1-Trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) Urea, a Selective and Potent Dual Inhibitor of Soluble Epoxide Hydrolase and p38 Kinase Intervenes in Alzheimer’s Signaling in Human Nerve Cells

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ABSTRACT: Alzheimer’s disease (AD) is the most common neurodegenerative disorder. Neuroinflammation is a prevalent pathogenic stress leading to neuronal death in AD. Targeting neuroinflammation to keep neurons alive is an attractive strategy for AD therapy. 1-Trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU) is a potent inhibitor of soluble epoxide hydrolase (sEH) and can enter into the brain. It has good efficacy on a wide range of chronic inflammatory diseases in preclinical animal models. However, the anti-neuroinflammatory effects and molecular mechanisms of TPPU for potential AD interventions remain elusive. With an aim to develop multitarget therapeutics for neurodegenerative diseases, we screened TPPU against sEH from different mammalian species and a broad panel of human kinases in vitro for potential new targets relevant to neuroinflammation in AD. TPPU inhibits both human sEH and p38β kinase, two key regulators of inflammation, with nanomolar potencies and distinct selectivity. To further elucidate the molecular mechanisms, differentiated SH-SY5Y human neuroblastoma cells were used as an AD cell model, and we investigated the neuroprotection of TPPU against amyloid oligomers. We found that TPPU effectively prevents neuronal death by mitigating amyloid neurotoxicity, tau hyperphosphorylation, and mitochondrial dysfunction, promoting neurite outgrowth and suppressing activation and nuclear translocation of NF-κB for inflammatory responses in human nerve cells. The results indicate that TPPU is a potent and selective dual inhibitor of sEH and p38β kinase, showing a synergistic action in multiple AD signaling pathways. Our study sheds light upon TPPU and other sEH/p38β dual inhibitors for potential pharmacological interventions in AD.

KEYWORDS: Alzheimer’s disease, soluble epoxide hydrolase, p38 mitogen-activated protein kinase, dual inhibitor, neuroinflammation, mitochondrial dysfunction, neuroprotection

INTRODUCTION

Alzheimer’s disease (AD) is the most common cause of dementia and is the fifth leading cause of global deaths. To date, no effective drugs can prevent, cure, or even slow this devastating disease.1 The accrued drug failures in AD clinical trials have amplified the calls for a more diverse drug pipeline. AD arises from a complexity of multifaceted mechanisms, including amyloid neurotoxicity, tau hyperphosphorylation, neuroinflammation, mitochondrial dysfunction, oxidative stress, synaptic loss, and ultimate death of neurons in the brains of AD patients.2−4 Hence, developing a multitarget drug to modulate diverse rather than a single pathogenic process of AD would increase the chances to find effective disease-modifying therapies.5

Inflammation associated with neurodegenerative conditions is a prevalent cellular stress in AD.6,7 Targeting neuroinflamma-
tion to keep neurons alive is an attractive therapeutic strategy. The soluble epoxide hydrolase (sEH) has been implicated in many chronic inflammatory conditions.\(^8,9\) The sEH is responsible for the conversion of epoxy fatty acids to their corresponding diols in lipid signaling pathways.\(^8\) In the arachidonic acid cascade, sEH hydrolizes epoxyeicosatrienoic acids (EETs) to dihydroxyeicosatrienoic acids (DHETs). EETs are known to mediate vascular relaxation responses and possess anti-inflammatory activities via attenuating endoplasmic reticulum (ER) stress and oxidative stress in vivo,\(^10\) while DHETs are relatively inactive in comparison to the EETs in most biological systems.\(^8,9,11,12\) Therefore, sEH inhibition can increase the endogenous concentration of EETs, leading to mitigation of inflammation. In recent years, there is ample evidence proving the therapeutic effects of sEH inhibitors in many rodent models for neuropsychiatric syndromes associated with inflammation such as stroke, seizure, depression, autism, and Parkinson’s disease.\(^9,10,13-16\) However, questions concerning whether sEH inhibitors can be used for AD intervention and the underlying molecular mechanisms remain elusive.

p38 mitogen-activated protein kinases (MAPKs) are another key regulator responsive to a variety of cellular stresses, and p38 MAPK signaling plays an important role in neuroinflammation.\(^6\) Previous studies revealed that p38 kinases are abnormally active in glial cells of AD brains, which enhances inflammatory gene expression and upregulates proinflammatory cytokines.\(^6,7,8\) In addition, an elevated p38 kinase activity in neurons contributes to tau hyperphosphorylation and tau-mediated neuroinflammation in AD.\(^9,10,14,16\) Hence, inhibiting aberrant p38 MAPK is also a promising approach for treating neurological diseases relevant to inflammation such as AD.\(^18,22,23\)

With a goal of developing novel therapeutics to target neuroinflammation for AD, we here tested a hypothesis that simultaneous modulation of sEH and p38 MAPK pathways in neurons by a urea-based inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), would provide synergistic outcomes of neuroprotection. TPPU (Figure 1) was originally developed as an inhibitor of sEH with a high potency and selectivity.\(^23\) Prior studies have shown that TPPU is effective in several animal models of neurological diseases.\(^10,13-16\) Given that the urea-based scaffold is a key pharmacophore in many inhibitors of sEH and protein kinases,\(^24-26\) we postulated that TPPU would also inhibit kinases relevant to neuroinflammation. Such dual inhibitory properties of TPPU and the resulting pharmacological effects have not been previously investigated.

We recently established a cell model of extracellular β-amyloid (Aβ) toxicity using differentiated SH-SYSY human neuroblastoma cells, which is specifically associated with AD.\(^27,28\) Herein, we tested pharmacological effects of TPPU in this cell model and demonstrated that TPPU is a highly potent and selective dual inhibitor of human sEH and p38β kinase with multifunctional actions in cell signaling of AD. The results support the studies of TPPU and development of new dual inhibitors of sEH/p38β kinase for potential AD therapy.

## RESULTS

TPPU Is a Potent Inhibitor Selectively Targeting Primate and Rodent sEHS. We previously demonstrated that TPPU is a slow and tight-binding inhibitor showing a high target occupancy with the sEH. It is highly potent against human and mouse sEHS.\(^23\) To further elaborate its species selectivity, we screened TPPU against sEHS in liver S-9 fractions from different mammals, including human, monkey, mouse, rat, dog, and mini-pig (Table 1). The results substantiated that TPPU is a potent and selective inhibitor for primate and rodent sEHS. Particularly, it has IC\(_{50}\) values less than 50 nM for the human, monkey, and rat sEH, and an IC\(_{50}\) of 90 nM for the mouse sEH. In contrast, TPPU is relatively less potent for the dog and mini-pig sEHS with IC\(_{50}\) values of 1800 nM and 220 nM, respectively.

| TPPU Selectively Inhibits Kinase Activities of p38β and p38γ Isoforms. Many urea-based compounds have been developed to target kinases because they can mimic ATP to block phosphate transfer in kinase-catalyzed reactions.\(^24-26\) Aberrant kinase activities have been implicated in many tauopathies including AD. With an aim at investigating whether TPPU could be a multitarget therapeutic for AD intervention, we screened TPPU at 1 μM against 40 human kinases (Figure 2A) that are pathologically relevant to AD.\(^19,28\) TPPU selectively inhibited kinase activities of p38β and p38γ isoforms (remaining kinase activities of 36% and 49%, respectively) among other kinases tested. In contrast, its inhibitory effects on p38α and p38δ isoforms were not significant. The results indicate good isoform selectivity of TPPU for p38β (IC\(_{50}\) 270 nM) and p38γ (IC\(_{50}\) 890 nM), where p38β is the most selective (approximately 3.3-fold over p38γ) (Figure 2B,C). TPPU weakly inhibited a handful of kinases such as GSK-3β, AMPKα2, and CK1ε/γ in comparison with p38β.

SH-SY5Y Human Nerve Cells Are a Valid Neuronal Model for the Study of sEH and p38 MAPK. SH-SY5Y human neuroblastoma cells are commonly used for the study of neurodegenerative diseases in vitro because they can be differentiated with morphological, biochemical, and functional features resembling human mature neurons.\(^27,29,30\) Western blotting on a whole-cell lysate showed that differentiated SH-SY5Y cells express a reasonable level of sEH and p38β kinase (Figure 3A) in comparison to the housekeeping protein β-actin. Treatment of the cells with TPPU (10, 100, and 1000 nM) for 24 h significantly decreased cellular sEH activities in a dose-dependent manner (Figure 3B). The results indicated that SH-SY5Y cells were a valid in vitro cell model suitable for the present study.

### Table 1. Inhibition of sEH Activity by TPPU in Liver S-9 Fractions from Various Species

<table>
<thead>
<tr>
<th>species</th>
<th>IC(_{50}) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>human (male)</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>monkey (male)</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>mouse (male)</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>rat (male)</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>dog (male)</td>
<td>1800 ± 300</td>
</tr>
<tr>
<td>mini-pig (male)</td>
<td>220 ± 40</td>
</tr>
</tbody>
</table>

*IC\(_{50}\) was determined with t-DPPO as a substrate. Results are average ± SD (n = 3).*

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**Figure 1.** Chemical structure and abbreviation of the sEH inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU).
TPPU Protects Neurite Outgrowth against Aβ_{42} Neurotoxicity in SH-SY5Y Cells. Chronic Aβ exposure in neuronal cells triggers AD-mimicking pathologies such as tau hyperphosphorylation, Ca^{2+} homeostatic dysregulation, activation of MAPK-linked toxicity, mitochondrial dysfunction, production of inflammatory proteins, and the ultimate loss of neuronal integrity.27,28,31,32 Because SH-SY5Y human neuronal cells express functional sEH and p38β kinase as well as mature tau isoforms with proper neuronal distribution in microtubules,33 we used differentiated SH-SY5Y cells under Aβ_{42} insults as a defined cell model of AD and evaluated the in vitro pharmacological effects of TPPU.

The results showed that treatment with 10 μM Aβ_{42} induced detrimental changes in neuronal morphology showing many dying and nondifferentiated cells with retracted neurites in comparison to the untreated control (Figure 4A,B). However, pretreatment with 100 nM TPPU effectively relieved Aβ_{42} toxicity in SH-SY5Y cells (Figure 4C,D). TPPU-treated cells maintained a healthy neuronal morphology in which they were well differentiated with extended neurites. Besides, the TPPU-treated cells tended to have a more pyramidal shaped soma and to become distinctly polarized. The cells also had longer and branched neurites and a detectable neuronal network in comparison to the control cells (Figure 4, panel A versus C).

Our results also demonstrated that pretreatment with either 0.1 μM EETs or 0.1 μM TPPU alone for 1 h followed by 10 μM Aβ_{42} co-incubation for 72 h regained cell viability from 30% to 100% in a dose-dependent manner. TPPU showed effective neuroprotection with an EC_{50} value of 48.6 nM (Figure 5B,C).

EETs are lipid metabolites and substrates of sEH. Our previous investigations demonstrated that EETs are neuroprotective and are capable of stimulating axonogenesis in rat PC12 pheochromocytoma cells and rat primary sensory and cortical neurons.33 In the present study, treatment with TPPU stimulated the SH-SY5Y cell proliferation (Figure 5A). Cotreatment with EETs and TPPU at 0.1 μM significantly increased cell proliferation by approximately 30% (p < 0.0001) (Figure 5D).
exposure for 72 h showed effective neuroprotection in SH-SY5Y cells (p < 0.0001). As expected, cotreatment with 0.1 μM EETs and 0.1 μM TPPU showed an enhanced effect that almost fully protected cell viability.

**Pairwise Treatment of Selective Inhibitors of sEH and p38 Kinase Synergizes Neuroprotection in SH-SY5Y Cells.** To further prove that TPPU is a dual inhibitor and that simultaneous inhibition of both sEH and p38β kinase would result in synergistic neuroprotection, we conducted a drug combination screening. A selective sEH inhibitor (t-AUCB) and a selective p38α/β kinase inhibitor (SB202190) were coadministered in a pairwise manner in SH-SY5Y cells under Aβ42 exposure. It is noteworthy that t-AUCB is a very potent urea-based sEH inhibitor (IC50, 2 nM) structurally distinct from TPPU. t-AUCB inhibits neither p38 MAPKs nor other kinases. SB202190 is a potent p38α/β inhibitor (IC50, 50 nM) capable of blocking MAPK pathways in cells. The combination effects of the two compounds were then assessed by the cell viability assay according to a screening method described by He et al.77

As shown in Figure 6, under treatment with SB202190 at a very low concentration (1 nM), treatment with t-AUCB from 0.1 to 100 nM protected the cells from Aβ42 toxicity in a dose-dependent manner. In parallel, under treatment with t-AUCB at 0.1 nM, treatment with SB202190 from 1 to 1000 nM increased neuroprotection in a dose-dependent manner as well. However, at the highest dosage tested, t-AUCB (100 nM) showed only a 27.3% neuroprotection, while that with SB202190 (1000 nM) was about 37.5%. Interestingly, an analysis of the combination dose–response matrices revealed that cotreatment with both compounds at certain ratios dramatically improved neuroprotection at different levels (e.g., cotreatment with t-AUCB at 1 nM and SB202190 at 1000 nM resulted in a 54.3% neuroprotection), which is a typical synergistic effect. The data provide compelling evidence that simultaneous inhibition of...
both sEH and p38β MAPK would offer synergistic benefits for potential AD therapy.

**TPPU Attenuates Tau Hyperphosphorylation Induced by Aβ42 in SH-SYSY Cells.** Aβ oligomers directly bind to the receptor for advanced glycation end-products (RAGE) or nicotinic acetylcholine receptors (e.g., α7 and α4/β2 nAChRs) resulting in activation of p38 MAPK signaling and tau hyperphosphorylation in neurons. Given that TPPU selectively inhibits p38β kinase in vitro (Figure 2), we further evaluated its effects on alleviating Aβ42-induced tau hyperphosphorylation in human SH-SYSY nerve cells. The tau phosphorylation level at the site S396 was monitored because it is a pathologic p-tau site in AD and is known to be susceptible to phosphorylation in human SH-SY5Y nerve cells. The tau phosphorylation level at the site S396 was monitored because it is a pathologic p-tau site in AD and is known to be susceptible to phosphorylation in human SH-SY5Y nerve cells. The results were normalized as the percentage of the neuroprotective activity relative to the Aβ42 free control (100%) and the 10 μM Aβ42 treatment (0%). Neuroprotection curve was analyzed by four-parameter regression. (D) Cotreatment of EETs with TPPU enhanced neuroprotection.

**TPPU and EETs Prevent Aβ-Induced Depolarization of Mitochondrial Membrane Potential and Mitochondrial Dysfunction.** Aβ oligomers induce oxidative stress by producing reactive oxygen species (ROS) and activate proinflammatory genes, which cause mitochondrial dysfunction. To assess the protective effects of TPPU against Aβ toxicity to maintain mitochondrial integrity and function, the changes of mitochondrial membrane potential (Δψm) in SH-SYSY human nerve cells were measured with a JC-10 assay. In this assay, the JC-10 dyes generate red fluorescent aggregates (Em = 590 nm) within healthy mitochondria upon membrane polarization. However, if mitochondria are dysfunctional, the dyes will be released in a green fluorescent monomer form (Em = 525 nm) through the collapsed and depolarized mitochondrial membrane. Because TPPU protected against Aβ42-induced neurotoxicity with an EC50 value of 48.6 nM (Figure 5B,C), we used relevant doses of TPPU (50 and 100 nM) to test its effect on Δψm. As shown in Figure 8, 10 μM Aβ42 treatment for 72 h decreased Δψm as the monomer/aggregate ratio (525/590 nm) increased by approximately 140% compared with the control (p < 0.0001). The results demonstrated that TPPU is pharmacologically equivalent to SB202190 in terms of the outcome of p-tau alleviation, which implicates the p38β inhibition by TPPU in the cells. In addition, the data showed that TPPU (0.01 μM) more significantly alleviates tau hyperphosphorylation than SB202190 does at a 5-fold higher dosage level (0.05 μM).
SH-SY5Y cells. The data substantiated the effect that pretreatment with EETs at 0.1 μM attenuated the loss of Δψₘ induced by Aβ₄₂ (p < 0.001) in SH-SY5Y cells, and cotreatment of 0.1 μM TPPU with 0.1 μM EETs synergistically restored Δψₘ to normal levels in the presence of Aβ₄₂ (p < 0.0001). Our data agreed with the other studies that EETs protect mitochondrial functions in rat hippocampal astrocytes.⁴¹ The results collectively suggest that sEH inhibition by TPPU or treatment with EETs results in prevention of Aβ-induced mitochondrial dysfunction in SH-SY5Y nerve cells.

TPPU Suppresses Activation and Nuclear Translocation of the Transcription Factor NF-κB in SH-SY5Y Cells. The transcription factor NF-κB is a master regulator of inflammatory responses. Aβ oligomers destabilize beneficial epoxy fatty acids like EETs and activate p38 MAPK/NF-κB pathways in neurons and glial cells,⁴¹,⁴²,⁴³ thereby promoting neuroinflammation and primarily microglial inflammation in AD brains.⁴⁵ NF-κB is inactive in the cytoplasm, bound to the inhibitory IκB proteins. Upon activation by p38 MAPK phosphorylation, the homo- or heterodimeric complexes of NF-κB will be released and translocate to the nucleus, where they activate proinflammatory gene expression.⁶ Given that TPPU is a potent inhibitor of both sEH and p38 kinase, we evaluated the effects on perturbation of the p38 MAPK/NF-κB...

Figure 6. A drug combination data analysis. Pairwise treatments of a selective sEH inhibitor (t-AUCB) and a selective p38α/β kinase inhibitor (SB202190) in differentiated SH-SYSY cells. Each compound was neuroprotective against 10 μM Aβ₄₂ in a dose-dependent manner. Combinations of both compounds in the dose–response matrices showed a synergistic effect. Colors in 3D mesh showed different levels of neuroprotection that were presented in percentage relative to the Aβ₄₂ free control (100%) and the Aβ₄₂ treatment (0%). Data are the mean of three independent experiments (n = 3).

Figure 7. TPPU attenuated Aβ₄₂ induced tau phosphorylation at site S396 in a dose dependent manner. Differentiated SH-SYSY cells were pretreated with various concentrations of TPPU or 0.2% PEG 400 vehicle for 1 h followed by treatment with 10 μM Aβ₄₂ for 72 h. The known selective p38 inhibitor, SB202190, at 0.05 μM was used as a reference control. ELISA analysis was performed with specific antibody against Tau pS396 to quantify cellular tau phosphorylation levels. Fold changes were calculated relative to the control with ± SEM (n = 6). Data were analyzed by one-way ANOVA with Tukey’s multiple comparison test. # p < 0.0001 relative to vehicle control; **** p < 0.0001 relative to the 10 μM Aβ₄₂ treatment.
signaling in response to Aβ1-42 stimuli in SH-SYSY cells. Aβ1-42 at 5 μM significantly increased nuclear concentrations of all five subunits of NF-κB (p50, p52, p65, RelB, and c-Rel) in SH-SYSY cells after 8 h (Figure 9). In particular, the nuclear p50 and p65 quantities were approximately 7.8-fold and 12.8-fold higher, respectively, than those of the untreated controls, while nuclear quantities were approximately 7.8-fold and 12.8-fold higher, respectively, than those of the untreated controls, while nuclear p50 and p65 decreases were comparable to DEX at 0.1 μM. To determine whether TPPU blocking of NF-κB activation is due to the p38 MAPK inhibition, we used dexamethasone (DEX) as a reference control that has been reported to inhibit both p38 MAPK and NF-κB.44 The effects of TPPU on suppressing activation and nuclear translocation of NF-κB were comparable to DEX at 0.1 μM.

**DISCUSSION**

sEH and p38 MAPKs are key mediators in inflammatory processes. sEH plays a pivotal role in the metabolism of lipid epoxides such as EETs. The cytochrome P450–epoxide hydrolase axis is clinically relevant to inflammatory responses. Therefore, sEH has been implicated in many human inflammatory diseases, including cardiovascular disease, hypertension, ischemia, diabetes, chronic kidney disease, cancer, neuropathic pain, and neurodegenerative disorders.5,9,11 p38 MAPKs are responsive to cellular stresses such as ROS, LPS, cytokines, UV light, DNA damage, and heat or osmotic shock. In AD, toxic Aβ oligomers bind to certain receptors such as RAGE and nAChR in brain cells, thereby stimulating abnormal activation of p38 MAPK signaling.41,36,39 causing hyperphosphorylation of tau proteins27 and excessively high activity of transcription factor NF-κB in neuroinflammation.18,20 These collective pathological events are believed to be key elements of the amyloid cascade in AD.45 Given that neuroinflammation is a major characteristic in most neurodegenerative diseases, the overall aim of this study was to investigate the neuroprotective effects and mechanistic understanding of a potent sEH inhibitor, TPPU, as a potential intervention for treating AD.

TPPU was originally developed as a urea-based sEH inhibitor to reduce inflammatory and neuropathic pain.23 The pharmacological efficacy of TPPU has been demonstrated in many preclinical animal models, including mice, rats, dogs, and non-human primates with chronic inflammatory conditions.9,15,23,46–49 In a previous study, the brain-to-plasma ratio of TPPU was 0.18, suggesting that it is brain penetrant.13 TPPU selectively targets primate sEH with a low nanomolar potency (human, IC50 = 45 nM; monkey, IC50 = 16 nM), whereas its potency in other mammalian species is relatively weaker (Table 1). The fine-tuned ADMET profiles and the blood–brain barrier permeability of TPPU make it a suitable CNS drug candidate for neurodegenerative diseases.10,13–16 Nevertheless, despite extensive pharmacological studies on inflammatory disorders targeting sEH, there is still limited knowledge regarding whether sEH is a viable therapeutic target in AD and whether inhibitors such as TPPU may be applicable in AD intervention.
To answer these questions, we used differentiated SH-SY5Y human cells as a defined nerve cell model of AD because they express functional sEH and p38β kinase that are of interest in our study (Figure 3). The results support our hypothesis that TPPU is a potent dual inhibitor of human sEH and human p38β kinase. Treatment with TPPU alone or a drug combination using t-AUCB (sEH inhibitor) and SB202190 (p38α/β inhibitor) exerts a synergistic neuroprotection against Aβ toxicity (Figures 5 and 6). In addition, TPPU being a p38β kinase inhibitor can alleviate tau hyperphosphorylation and maintain healthy tau−microtubule association for neuronal differentiation and axonogenesis in SH-SY5Y cells. Although both p38 MAPK and GSK-3β are tau protein kinases,19,50 TPPU selectively inhibits p38β (IC50 = 270 nM) over GSK-3β (IC50 > 10 μM) (Figure 2) and alleviates tau hyperphosphorylation at the site S396 that is found to be aberrant in AD brains (Figure 7).

Mitochondria are the primary energy source crucial for the viability of human cells. Their dysfunction has been implicated in many disease conditions ranging from cancer, diabetes, and cardiovascular diseases to neurodegenerative disorders.2,51 In AD, toxic Aβ oligomers increase oxidative stress and ER stress, impair mitochondrial function, and trigger programmed cell death of neurons. Particularly in mitochondria, Aβ causes formation of mitochondrial permeability transition pores and disrupts the electron transport chain, consequently leading to pathological ROS generation.52 Such ROS feed back to exacerbate mitochondrial dysfunction. As shown in Figure 8, sEH inhibition by TPPU protects nerve cells against Aβ42 at least in part through maintaining the membrane integrity, intracellular ionic charges, and mitochondrial function, which in turn increase neuronal survival. Additionally, the p38β inhibition by TPPU might contribute to reducing mitochondrial oxidative stress downstream of the AD signaling pathway (Figure 10), thereby resulting in neuroprotection. These data collectively suggest that sEH/p38β dual inhibitors could be a potential therapy in regulating mitochondrial homeostasis and preventing AD. They also support a hypothesis that mitochondrial dysfunction leads to a pathological increase of ROS levels, a feedback loop of ROS on mitochondria, and a shift in the ER stress response from cell homeostasis to inflammatory status.9,53 Modulating the mitochondrial−ROS−ER stress axis by sEH/p38β dual inhibitors is an attractive strategy for treating AD.

Importantly, the results indicate that TPPU mitigates neuroinflammation. By inhibiting p38 MAPK, TPPU effectively suppresses activation and nuclear translocation of NF-κB responsive to amyloid stimuli (Figure 9), suggesting a downstream repressive effect on proinflammatory genes by blocking NF-κB signaling. Such anti-inflammatory properties could also be reinforced through inhibition of neuronal sEH to

Figure 10. Proposed neuroprotective mechanisms of TPPU against Alzheimer’s disease.

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stabilize the endogenous EETs level. While all of the primary
targets of EETs are still unknown, increasing evidence shows
that EETs inhibit ER and oxidative stresses and NF-κB
activation to modulate inflammatory responses in cells.\cite{3,12,54}
On the other hand, it has become apparent that toxic p-tau
oligomers can spread through extracellular vesicles from
neurons to astrocytes and microglia and in turn trigger release
of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in
glial, thereby aggravating chronic neuroinflammation.\cite{5,20,55}
Since elevated p38 MAPK activity is prone to increase tauopathy
in neurons, inhibition of p38/β kinase by TPPU may help reduce
tau-mediated neuroinflammation in the brain.

As illustrated above, TPPU intervenes in the canonical
amyloid cascade of AD, offering multiple benefits in alleviating
neuroinflammation, mitochondrial dysfunction, tau hyper-
phosphorylation, and eventual death of neurons. A plausible
proposal of neuroprotective mechanisms of TPPU against AD is
summarized in Figure 10. Since no disease-modifying therapies
for AD have been approved by the US-FDA, investigating drug
treatment. Neuroinflammation is a prominent feature in most
neurodegenerative diseases including AD, where sEH and
p38 MAPK regulate distinct but separate inflammatory path-
ways.\cite{3,6,7} Interestingly, TPPU being a urea-based small-
molecule inhibitor shows good selectivity for p38/β kinase over
the other three isoforms (i.e., p38α, p38δ, and p38γ). The p38
isoform selectivity of TPPU is particularly advantageous to
potentially reduce the risk of off-target effects. In fact, a p38α
kinase selective inhibitor, neflamapimod (VX-745),\cite{3,6}
is currently in phase II clinical trials in AD patients, which
highlights the therapeutic relevance of p38 MAPKs in AD
pathology. While TPPU shows approximately 3-fold selectivity
for p38/β over p38γ, it serves as a promising drug lead for
developing new selective sEH/p38/β dual inhibitors through
medicinal chemistry approaches in the future.\cite{6,57}

**CONCLUSIONS**

The present study in conjunction with the prior evidence gives
new insights into an investigational small-molecule inhibitor,
TTPU, for potential AD therapy. We conducted in vitro
enzymatic assay screening to identify new targets of TTPU
and then functionally validated it in a cell model of AD, with
a primary aim of understanding the molecular mechanisms and
pharmacology of TTPU in human nerve cells. The present
investigation supports the argument that sEH plays a key role in
AD pathology and is a novel target worth pursuing. The new
findings on TTPU regarding the dual inhibitory properties, the
molecular and cellular mechanisms, and our curated knowledge
in sEH biochemistry and pharmacology would pave a road for
developing new selective sEH/p38/β dual inhibitors with
improved blood–brain barrier permeability via structure-based
drug design and optimization for potential therapies in AD and
related neurodegenerative diseases. Notwithstanding, because
different brain cells may have distinct drug responses, it would
be interesting to expand the study to microglia and astrocytes as
they are the main contributors of neuroinflammation. In
addition, it remains essential to conduct in vivo pharmacological
studies of TTPU to ascertain its neuroprotective and anti-
-inflammatory outcomes as well as its target engagement in
preclinical animal models of AD in the future.

**METHODS**

**Chemicals and Reagents.** All solvents and reagents were
purchased from commercial sources and were used without further
purification. TPPU, t-AUCB, and EETs were synthesized in-house as
previously described.\cite{3,12} Liver S-9 fractions were acquired from
Xenotech-LLC (Kansas City, KS). Staurosporine, 4-(4-fluorophenyl)-
2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole (SB202190), PEC
Dex, phenylmethanesulfonyl fluoride (PMSF), protease inhibitor
cocktail, retinoic acid, PEG 400, hexafluoroisopropyl alcohol, and Nonidet
P-40 were from Sigma-Aldrich (Saint Louis, MO). Aβ42 peptide was from
AnaSpec (Fremont, CA). Kinase Selectivity Profiling Assay Kit (cat.
no. V6854, V6856, V6858, V6924, V6918), ADP-Glo Kinase Assay Kit
(cat. no. V6930), and CellTiter 96 AQueous One Solution Cell
Proliferation MTS Assay Kit (cat. no. G3580) were from Promega
(Madison, WI). Human Tau pS396 ELISA Kit (cat. no. KHB7031) and
Cell Extraction Buffer were from Invitrogen (Camarillo, CA). Bradford
Protein Assay Kit (cat. no. 5000002) was from Bio-Rad (Hercules, CA).
JC-10 Mitochondrial Membrane Potential Assay Kit (cat. no.
ab121134) was from Abcam (Burlingame, CA). TransAM NF-κB
Family ELISA Assay Kit (cat. no. 43296) was from Active Motif
(Carlsbad, CA). Primary antibody anti-EPHX2 (cat. no. ab155280)
and secondary antibodies were obtained from Bio-Rad (Hercules, CA).

**sEH Enzyme Assay.** Liver S9 fractions were diluted with sodium
phosphate buffer (0.1 M, pH 7.4) containing 0.1 mg/mL bovine serum
albumin (BSA) to ensure that the activity was in the linear range of
the assay (<20% hydrolysis of the substrate). The enzyme activity was
measured with [3H]-trans-diphenylpropene oxide (t-DPPO) as a
substrate as described previously.\cite{3,20} To 100 μL of diluted extract, 1 μL
of inhibitor solution in DMSO was added ([I]_{inact} = 2.5–10 000 nM).
The mixture was incubated at 37 °C for 10 min. The enzymatic reaction
was started by adding 1 μL of t-DPPO ([S]_{final} = 50 μM). The reaction
was performed at 37 °C for 15 min. The reaction was stopped by adding
60 μL of methanol and extracted with 200 μL of isooctane. The diol
product was measured by quantifying the amount of radioactivity in 40
μL of the water phase using a liquid scintillation counter (TriCarb
2810TR, PerkinElmer, Shelton, CT). Each assay was done in triplicate
(n = 3). Data were plotted as a percent of inhibition (100% activity
measured in the absence of inhibitor but in the presence of 1% DMSO) as
a function of the concentration of inhibitor. The IC_{50} values were
determined by nonlinear regression of the data, using a curve fitted onto
a sigmoidal, logistic three-parameter equation.

To measure the sEH activities in SH-SYSY cells, the cells were
seeded at a density of 3 × 10^5 cells/mL. When they reached 80% cell
confluence, the cells were treated with different concentrations of
inhibitor for 24 h. After treatment, the cells were harvested and pelleted by
centrifugation at 600 g, 4 °C, for 15 min. The cell pellet was then
resuspended in 0.5 mL of sodium phosphate buffer (20 mM, pH 7.4)
containing 5 mM of EDTA. In a glass tube containing 10 μg of BSA, 100
μL of the cell suspension was added. The sEH activity was measured
using [3H]-t-DPPO as a substrate ([S] = 50 μM). The mixture was
incubated at 37 °C for 60 min. The remaining substrate was extracted
with 200 μL of isooctane. The diol product was quantified by measuring
radioactivity remaining in water phase by liquid scintillation (TriCarb
2810TR, PerkinElmer, Shelton, CT). The sEH activities were
normalized by the total protein concentration using a BCA assay.
Each assay was done in triplicate (n = 3).

**Kinase Luminescent Assay.** Kinase inhibition was assessed with the
ADP-Glo Kinase Assay according to the procedure described
previously.\cite{25,28} For screening, 5 ng/μL kinase solutions were assayed in
a 20 μL reaction mixture containing 50 ng/μL substrate, 40 μM Tris,
pH 7.5, 20 mM MgCl₂, 0.1 mg/mL BSA, 50 μM diithiothreitol (DTT),
25 μM ATP, and varying concentrations of TPPU solutions or 0.2%
PEG 400 vehicle in a 384-well microplate. The reaction mixture was
incubated for 1 h at room temperature followed by the addition of the
ADP-Glo reagents according to the manufacturer's protocol. The
pan kinase inhibitor staurosporine was used at 1 μM as a reference control.
Inhibition is presented as the percentage of the kinase activity relative to the vehicle control. Inhibition curves were analyzed by four-parameter regression. Samples were analyzed in duplicate in six independent experiments ($n = 6$).

**Cell Culture and Differentiation.** An SH-SY5Y human neuroblastoma cell line (ATCC CRL-2266; Sigma-Aldrich, Saint Louis, MO) was cultured in 1:1 (v/v) DMEM/F12 media supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS), and 1% antibiotics including penicillin and streptomycin. Cell cultures with three to four passages were used for the experiments in compliance with ATTC recommendation. After reaching 70–80% confluence, cells were subcultured on poly(-lysine) plates with 10 μM retinoic acid in a reduced serum media (1% FBS) to induce postmitotic differentiation. 7,25 Differentiation was confirmed by the change to polygonal morphology and extension of long neurites. Cells were cultured at 37 °C in a fully humidified atmosphere containing 5% CO₂.

**Aβ$_{1-40}$ Oligomer Preparation.** The toxic oligomers of Aβ$_{1-40}$ were prepared as described previously. 27 Briefly, lyophilized Aβ$_{1-40}$ peptide was dissolved in hexafluoroisopropanol, dried under vacuum, and stored at −20 °C. Immediately prior to use, the peptide residue was reconstituted in the DMEM/F12 media to make a 0.1 mM stock solution and incubated at 4 °C for 24 h to form diffusible oligomers. The toxicity of Aβ$_{1-40}$ oligomers at a final concentration of 5 or 10 μM was confirmed by cell viability assay.

**Chemical Treatment in SH-SY5Y Cells.** SH-SY5Y human neuroblastoma cells were seeded at a density of 3 × 10⁵ cells/mL in a 6-well or 96-well poly(-lysine) plate or a 10 cm poly(-lysine) culture dish in DMEM/F12 media containing 10 μM retinoic acid and 1% FBS to induce postmitotic differentiation. The cells were incubated under regular culture conditions for attachment. After 24 h of plating, the cells were pretreated with different concentrations of test chemicals or a reference control for 1 h before co-incubation with 5 or 10 μM Aβ$_{1-40}$ during a desired time period.

**Anti-Aβ$_{1-42}$ Neurotoxicity Assay and Neuronal Morphology Analysis.** The assay procedure was previously described. 27–29 SH-SY5Y cells were treated with chemicals in a 6-well plate under the culture conditions described above. Staurosporine at 1 μM was used as a reference control for cytotoxicity, while 0.05 μM SB202190 was used as a reference control for p38 inhibition. After experimental treatment for 72 h, the cells were subjected to microscopic analyses of neuronal morphology and a CellTiter 96 AQueous One Solution Cell Proliferation MTS assay according to the manufacturer’s instruction. Cell viability was normalized by comparing the colorimetric intensity of the nontreated control (100%) and 10 μM NaF, 20 mM Na₂PO₄, 2 mM NaN₃VO₃, 1% Triton X-100, 10% glycerol, 10% DMSO, and a protease inhibitor cocktail (Sigma-Aldrich). Total protein concentrations were determined with the Bradford assay (Bio-Rad). The phosphorylated human tau at the S396 site (a specific site of p38 kinase found in AD) 25 in the cell lysate was quantified with the human Tau (Phospho) [pS396] ELISA kit according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Absorbance at 450 nm was read with a Multiskan Go microplate reader (Thermo Scientific, Waltham, MA). Samples were analyzed in duplicate in six independent experiments ($n = 6$).

**JC-10 Mitochondrial Membrane Potential Assay.** The assay procedure was previously described. 30 SH-SY5Y cells were treated with chemicals in a 96-well plate under the culture conditions described above. The mitochondrial ionophore carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine (FCCP) at 50 μM was used as a reference control. After incubation at 37 °C for 72 h, the cells were subjected to a JC-10 mitochondrial membrane potential potential assay according to the manufacturer’s instruction (Abcam, Burlingame, CA). Briefly, JC-10 dye-loading solution was added to each well and incubated at 37 °C, 5% CO₂ for 1 h. Fluorescence intensities (Ex/Em = 490/525 nm and Ex/Em = 540/590 nm) of each well were monitored. The ratio of fluorescence intensity (S25/S90 nm) was used to determine the mitochondrial membrane potential (Δψₘ). Increasing ratios indicate mitochondrial membrane depolarization. Samples were analyzed in duplicate in six independent experiments ($n = 6$).

**Nuclear Extraction of Cell Lysate.** The harvested SH-SY5Y cells were washed with ice-cold PBS, incubated with a hypotonic buffer containing 20 mM Tris-HCl, pH 7.4; 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, 1 mM PMSF, and a protease inhibitor cocktail on ice for 15 min, and vortexed. The cell homogenate was centrifuged (1000g) at 10 min at 4 °C. The resulting supernatant containing the cytosolic fraction was removed. The nuclear pellet was lysed with cell extraction buffer containing 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₂PO₄, 2 mM NaN₃VO₃, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, and a protease inhibitor cocktail followed by centrifugation (14000g) for 30 min at 4 °C. Nuclear protein concentrations were determined with the Bradford assay.

**Nuclear NF-κB DNA-Binding ELISA Analysis.** The assay procedure was previously described. 31–36 SH-SY5Y cells were treated with chemicals in a 6-well plate under the culture conditions described above. Dexamethasone (DEX, 0.1 μM) known as a MAFK/NF-κB inhibitor with anti-inflammatory activities was used as a reference control. After the experimental treatment for 8 h, nuclear proteins were extracted from harvested cells. TransAM NF-κB Family Assay Kit (Active Motif, Carlsbad, CA) was used to determine concentrations of p50, p52, p65, RelB, and c-Rel in cellular nuclear fractions via a colorimetric DNA-binding ELISA in response to the NF-κB consensus sequence (5′-GGGACTTCCC-3′). Wild-type and mutated consensus oligonucleotides were used to monitor the NF-κB binding specificity. Nuclear NF-κB levels were quantified by measuring the absorbance at 450 nm on a Multiskan Go microplate reader. Samples were analyzed in duplicate in six independent experiments ($n = 6$).

**General Procedures for Instrumental Analysis.** Fluorescent and luminescent measurements were performed on an Agilent Cary Eclipse fluorescence spectrophotometer. Optical absorbance was measured on a Multiskan GO microplate reader. Microscopic images were taken under a Nikon Diaphot inverted tissue culture microscope with Optronics MicroFire microscope camera (Nikon Precision Inc., Belmont, CA).

**Statistical Analysis.** The data and statistical analyses were complied with the recommendations on experimental design and analysis in pharmacology. Data were presented as mean ± SEM or mean ± SD of three to six independent experiments performed in duplicate. The data were analyzed by one-way ANOVA with Tukey’s multiple comparison post hoc test as well as Student’s t-test; p values less than 0.05 were considered statistically significant. Analysis was performed using GraphPad Prism 6 and SigmaPlot 13.0.
Author Contributions
Z.L., B.D.H., and Q.X.L. conceived the project, designed experiments, and performed data analysis and interpretation. Z.L. and B.Z. performed kinase and most cellular assays. M.X. acquired the sEH assay data. The manuscript was written through contributions of all authors.

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Notes
The authors declare the following competing financial interest(s): B.D.H. is a founder of EicOsis Human Health, which is developing sEH inhibitors for treatment of inflammatory and neuropathic pain.

Abbreviations
AD, Alzheimer’s disease; Aβ1-42, β-amyloid fragment peptide 1–42; TPUU, 1-trifluoromethoxyphenyl-3-[(propionylpiperidin-4-yl) urea; t-AUCB, trans-4-[(3-adamantan-1-yl-ureido)-cyclohexyl]-benzoic acid; EETs, epoxycisatrienioic acids; DHETs, dihydroxyicosatrienioic acids; [3H]-t-DPPO, [3H]-trans-diphenylpropene oxide; DEX, dexamethasone; FCCP, carbonyl cyanide-4-[trifluoromethoxy] phenylhydrazone; PEG 400, poly(ethylene glycol) 400; sEH, soluble epoxide hydrolases; p38 MAPK, p38 mitogen-activated protein kinase; GSK-3β, glycogen synthase kinase-3β; ERK2, extracellular signal regulated kinase 2; JNK1, c-Jun N-terminal kinase 1; JNK3, c-Jun N-terminal kinase 3; CDK1/cyclin A, cyclin-dependent kinase 1 with subunit cyclin A; CDK2/cyclin E, cyclin-dependent kinase 2 with subunit cyclin E; CDK3/cyclin E, cyclin-dependent kinase 3 with subunit cyclin E; CDK5/p25, cyclin-dependent kinase 5 with subunit p25; CDKS/p35, cyclin-dependent kinase 5 with subunit p35; CDK6/cyclin D, cyclin-dependent kinase 6 with subunit cyclin D; CDK9/cyclin K, cyclin-dependent kinase 9 with subunit cyclin K; CLK1, dual specificity protein kinase 1; AKT1, v-akt murine thymoma viral oncogene homolog 1; p70S6K, p70 ribosomal protein S6 kinase beta; PDK1, phosphoinositide-dependent kinase 1; PKA, protein kinase A; PKC, protein kinase C; PRKG1, cGMP-dependent protein kinase 1; ROCK1, Rho-associated, coiled-coil containing protein kinase 1; RSK2, ribosomal protein S6 kinase 2; AMPK, AMP-activated protein kinase with subunits; CAMKIIα, Ca2+/calmodulin-dependent protein kinase IIα; CAMKIIγ, Ca2+/calmodulin-dependent protein kinase IIγ; CAMKV, Ca2+/calmodulin-dependent protein kinase IV; DAPK1, death-associated protein kinase 1; STK33, serine/threonine-protein kinase 33; CK2α1, casein kinase 2α1; DNA-PK, DNA-dependent protein kinase; CK1ζ, casein kinase 1ζ; CK1ε, casein kinase 1ε; CK1γ1, casein kinase 1γ1; VRK2, vaccinia related kinase 2; RAGE, receptor for advanced glycation end-products; nAChR, nicotinic acetylcholine receptor; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; ROS, reactive oxygen species; ER, endoplasmic reticulum

REFERENCES


