite the loss of AMPK. These studies suggest that the effects of TAK1 in stroke are mediated through pathways independent of AMPK signaling.

Once it was recognized that AMPK may not be the signaling pathway by which TAK inhibition exerts its neuroprotective effects, we evaluated JNK, a major pro-apoptotic pathway recently found to be activated by TAK1. It is unclear whether activation of TAK1 is anti- or pro-apoptotic, and the overall effect appears to be tissue specific. Mice deficient in TAK1 are embryonic lethal due to apoptosis of hematopoietic cells and hepatocytes (Tang, et al., 2008). In the heart, unlike our findings in the brain, inhibition of TAK1 by 5Z-7-oxozeaenol led to increased cardiomyocyte death after cardiac ischemia (Sicard, et al., 2009), suggesting that TAK1 has anti-apoptotic effects. In contrast, several studies have shown that activation of TAK1 has enhanced apoptosis via activation of p38 mitogen-activated protein kinase (p38 MAPK) and JNK in fibroblasts (Matluk, et al., 2010, Resch, et

al., 2009, Sorrentino, et al., 2008) and both JNK and its downstream target c-Jun, are well recognized pro-apoptotic factors in ischemic brain (Neubert, et al., 2011, Nijboer, et al., 2010) consistent with our results demonstrating neuroprotection and the significant decrease in stroke-induced pJNK levels with TAK inhibition. Therefore, the effects of TAK1 on apoptosis may be dependent on the duration of ischemia, reperfusion status, and the tissue examined, similar to what has been seen with AMPK activation (Weisova, et al., 2011).

TAK1 activation is also known to induce pro-inflammatory gene expression (Zeng, et al., 2010, Zhang, et al., 2010) leading to the production of pro-inflammatory cytokines and chemokines (Jang, et al., 2002, Ninomiya-Tsuji, et al., 2003) and activation of immune cells (Sato, et al., 2005). Accordingly, topical 5Z-7-oxozeaenol reduced picric acid induced inflammation in the mouse ear via an inhibitory action on TAK1 and subsequent COX-2 expression (Ninomiya-Tsuji, et al., 2003). As COX-2 is induced after stroke and leads to an exacerbation of ischemic damage by the promotion of inflammation (Candelario-Jalil and Fiebich, 2008, Iadecola and Ross, 1997), we also examined COX-2. Consistent with previous work, stroke induced a significant increase in COX-2 levels. However, there were no differences in COX-2 levels between vehicle and drug-treated stroke animals, suggesting that the anti-inflammatory properties of 5Z-7-oxozeaenol play a less significant role in neuroprotection. Importantly, the fact that COX-2 levels did not change, despite a significant reduction in infarct in drug-treated animals, suggests that the effects of 5Z-7-oxozeaenol on JNK and c-JUN signaling (which did decrease after treatment) are specific, and are not merely an epiphenomenon of changes in infarct size.

In addition to reducing infarct damage, TAK1 inhibition also led to improvements in behavioral deficits. Although no effect was seen on total locomotor activity or on forepaw usage between drug and vehicle treated mice, a significant improvement was seen on the novel object recognition test (NORT). In this test, the interest of an animal in a novel object versus a familiar one is measured and compared. If the exploration of the novel and the familiar object is equal (as normal uninjured animals prefer to explore the novel object), this is interpreted as a deficit in memory and cognition. Cognitive deficits have been well described in the MCAO model, and may be more of a “translatable” deficit to measure than pure motor deficits. In our experiment, sham mice had a preference for the novel object spending more than 50% of the test time examining the novel object, as expected. Drug treated MCAO mice were indistinguishable from shams, whereas vehicle-treated MCAO mice had a significant reduction in novel object preference. This deficit is not secondary to enhanced motor impairments in vehicle treated stroke mice, as their overall locomotor function was equivalent to sham. These results are consistent with a recent study (Dhawan, et al., 2011), that also demonstrated a preference for the familiar object in this task after stroke, although the exact underlying mechanisms and neuroanatomical substrates are unknown. Interestingly, we did not see an effect of drug treatment on stroke-induced motor deficits. This may be due to the mechanism of action of this agent, as we expect the effects of TAK1 inhibition to be greatest in the ischemic penumbra, where apoptosis predominates, rather than salvaging the ischemic core, which in the focal MCAO model primarily involves the striatum. The memory dysfunction created by MCAO may reflect diffuse cortical dysfunction which are reflected by the deficits seen in the NORT. Reversing these deficits may have greater relevance in clinical populations as memory and cognitive deficits lead to considerable disability and loss of independence for stroke patients.

There are several potential limitations of this work, and the data must be interpreted with these in mind. As with all pharmacological therapies, possible off-target effects of 5Z-7-oxozeaenol cannot be completely excluded. It is clear that the drug does act on TAK based on the significant reduction of pTAK1 with treatment, and currently other approaches are not feasible as TAK1 KO mice are embryonic lethal. Alternative approaches such as



development of cell type selective or conditional KO mice could address some of these mechanistic questions. 5Z-7-oxozeaenol is one of the few pharmacological agents that appears to have efficacy on both histological and functional outcomes, has long-term durable effects, can be given systemically and most importantly, retains efficacy even when administered several hours after stroke onset. Interestingly, functional improvement was significant when the drug was given in the post-stroke treatment, but not when given immediately at stroke onset, despite robust neuroprotective effects in both paradigms. It is possible that later anti-inflammatory effects may mediate the behavioral recovery, whereas immediate anti-apoptotic effects are responsible for histological improvements. Although the half-life of the drug is yet unknown, later effects on inflammatory pathways remain to be investigated. Prior to moving forward in translational efforts, future studies to determine dose response curves, toxicity, to evaluate efficacy in both sexes and in models replicating the disease population (hypertensive, diabetic, aging etc.) need to be undertaken.

## CONCLUSION

TAK1 inhibition is neuroprotective after induced cerebral ischemia and the mechanism may be independent of AMPK signaling. We demonstrated that 5Z-7-oxozeaenol has the potential to reduce the severity of ischemic stroke through its effects on TAK1 and downstream signaling via JNK. This work has uncovered a new, yet unexplored signaling pathway involved in ischemic cell death.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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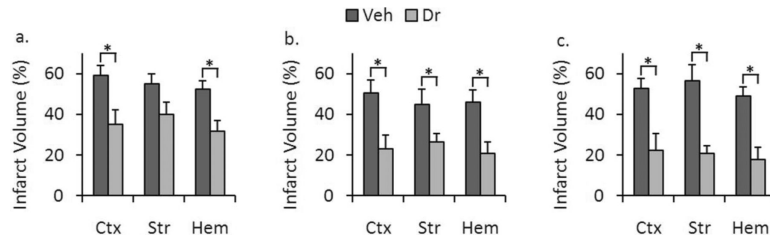
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**Highlights**

Stroke injury propagation involves complex molecular processes.

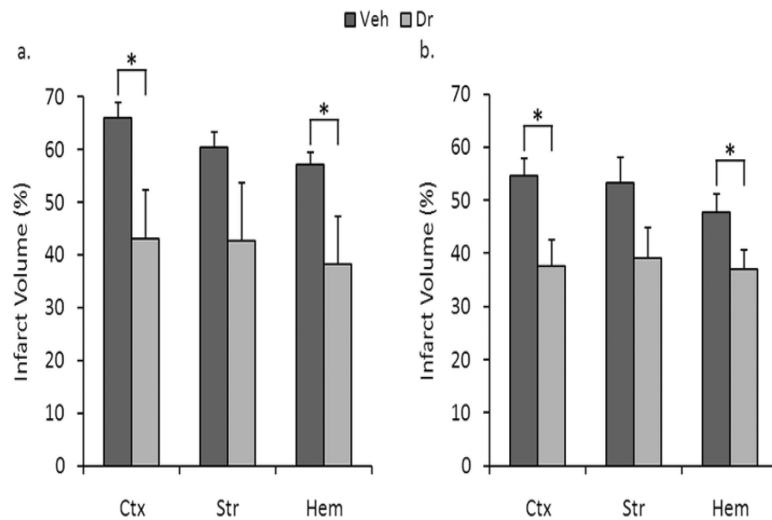
Inhibiting transforming growth factor- $\beta$ -activated kinase 1 (TAK1) reduced injury.

TAK1 is a critical molecule in deciding neuronal fate in stroke.



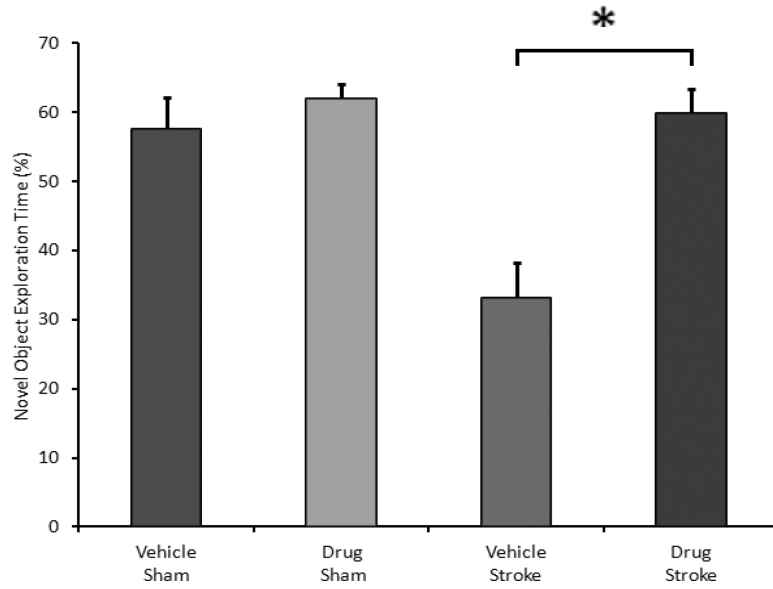
**Figure 1.**

Administration of 5Z-7-oxozeaenol decreased stroke severity in WT mice. **a.** ICV administration of 1.6 ug of 5Z-7-oxozeaenol 20 minutes prior to stroke led to a statistically significant reduction in stroke volume in cortex ( $p = 0.014$ ) and total hemisphere ( $p = 0.008$ ) but not in striatum ( $p = 0.066$ ) ( $n = 6$  vehicle, 7 drug). **b.** IP administration of 5Z-7-oxozeaenol (5 mg/kg) at stroke onset led to a statistically significant reduction in stroke volume in cortex ( $p = 0.011$ ), striatum ( $p = 0.044$ ), and total hemisphere ( $p = 0.010$ ) ( $n = 7$  vehicle, 8 drug). **c.** IP administration of 5Z-7-oxozeaenol (5 mg/kg) 2 hours after onset of stroke led to a statistically significant reduction in stroke volume in cortex ( $p = 0.007$ ), striatum ( $p = 0.001$ ), and total hemisphere ( $p = 0.001$ ) ( $n = 7$  vehicle, 8 drug). Data presented as mean  $\pm$  s.e.m. \*  $p < 0.05$ .



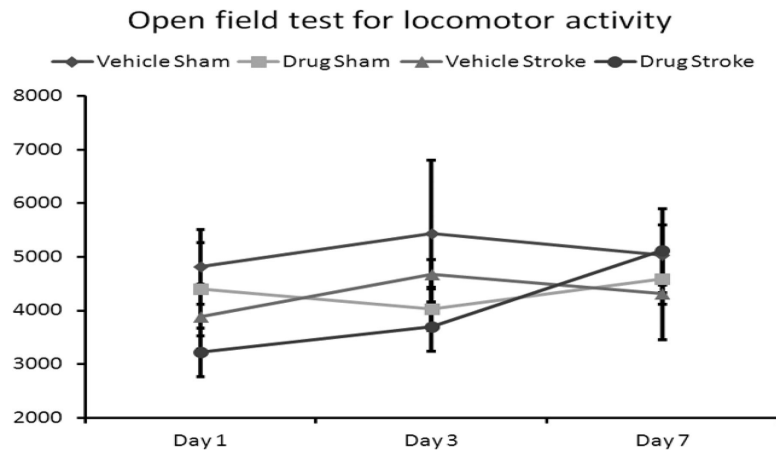
**Figure 2.**

Administration of 5Z-7-oxozeaenol decreased stroke severity when measured at 7 days following stroke in both WT and AMPK KO mice. **a.** IP administration of 5Z-7-oxozeaenol (5 mg/kg) to WT mice after stroke led to a statistically significant reduction in cortical ( $p = 0.028$ ) and whole-hemisphere ( $p = 0.049$ ), but not striatal ( $p = 0.117$ ) stroke volume ( $n = 8$  vehicle, 8 drug). **b.** IP administration of 5Z-7-oxozeaenol (5 mg/kg) to AMPK KO mice after stroke led to a statistically significant reduction in cortical ( $p = 0.008$ ) and whole-hemisphere ( $p = 0.037$ ) infarct volume ( $n = 11$  vehicle, 9 drug), but not striatal ( $p = 0.066$ ,  $n = 11$  vehicle, 9 drug). Data presented as mean  $\pm$  s.e.m. \*  $p < 0.05$ .



**Figure 3.**

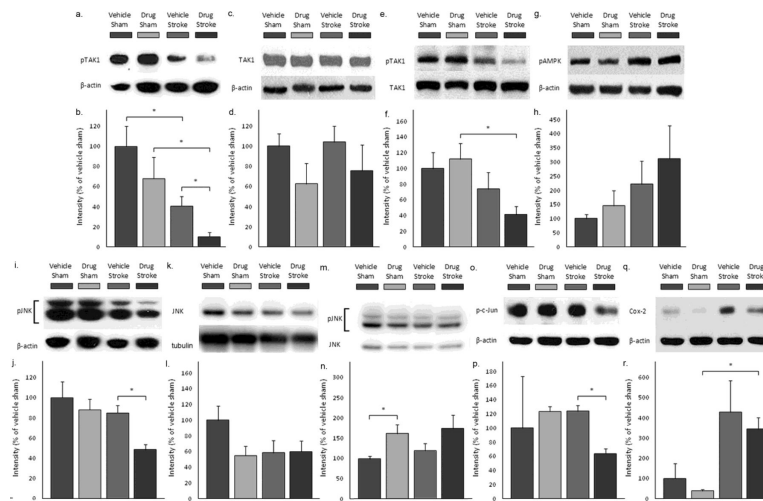
Administration of the drug to WT mice 3.5 hours after onset of MCAO lead to a statistically-significant improvement in the novel object recognition test ( $33.15 \pm 5.04\%$  for vehicle versus  $59.85 \pm 3.62\%$  for drug,  $p = 0.001$ ,  $n = 7$  vehicle, 7 drug). There was no difference between drug- and vehicle-treated mice subjected to sham surgery ( $p = 0.359$ ,  $n = 5$  vehicle, 5 drug). Data presented as mean  $\pm$  s.e.m. \*  $p < 0.05$ .



**Figure 4.**

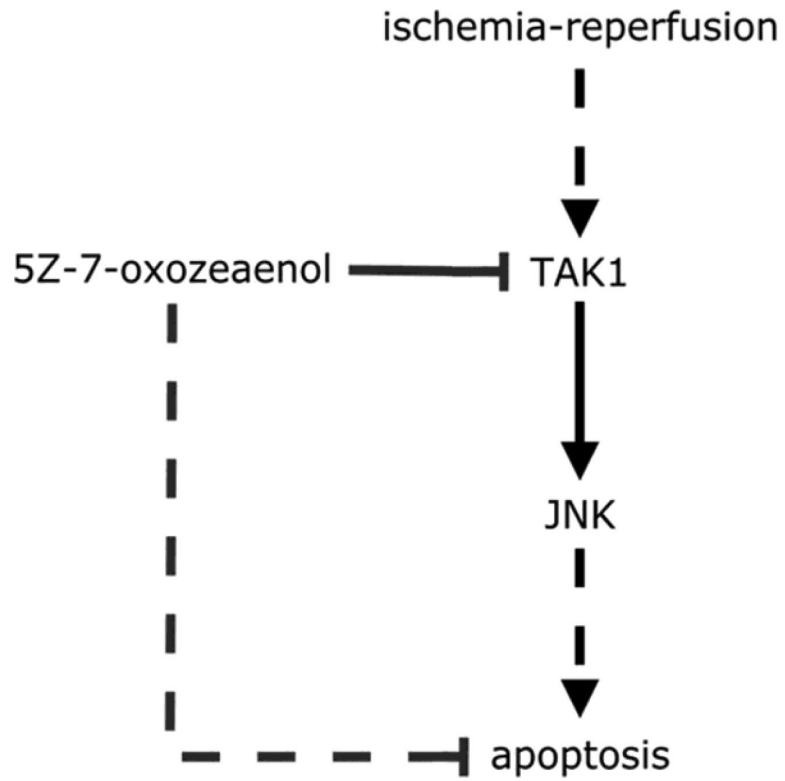
Delayed administration of the drug had no effect on the average locomotor activity of stroke mice as measured by total beam breaks by the mice 1 day after MCAO ( $p = 0.366$ ), 3 days after MCAO ( $p = 0.07$ ), or 7 days after MCAO ( $p = 0.488$ ,  $n = 8$  vehicle, 7 drug). Drug administration had no effect in mice subjected to sham surgery 1 day after MCAO ( $p = 0.690$ ), 3 days after MCAO ( $p = 0.298$ ), or 7 days after MCAO ( $p = 0.523$ ,  $n = 5$  vehicle, 5 drug). Data presented as mean  $\pm$  s.e.m. \*  $p < 0.05$ .





**Figure 5.**

Drug treatment reduced levels of activated TAK1, activated JNK, total JNK, and activated c-Jun, but not total TAK1, activated AMPK, or COX-2. **a.** Levels of phosphorylated TAK1 (activated form) were decreased by drug treatment during stroke (n = 5 vehicle shams, 6 vehicle strokes, 5 drug shams, 5 drug strokes). **b.** Quantitation of phospho-TAK1 western blots. **c.** Levels of total TAK1 were unchanged following stroke or drug administration (n = 5 vehicle shams, 6 vehicle strokes, 5 drug shams, 5 drug strokes). **d.** Quantitation of TAK1 western blots. **e.** Levels of pTAK1/TAK1 were decreased by stroke in drug-treated groups. Levels were unchanged by drug treatment (n = 8 vehicle shams, 10 vehicle strokes, 8 drug shams, 8 drug strokes). **f.** Quantitation of pTAK1/TAK1 western blots. **g.** Levels of phosphorylated AMPK (activated form) were unchanged by drug treatment before stroke (n = 7 vehicle shams, 9 vehicle strokes, 7 drug shams, 9 drug strokes). **h.** Quantitation of phospho-AMPK western blots. **i.** Levels of phosphorylated JNK (activated form) were decreased by drug treatment before stroke (n = 5 vehicle shams, 6 vehicle strokes, 5 drug shams, 5 drug strokes). **j.** Quantitation of phospho-JNK western blots. **k.** Levels of total JNK were not significantly changed by drug treatment or by stroke (n = 8 vehicle shams, 10 vehicle strokes, 8 drug shams, 8 drug strokes). **l.** Quantitation of JNK western blots. **m.** Levels of pJNK/JNK were increased by drug treatment in sham groups (n = 8 vehicle shams, 10 vehicle strokes, 8 drug shams, 8 drug strokes). **n.** Quantitation of pJNK/JNK western blots. **o.** Levels of phosphorylated c-Jun (activated form) were decreased by drug treatment before stroke. (n = 2 vehicle shams, 2 drug shams, 3 vehicle strokes, 3 drug strokes). **p.** Quantitation of phospho-c-Jun western blots. **q.** Levels of COX-2 were increased following stroke, but were not affected by drug treatment (n = 2 vehicle shams, 2 drug shams, 3 vehicle strokes, 3 drug strokes). **r.** Quantitation of COX-2 western blots. Data presented as mean ± s.e.m. \* p < 0.05.



**Figure 6.** Proposed mechanism for neuroprotection by TAK1 inhibition. Inhibition of TAK1 by 5Z-7-oxozeaenol reduces JNK activation, leading to reduced apoptosis following ischemic stroke.

**Table 1**

There is no difference in physiological parameters between the vehicle and drug treated mice

Group	Condition	pH	pCO <sub>2</sub>	pO <sub>2</sub>	Glucose	MABP (mmHg)
Vehicle	Pre-ischemic	7.37 ± 0.01	29.3 ± 3.2	118 ± 2.9	153 ± 9.2	73 ± 5.6
	Intra-ischemic	7.25 ± 0.01	31.8 ± 3.1	112 ± 10	159 ± 11	74 ± 2.5
Drug	Pre-ischemic	7.39 ± 0.01	22.8 ± 2.2	119 ± 9.4	159 ± 23	72 ± 2.3
	Intra-ischemic	7.29 ± 0.03	29.8 ± 1.9	129 ± 7.6	170 ± 40	78 ± 1.7

Blood samples were collected from the femoral artery at the onset of MCAO or 60 min from the onset of MCAO. Data are expressed as Mean ± SEM. n = 4 per group. Student's t-test was used to compare the difference of each parameter between vehicle and drug treated groups.

**Table 2**

There is no difference in cerebral blood flow (LDF) between the vehicle and drug treated mice

Group	LDF (% of baseline)	
	Intra-ischemia	Reperfusion
Vehicle	13.2 ± 1.3	82.3 ± 1.5
Drug	13.7 ± 3.1	83.4 ± 5.4

Cerebral blood flow was measured by using Laser Doppler Flow (LDF). Data are expressed as Mean±SEM. n = 4 per group. Student's t-test was used to compare the difference of each parameter between vehicle and drug treated groups.

**Table 3**

TAK1 inhibitor treatment reduced neurological deficit score after stroke

Route	ICV		IP	
	Vehicle	Drug	Vehicle	Drug
Median (interquartile range)	3 (0)	2 (0)	3 (1)	2 (0.5)

ICV injection of the drug (1.6 $\mu$ g) resulted in decreased neurological deficits (n = 7 vehicle, 9 drug, p = 0.038). IP injection of the drug (5.0 mg/kg) showed a similar trend but did not reach statistical significance (n = 7 vehicle, 8 drug, p = 0.073). Data was presented as median (interquartile range). The Mann-Whitney U test was used to detect any statistical significance between drug and vehicle groups.