Anthranilate Derivative Inhibits Glutamate Release and Glutamate Excitotoxicity In Rats

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Research Article

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Abstract

The neurotransmitter glutamate plays an essential role in excitatory neurotransmission; however, excessive glutamate leads to excitotoxicity, which is the most common pathogenic feature of numerous brain disorders. This study aimed to investigate the role of butyl 2-[2-(2-fluorophenyl)acetamido]benzoate (HFP034), a synthesized anthranilate derivative, in the central glutamatergic system. We used rat cerebrocortical synaptosomes to examine the effect of HFP034 on glutamate release. In addition, we used a rat model of kainic acid (KA)-induced glutamate excitotoxicity to evaluate the neuroprotective potential of HFP034. We showed that HFP034 inhibited 4-aminopyridine (4-AP)-induced glutamate release from the synaptosomes, and this inhibition was abolished in the absence of extracellular calcium. HFP034-mediated inhibition of glutamate release was associated with a decreased 4-AP-evoked Ca$^{2+}$ level elevation, and had no effect on synaptosomal membrane potential. The inhibitory effect of HFP034 on evoked glutamate release was suppressed by blocking P/Q-type Ca$^{2+}$ channels and protein kinase C (PKC). Furthermore, HFP034 inhibited the phosphorylation of PKC and its substrate, myristoylated alanine-rich C kinase substrate (MARCKS), in the synaptosomes. We also observed that HFP034 pretreatment reduced neuronal death, glutamate concentration, glial activation, and the levels of endoplasmic reticulum stress-related proteins, calpains, glucose-regulated protein 78 (GRP 78), C/EBP homologous protein (CHOP), and caspase-12 in the hippocampus of KA-injected rats. We concluded that HFP034 is a neuroprotective agent that prevents glutamate excitotoxicity, and we suggest that this effect involves the inhibition of presynaptic glutamate release by suppressing P/Q-type Ca$^{2+}$ channels and PKC/MARCKS pathways.

Introduction

Glutamate is a key excitatory neurotransmitter in brain development, synaptic transmission, synaptic plasticity, and learning and memory processes [1, 2]. However, excess glutamate leads to the excessive activation of glutamate receptors, which increases the concentration of intracellular calcium and results in the activation of proteases, production of free radicals, induction of the mitochondrial dysfunction, and activation of pro-apoptotic factors. These effects ultimately lead to neurodegeneration and neuronal death. Indeed, glutamatergic excitotoxicity is the most common pathogenic feature of many brain disorders, such as ischemia, epilepsy, and psychiatric and neurodegenerative diseases [3, 4]. Therefore, modulating glutamate levels may be a valuable strategy for reducing neurotoxicity and protecting the brain.

Anthranilate derivatives have gained much attention in drug discovery and development because of their diverse pharmacological activities, including anti-inflammatory, antiviral, immunosuppressive, anticancer, antithrombotic, antidiabetic, and analgesic activities [5–14]. Anthranilate derivatives was also reported to have antidepressant and anticonvulsant effects, as described in different animal models [14, 15], indicating that they are potential candidates for drug therapy for related diseases such as anxiety, depression, and epilepsy. Butyl 2-[2-(2-fluorophenyl)acetamido]benzoate (HFP034) is a synthetic anthranilate derivative with reported anti-inflammatory effects [16, 17]. However, no studies on the role of
HFP034 in the central nervous system (CNS), especially regarding synaptic glutamate release, are available. Therefore, this study aimed to investigate the effect of HFP034 on glutamate release from nerve terminals of the rat cerebral cortex and evaluate its neuroprotective effect in a rat model of excitotoxicity induced by the systemic administration of kainic acid (KA), a glutamate analog [18].

**Materials And Methods**

The experimental protocol was approved by the Fu Jen Institutional Animal Care and Utilization Committee with code A11009. Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals. The minimal number of animals to obtain consistent data were employed.

**Materials**

HFP034 was synthesized by one of the authors (Pei-Wen Hsieh) [16]. dl-threo-β-benzylloxyaspartate (dl-TBOA), bafilomycin A1, dantrolene, 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), and bisindolylmaleimide I (GF109203X) were purchased from Tocris (Bristol, UK). 3,3,3-Dipropylthiadicarbocyanine iodide [DiSC$_3$(5)], and fura-2-acetoxymethyl ester (Fura-2-AM) were purchased from Thermo (Waltham, USA). ω-conotoxin GVIA (ω-CgTX GVIA) and ω-agatoxin IVA (ω-Aga IVA) were purchased from Alomone lab (Jerusalem, Israel). 4-aminopyridine (4-AP), dimethylsulfoxide (DMSO), kainic acid (KA) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Adult male Sprague-Dawley rats (n = 42, 150-200 g) were purchased from BioLASCO (Taipei, Taiwan).

**Synaptosome preparation**

Rats (n = 18) were sacrificed via cervical dislocation and the cerebral cortex were rapidly removed. The brain tissue was homogenized in 320 mM sucrose solution and centrifuged at 300 0 g rpm for 10 min. The supernatant was stratified on a Percoll discontinuous gradients and centrifuged at 32,500 g for 7 min. The synaptosomal fraction was collected and centrifuged for 10 min at 27,000 g. Protein concentration was determined using the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets with 0.5 mg protein, as previously described [19-21].

**Glutamate release analysis**

For the glutamate release experiments, the synaptosomal pellet (0.5 mg protein) was resuspended in the hepes-buffered solution and glutamate release was assayed by on-line fluorimetry [22]. CaCl$_2$ (1.2 mM), glutamate dehydrogenase (GDH, 50 units/ml) and NADP$^+$ (2 mM) were added at the start of incubation. Glutamate release was induced with 4-AP (1 mM) and monitored by measuring the increase of fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively) resulting from NADPH being produced by the oxidative deamination of released glutamate by GDH. Released glutamate
was calibrated by a standard of exogenous glutamate (5 nmol) and expressed as nanomoles glutamate per milligram synaptosomal protein (nmol/mg).

**Intrasynaptosomal Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\))**

Synaptosomes (0.5 mg protein) were incubated in the hepes-buffered solution containing Fura 2-AM (5 μM) and CaCl\(_2\) (0.1 mM) for 30 min at 37°C. Samples were centrifuged for 1 min at 5000 rpm, and pellets were resuspended in hepes-buffered medium containing CaCl\(_2\) (1.2 mM). Fura-2-Ca fluorescence was monitored at 5 s intervals for 5 min. [Ca\(^{2+}\)]\(_i\) (nM) was calculated by using calibration procedures and equations described previously [23].

**Membrane potential**

The synaptosomal membrane potential was assayed with a positively charged membrane potential-sensitive carbocyanine dye DiSC\(_3\)\(_{5}\). DiSC\(_3\)\(_{5}\) fluorescence was monitored at 2 s intervals and data are expressed in fluorescence units [24].

**Histological analysis**

The rats (n = 24) were divided into four experimental groups: DMSO-treated group (control), KA-treated group, HFP034 10 mg/kg + KA group, and HFP034 30 mg/kg + KA group. HFP034 was dissolved in a saline solution containing 1% DMSO and was administered (i.p.) 30 min before KA injection (15 mg/kg in 0.9% NaCl, pH 7.0, i.p.). For Nissl staining, rats (n = 3 per group) were euthanized at 72 h after KA injection by transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) under ether anesthesia. The brains were removed, fixed overnight with 4% paraformaldehyde solution, cryoprotected in sucrose phosphate buffer at 4°C. The brains were cut into 30 μm coronal sections and mounted on gelatinized slides, air-dried and stained with 0.1% aqueous crystal violet stain (Sigma Chemicals, St. Louis, MO, USA) for 20 min. Then, the slides were washed in distilled water, differentiated in 70% ethyl alcohol and dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and mounted with DPX (Sigma Chemicals, St. Louis, MO, USA). For immunofluorescence staining, the brain sections were blocked with 2% bovine serum albumin (BSA) in PBS for 30 min, and then incubated overnight at 4°C with primary antibody for anti-NeuN (1:500, Merckmillipore), anti-OX42 (1:500, Merckmillipore), and anti-GFAP (1:1000, Cell signaling). The sections were incubated for 90 min at room temperature with corresponding secondary antibodies (1:1000, Alexa Fluor 488, DyLight 594, Invitrogen), and then mounted on gelatin-coated slides and coverslipped with VectaShield medium (Vector Labs, Burlingame, CA). Cells were stained with nuclear staining dye DAPI (1 μg/ml, Sigma-Aldrich) for 20 sec. Images were captured with an upright fluorescence microscope (Zeiss Axioskop 40, Goettingen, Germany) using ×4 (aperture is 0.1) and ×10 (aperture is 0.25) objectives. The numbers of living neurons, NeuN\(^+\), OX42\(^+\), and
GFAP+ cells were counted in a 255×255µm² area of the hippocampal CA1 and CA3 using Image J software (Synoptics, Cambridge, UK).

**High-performance liquid chromatography**

Determination of glutamate concentrations in brain tissue was performed by high-performance liquid chromatography (HPLC) system with electrochemical detection (HTEC-500). Briefly, the frozen hippocampal tissue was prepared by homogenizing in 5 ml hepes-buffered medium. The homogenate was centrifuged at 1500g at 4°C for 10 min then the supernatant was filtered through 0.22 µm filters before injection into HPLC. The relative free glutamate concentration was determined using peak areas by an external standard method. Serial dilutions of the standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus corresponding concentrations of each standard [21].

**Western blot**

Synaptosome or the hippocampal tissue was lysed in an ice-cold Tris–HCl buffer solution, centrifuged for 10 min at 13000  g at 4°C. The supernatant was measured the protein concentration using the Bradford protein assay (Bio-Rad laboratories, Hercules, CA, USA). Equal amounts (30 µg) of protein were loaded per lane onto 10% polyacrylamide gel, and then transferred to a polyvinylidenedifluoride (PVDF) membrane in a semi-dry system (Bio-Rad, Hercules, USA) for 120 min. Transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, and 0.05% Tween 20) and incubated overnight at 4 °C with specific primary antibodies [anti-protein kinase C (PKC), 1:700, Abcam); phospho-PKC (1:2000, Cell signaling; PKCa (1:600, Cell signaling), phospho-PKCa (1:2000, Abcam); phospho-MARCKS (1:250, Cell signaling); calpain 1 (1:2000, Abcam), calpain 2 (1:800, Millipore); caspase 12 (1:3000, Abcam); C/EBP homologous protein (CHOP) (1:300, Santa Cruz); glucose-regulated protein 78 (GRP 78) (1:1500, Abcam); β-actin (1:8000, Cell signaling)]. Membranes were washed with TBST for 15 min and incubated with horseradish peroxidase-coupled secondary antibodies (1:16000, GeneTex) for 1 h at room temperature. Next, the specific protein bands were visualized using a film exposure with the chemiluminescence detection system (GeneTex, CA, USA) and quantified using Image J software (Synoptics, Cambridge, UK).

**Statistical analyses**

Results are expressed as mean ± standard error of the mean (S.E.M.). Statistical analysis was performed using GraphPad Prism-8 software (GraphPad Inc., San Diego, CA). When testing the significance of the effect of HFP034 versus control, a Student's t-test was used. When comparing the effect of HFP034 in different experimental conditions, one-way analysis of variance (ANOVA) was used followed by Tukey's post hoc test. p < 0.05 was considered to indicate a statistically significant difference between groups.
Results

Effect of HFP034 on 4-AP-evoked glutamate release from rat cerebrocortical synaptosomes

To investigate the effect of HFP034 on glutamate release, synaptosomes isolated from rat cerebral cortex were stimulated by 4-AP (1 mM), which opens voltage-dependent Ca$^{2+}$ channels and induces the release of glutamate [25]. As shown in Fig. 1B, preincubation of synaptosomes with HFP034 (10 μM) for 10 min before 4-AP addition did not produce any significant effect on the basal release of glutamate, but markedly reduced the 4-AP-induced release of glutamate release in the presence of 1.2 mM CaCl$_2$ [t(9) = 36.77, p < 0.001 vs. control group]. HFP034 (1–30 μM) caused a concentration-dependent inhibition of 4-AP-evoked glutamate release, reaching a maximal effect at 10 μM (Fig. 1C). In addition, 4-AP-evoked glutamate release was reduced in extracellular-Ca$^{2+}$-free solution that contained 300 μM EGTA [F(2,11) = 563.01, p < 0.001]. This Ca$^{2+}$-independent glutamate release evoked by 4-AP was, however, not affected by HFP034 (10 μM) (p = 0.92; Fig. 1C). dl-TBOA, an inhibitor of the plasma membrane glutamate transporters, which blocks the Ca$^{2+}$-independent nonvesicular efflux by transporter reversal, increased 4-AP-evoked glutamate release [F(2,11) = 274.59, p < 0.001 vs. control group]. When dl-TBOA we present, the inhibitory effect of HFP034 on 4-AP-evoked glutamate release was not changed (p < 0.05 vs. dl-TBOA-treated group; Fig. 1C). By contrast, bafilomycin A1, an inhibitor of vesicular glutamate transporters, reduced 4-AP-evoked glutamate release [F(2,11) = 323.16, p < 0.001 vs. control group]. In the presence of bafilomycin A1, HFP034 failed to produce significant inhibition (p = 0.94 vs. bafilomycin A1-treated group; Fig. 1C). Furthermore, HFP034 (10 μM) preincubation efficiently decreased 15 mM KCl-evoked glutamate release [t(9) = 16.5, p < 0.001 vs. control group; Fig. 1D], a process that involves Ca$^{2+}$ influx primarily through voltage-dependent Ca$^{2+}$ channel opening [26].

Effect of HFP034 on 4-AP-induced [Ca$^{2+}$]$_i$ elevation and synaptosomal membrane potential

Figure 2A shows that 4-AP (1 mM) elicited a rise in [Ca$^{2+}$]$_i$ and HFP034 (10 μM) preincubation reduced the 4-AP-induced [Ca$^{2+}$]$_i$ increase by 33% [t(9) = 9.89, p < 0.001 vs. control group]. HFP034 (10 μM) had no significant effect on the basal [Ca$^{2+}$]$_i$ (p = 1). In addition, 4-AP (1 mM) evoked DiSC$_3$(5) fluorescence increase and this phenomenon was not affected by HFP034 (10 μM) preincubation [t(9) = −0.09, p = 0.93 vs. control group; Fig. 2B].

Effect of HFP034 on glutamate release in the presence of voltage-dependent Ca$^{2+}$ channel blockers or intracellular Ca$^{2+}$ release inhibitors

Either voltage-gated Ca$^{2+}$ channels (VGCCs) or intracellular Ca$^{2+}$ stores is responsible for the release of glutamate evoked by depolarization [27, 28]. As shown in Fig. 3, 4-AP-evoked glutamate release was
reduced by 2 µM ω-conotoxin GVIA \([\omega-\text{CgTX GVIA}, F(2,11) = 523.85, p < 0.001 \text{ vs. control group}]\) and 0.5 µM ω-agatoxin IVA \([\omega-\text{AgTX IVA}, F(2,11) = 476.69, p < 0.001 \text{ vs. control group}]\), which selectively block N- and P/Q-type Ca\(^{2+}\) channels, respectively [29]. When ω-CgTX GVIA was present, 4-AP-evoked glutamate release was further inhibited by HPF (10µM) \((p < 0.05 \text{ vs. ω-CgTX GVIA-treated group})\). However, the inhibitory action of HPF034 was abolished in the presence of ω-Aga IVA. The release measured in the presence of ω-Aga IV and HPF034 being similar to that obtained in the presence of ω-Aga IV \((p = 0.83 \text{ vs. ω-Aga IVA-treated group})\). In addition, 4-AP-evoked glutamate release was reduced by 10 µM dantrolene, an inhibitor of intracellular Ca\(^{2+}\) release from the endoplasmic reticulum (ER) \([F(2,11) = 675.25, p < 0.001 \text{ vs. control group}]\), and 10 µM CGP37157, an inhibitor of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange \([F(2,11) = 125.84, p < 0.001 \text{ vs. control group}]\). When dantrolene or CGP37157 was present, HFP034 (10 µM) was able to reduce 4-AP-evoked glutamate release \((p < 0.05 \text{ vs. dantrolene- or CGP37157-treated group}; \text{Fig. 3})\).

**Contribution of decreased protein kinase C/MARCKS pathway to the HFP034-mediated inhibition of glutamate release**

The involvement of protein kinase C (PKC)-dependent pathway in the HFP034-mediated inhibition of glutamate release was studied using the selective PKC inhibitor, GF109203X (10 µM) [30]. As shown in Fig. 4A, GF109203X (10 µM) reduced the glutamate release induced by 4-AP \([F(2,11) = 113.84, p < 0.001 \text{ vs. control group}]\). When GF109203X was present, HFP034 (10 µM) did not produce any significant inhibition of glutamate release \((p = 0.98 \text{ vs. GF109203X-treated group})\). In addition, we determined the effect of HFP034 on the phosphorylation of PKC and its substrate MARCKS in the synaptosomes. Compared with the control group, a statistically significant increase in the phosphorylation of PKC \([F(2,12) = 154.66, p<0.001]\), PKC\(\alpha\) \([F(2,12) = 40.59, p<0.001]\), and MARCKS \([F(2,12) = 43.52, p<0.001]\) was observed in the 4-AP group. After pretreatment with HFP034 (10 µM), no significant increase in PKC, PKC\(\alpha\), and MARCKS phosphorylation was observed after exposure to 1 mM 4-AP compared with the control group \((p > 0.05; \text{Fig. 4B})\).

**Effect of HFP034 on the neuronal death and glutamate levels in the hippocampus of rats with KA**

We also studied the effect of HFP034 in a rat model of KA-induced glutamate excitotoxicity. As shown in Fig. 5A, rats were treated with HPF034 or DMSO 30 min before KA injection (15 mg/kg, i.p.). Three days after the KA injection, Nissl staining of the hippocampal sections showed decreased neurons in the CA1 \([F(3,8) =153.35, p < 0.001]\) and CA3 \([F(3,8) = 46.12, p < 0.001]\) regions in the KA group compared with the control group. Pretreatment with HFP034 (10 and 30 mg/kg, i.p.) protected the neurons from damage both in the CA1 and CA3 regions compared to that in the KA group. Quantitative studies showed a significant increase in the number of living neurons in the CA1 and CA3 of HFP034-pretreated rats, compared with KA-treated rats \((p < 0.05, \text{Fig. 5B})\). Similar results were observed with NeuN staining of the hippocampal section. Quantification of neurons in the CA1 and CA3 regions showed significantly
decreased neurons in the CA1 \[F(3,8) = 54.65, p < 0.001\] and CA3 \[F(3,8) = 39.53, p < 0.001\] regions in the KA group compared with the control group. Pretreatment with HFP034 significantly increased the number of neurons in both the CA1 and CA3 regions compared to that in the KA group \(p < 0.05\); Fig. 5C). In addition, we examined the effect of HFP034 on the concentration of glutamate in the hippocampus of rats with KA (Fig. 5D). Compared to the control group, statistical analysis revealed that KA significantly increased glutamate levels in the hippocampus \[F(3, 7) = 60.17, p < 0.001\]. However, groups that received KA and were pretreated with HFP034 had a decrease in glutamate levels in the hippocampus, data that was significantly different from the KA group \(p < 0.05\).

Effect of HFP034 on the levels of endoplasmic reticulum (ER) stress-related proteins in the hippocampus of rats with KA

To investigate how HFP034 attenuated the KA-induced neuronal death, we examined whether pretreatment with HFP034 contributed to reduction of KA-induced ER stress, resulting in neuronal damage [31]. As shown in Fig. 6, the levels of the ER stress signature molecules including calpains \[F(3,8) = 25.24, p < 0.001 \text{ vs. control group}\], GRP 78 \[F(3,8) = 216.04, p <0.001 \text{ vs. control group}\], CHOP \[F(3,8) = 373.71, p < 0.001 \text{ vs. control group}\], and caspase-12 \[F(3,8) = 55.94, p < 0.001 \text{ vs. control group}\] were increased in the hippocampus of rats treated with KA compared to the control group, but the increased levels were decreased by HFP034 pretreatment \(p < 0.05 \text{ vs. KA group}\).

Effect of HFP034 on the activation of microglia and astrocyte in the hippocampus of rats with KA

Microglia and astrocyte activation is a common pathological feature following KA-induced excitotoxic injury [32]. We next examined the effect of HFP034 on KA-induced microglia and astrocyte activation in hippocampus sections using anti-OX42 and anti-GFAP antibodies, respectively. In the Fig. 7A, we observed a significant increase in the number of OX42\(^+\) microglial cells in the hippocampal CA1 \[F(3,8) = 82.69, p < 0.001\] and CA3 \[F(3,8) = 164.19, p < 0.001\] regions of KA group compared with the control group. In contrast, HFP034-pretreated rats displayed much less staining for OX42, indicating a reduced microglial response to KA-induced injury. Quantification of the results showed a significant decrease in the number of OX42\(^+\) cells \(p < 0.05 \text{ vs. KA group}; \text{Fig. 7B}\). In addition, the evaluation of GFAP staining for astrocyte activation showed a considerably response in KA-treated rats compared with control group \(\text{CA1, } F(3,8) = 124.93, p < 0.001; \text{CA3, } F(3,8) = 129.61, p < 0.001; \text{Fig. 7A}\). Similarly, HFP034 pretreatment significantly reduced astrocyte activation induced by KA, as observed by the reduction in the number of GFAP\(^+\) cells in the CA1 and CA3 \(p < 0.05 \text{ vs. KA group}; \text{Fig. 7C}\).

Discussion

The excessive release and accumulation of glutamate in the brain is associated with excitotoxicity, a key mechanism that contributes to neuronal degeneration in several acute and chronic CNS diseases [3, 33]. Finding new drugs that regulate glutamate release and provide protection against glutamate excitotoxicity is therefore crucial [4, 34]. In the current study, we investigate the effect of the anthranilate
derivative HFP034 on glutamate release in vitro and its neuroprotective potential against KA-induced glutamate excitotoxicity in vivo. We also examine possible mechanisms underlying the effects of HFP034.

The influence of HFP034 on glutamate release has not been previously explored in synaptosomal preparations. Here, we demonstrate that HFP034 reduces the release of glutamate induced by 4-AP in rat cerebrocortical synaptosomes. The reduction of glutamate release by HFP034 revealed several notable features. First, when extracellular Ca\(^{2+}\) ions were removed, HFP034 failed to inhibit the 4-AP-induced release of glutamate, suggesting a possible dependency of the drug's action on Ca\(^{2+}\) influx. Second, the inhibition was prevented by the vesicular glutamate transporter blocker bafilomycin A1, not dl-TBOA, an inhibitor of the plasma membrane glutamate transporter that blocks the Ca\(^{2+}\)-independent nonvesicular efflux by transporter reversal [35]. This finding suggests that a reduction of Ca\(^{2+}\)-independent glutamate release through the glutamate transporter is not involved in the effect of HFP034. Third, HFP034 reduced the 4-AP-induced increase in intrasynaptosomal Ca\(^{2+}\) levels, which resulted from both an increase in calcium entry through VDCCs and calcium release from ER or mitochondria [27, 29]. However, HFP034 did not inhibit glutamate release when P/Q-type Ca\(^{2+}\) channels were blocked, and intracellular Ca\(^{2+}\) release inhibitors had no effect, suggesting the involvement of a reduction in Ca\(^{2+}\) influx mediated by P/Q-type Ca\(^{2+}\) channels. Supporting this, we observed that HFP034 also considerably inhibited KCl-induced glutamate release, which involves only Ca\(^{2+}\) channels [25]. Fourth, HFP034 had no effect on synaptosomal membrane potential. This indicates that HFP034 does not reduce synaptosomal excitability, which would reduce the influx of Ca\(^{2+}\) and thus reduce glutamate release. Finally, the inhibition of PKC prevented the effect of HFP034 on evoked glutamate release. Furthermore, HFP034 suppressed the 4-AP-evoked PKC and its substrate MARCKS phosphorylation. PKC present in nerve terminals is activated by Ca\(^{2+}\) thereby phosphorylating MARCKS. This phosphorylation reaction causes cytoskeletal disassembly, which increases synaptic vesicle availability and glutamate release [36, 37]. Therefore, our data suggest that HFP034 suppresses P/Q-type Ca\(^{2+}\) channels, which would reduce Ca\(^{2+}\)-induced activation of PKC/MARCKS and subsequently reduces synaptic vesicle availability and glutamate release from rat cerebrocortical synaptosomes. However, how HFP034 affects P/Q-type Ca\(^{2+}\) channels remains to be elucidated.

We also found that HFP034 exerted neuroprotective efficacy in a rat model of KA-induced glutamate excitotoxicity. KA, a glutamate analog, is a powerful neurotoxic agent that stimulates glutamate release [38]. Systemic KA injection induces neuronal damage in several brain regions, especially in the hippocampus [39]. Neuronal damage induced by KA resembles that of some forms of neurological diseases; thus, KA is an ideal agent for clarifying the mechanisms underlying neurodegeneration and neuroprotection [18]. In this study, compared with the control group, KA significantly increased glutamate levels in the hippocampus and caused substantial neuronal loss in the hippocampal CA1 and CA3 regions, as has been reported previously [40, 41, 21]. These KA-induced alternations were significantly counteracted in the HFP034 pretreatment group, indicating that HFP034 exerts anti-excitotoxic and neuroprotective effects. In addition, we observed that KA increased the expression levels of ER stress-
associated proteins, including calpain, GRP78, CHOP, and caspase-12 in the hippocampus; these changes were also reduced by HFP034 pretreatment. These results suggest that HFP034 can reduce ER stress, a key factor of neuronal death in numerous neurological diseases [42, 43]. In particularly, ER stress can cause neuronal death either by triggering ER Ca\(^{2+}\) release, resulting in calpain and caspase-12 activation, or by activating CHOP and GRP78-mediated pro-apoptotic pathways [44–46], which have been suggested to be involved in neuronal cell death after KA-induced excitotoxicity [43]. Furthermore, ER stress inhibition can protect against KA-induced hippocampal neuronal damage [47, 48]. Thus, suppressed KA-induced ER stress might partly explain the neuroprotective effect of HFP034.

In addition to ER stress, inflammatory responses, including the activation of microglia and astrocytes, are often associated with KA-induced excitotoxic injury. Activated glial cells increase the production of toxic substances, which in turn contribute to the expansion of brain injury and an increased neuron loss. This evidence suggests that the control of KA-induced neuroinflammation is vital to protect the hippocampal neurons [32, 49]. In the present study, we observed that KA substantially increased in the number of activated microglia and astrocytes in the hippocampus, and these increases were suppressed by HFP034 pretreatment. Thus, the suppression of neuroinflammation, in addition to ER stress, could be another mechanism explaining the neuroprotection provided by HFP034 against KA-induced glutamate excitotoxicity. Our finding is consistent with that of previous studies that have reported the anti-inflammatory activities of HFP034 [16, 17]. Although how HFP034 suppresses glial activation was not explored in this study, toll-like receptors (TLRs) have been shown to play a critical role in glial cell activation and subsequent hippocampal neuron excitotoxicity [50, 51]. Whether HFP034 inhibits the activation of TLRs, thereby reducing glial cell activation and thereby further suppressing KA-induced excitotoxic insults, is a possibility that should be addressed in future studies.

The ability of HFP034 to reduce glutamate release from nerve terminals may partially explain its neuroprotective mechanism against KA-induced excitotoxicity in vivo. This hypothesis is based on the association between KA-induced neurotoxicity and excessive glutamate release [38, 52]. Apart from excessive glutamate release, KA also causes glutamate receptor overstimulation. This overstimulation results in calcium elevation and subsequently triggers intracellular cascade reactions, including reactive oxygen species production, lipid peroxidation, ER stress, and mitochondrial dysfunction and inflammation, eventually leading to cell death [18]. On the basis of these considerations, HFP034 might suppress ER stress and neuroinflammation to prevent KA-induced insults to neuronal integrity, which may be associated with the inhibition of released glutamate.

In summary, we demonstrated that the anthranilate derivative HFP034 inhibits glutamate release from rat cerebrocortical nerve terminals by suppressing P/Q-type Ca\(^{2+}\) channels and the PKC/MARCKS pathways and that HFP034 prevents KA-induced glutamate neurotoxicity in vivo by inhibiting ER stress and neuroinflammation (Fig. 8). To our knowledge, this is the first report to demonstrate the inhibitory role of HFP034 in glutamate release and glutamate excitotoxicity. This finding may provide a pharmacological basis for the clinical use of HFP034 in the treatment of CNS diseases involving glutamate excitotoxicity.
Declarations

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Author Contributions Conceptualization, Tzu-Yu Lin and Cheng-Wei Lu; data curation, Pei-Wen Hsieh and Kuan-Ming Chiu; formal analysis, Tzu-Yu Lin, Cheng-Wei Lu and Pei-Wen Hsieh; funding acquisition, Tzu-Yu Lin, Cheng-Wei Lu and Su-Jane Wang; investigation, Tzu-Yu Lin, Cheng-Wei Lu and Su-Jane Wang; project administration, Kuan-Ming Chiu and Ming-Yi Lee; resources, Pei-Wen Hsieh and Kuan-Ming Chiu; supervision, Su-Jane Wang; writing—original draft, Su-Jane Wang; writing—review and editing, Ming-Yi Lee and Su-Jane Wang. All authors have read and agreed to the published version of the manuscript.

Ethics approval Ethical approval was granted by the Fu Jen Catholic University (No. A11009) and, therefore, experiments were performed in accordance with the ethical standards laid out by the IACUC.

Data Availability All data generated or analyzed during this study are included in the published article and its supplementary information files.

Consent for Participate Not applicable.

Consent for Publication All authors have given consent for publishing in the journal Molecular Neurobiology.

Conflict of Interest The authors declare no competing interests.

References


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Figures
Figure 1

Effect of HFP034 on 4-AP-evoked glutamate release from rat cerebrocortical nerve terminals. (A) The chemical structure of HFP034. (B) Glutamate release was measured under control conditions or in the presence of 10 µM HFP034 added 10 min prior to the addition of 4-AP (1 mM). (C) Effect of HFP034 at different concentrations on 4-AP-evoked glutamate release, and extracellular Ca2+-free solution, glutamate transporter inhibitor DL-TBOA or vesicular glutamate transporter inhibitor balomycin A1 on
the effect of HFP034. Effect of HFP034 on the release of glutamate evoked by 15 mM KCl was showed in the Fig. 1C. Data are presented as mean ± S.E.M. (n = 5 per group). ***p < 0.001 vs control group; #p < 0.001 vs DL-TBOA-treated group.

**Figure 2**

Effect of HFP034 on [Ca2+]i (A) and the synaptosomal membrane potential (B). HFP034 (10 µM) was added 10 min before the addition of 4-AP. Data are presented as mean ± S.E.M. (n = 5 per group). ***p <
0.001 vs control group.

Figure 3

Effect of HFP034 on 4-AP-evoked glutamate release in the presence of N-type Ca2+ channel blocker ω-CgTX GVIA, P/Q-type Ca2+ channel blocker ω-AgTX IVA, ryanodine receptor inhibitor dantrolene, or mitochondrial Na+/Ca2+ exchanger inhibitor CGP37157. HFP034 was added 10 min before the addition of 4-AP, and other drugs were added 10 min before this. Data are presented as mean ± S.E.M. (n = 5 per group). ***p < 0.001 vs control group; #p < 0.001 vs dantrolene- or CGP37157-treated group.
Figure 4

(A) Effect of the PKC inhibitor GF109203X on the HFP034-mediated inhibition of 4-AP-evoked glutamate release. (B) Effect of HFP034 on PKC and MARCKS phosphorylation evoked by 4-AP. HFP034 or GF109203X was added 10 min before the addition of 4-AP. Data are presented as mean ± S.E.M. (n = 5 per group). ***p < 0.001 vs control group; #p < 0.001 vs GF109203X- or 4-AP-treated group.
Figure 5

Effect of HFP034 pretreatment on the neuronal cell death and glutamate levels in the hippocampus of rats with KA. (A) Representative images of crystal violet and NeuN staining at 3 d after i.p. KA. (B, C) Quantitative data of A showing the number of living neurons and NeuN+ cells in the hippocampal CA1 and CA3 regions. (D) The effect of HFP034 on the concentration of glutamate in the hippocampus of rats.
with KA. Data are presented as mean ± S.E.M. (n = 3 rats for each group). ***p < 0.001 vs control group; #p < 0.001 vs KA group.

**Figure 6**

Effect of HFP034 pretreatment on the expression levels of ER stress-associated proteins, calpains, GRP78, CHOP, and caspase-12 in the hippocampus of rats with KA. Data are presented as mean ± S.E.M. (n = 3 rats for each group). ***p < 0.001,**p < 0.01 vs control group; *p < 0.01, #p < 0.001 vs KA group.
Figure 7

Effect of HFP034 pretreatment on the activation of microglia and astrocyte in the hippocampus of rats with KA. (A) Representative images of OX42 and GFAP staining at 3 d after i.p. KA. (B, C) Quantitative data of A and C showing the number of OX42+ and GFAP+ cells in the hippocampal CA1 and CA3 regions. Data are presented as mean ± S.E.M. (n = 3 rats for each group). ***p < 0.001 vs control group; #p < 0.001 vs KA group.
Figure 8

A proposed mechanism underlying the inhibition of glutamate release and glutamate excitotoxicity by HFP034 in rat. HFP034 depresses glutamate release via suppressing presynaptic P/Q-type Ca2+ channels and PKC/MARCKS pathway, as well as protects KA-induced neuronal death via ER stress and neuroinflammation inhibition. Black arrows indicate positive regulation, and red arrows indicate negative regulation.