A novel non-selective atypical PKC agonist could protect neuronal cell line from Aβ-oligomer induced toxicity by suppressing Aβ generation

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Abstract. Atypical protein kinase C (aPKCs) serve key functions in embryonic development by regulating apical-basal polarity. Previous studies have shed light on their roles during adulthood, especially in the development of Alzheimer’s disease (AD). Although the crystal structure of PKCζ has been resolved, an agonist of aPKCs remains to be discovered. In this study, by using the Discovery Studio program and LibDock methodology, a small molecule library (K66-X4436 KINA Set) of compounds were screened for potential binding to PKCζ. Subsequently, the computational docking results were validated using affinity selection-mass spectrometry, before in vitro kinase activity was used to determine the function of the hit compounds. A cell-based model assay that can mimic the pathology of AD was then established and used to assess the function of these hit compounds. As a result, the aPKC agonist Z640 was identified, which could bind to PKCζ in silico, in vitro and in this cell-based model. Z640 was further confirmed as a non-selective aPKC agonist that can activate the kinase activity of both PKCζ and PKCζ. In the cell-based assay, Z640 was found to protect neuronal cell lines from amyloid-β (Aβ) oligomer-induced cell death by reducing reactive oxygen species production and restore mitochondrial function. In addition, Z640 could reduce Aβ40 generation in a dose-dependent manner and shift amyloid precursor protein processing towards the non-amyloid pathway. To conclude, the present study is the first, to the best of the authors’ knowledge to identify an aPKC agonist by combining computer-assisted drug discovery and cell-based assays. The present study also revealed that aPKC agonists have therapeutic potential for the treatment of AD.

Introduction

The protein kinase C (PKC) family of kinases is comprised of 11 members. Based on their molecular structures and modes of activation, they are divided into the following three major categories: Conventional PKCs; novel PKCs; and atypical PKCs (aPKC, ζ and ι/λ) (1,2). aPKCs serve key roles during embryonic development. aPKCs form polarity complexes with other components and translocate between the apical or basolateral membranes to regulate the direction of epithelial cell division (3,4). Furthermore, aPKCs can interact with partitioning-defective (Par)-6 in the subapical epithelial region alongside the aPKC substrate Par-3 (bazooka in Drosophila) (3). This aPKC/Par-6/Par-3 complex is key for the establishment of apical-basal polarity and for the maturation of epithelial junctions in both Drosophila and mammals (4). In turn, this complex regulates the direction of asymmetric cell division during development (5), which is a key step in determining the fate of the majority of tissues during the embryonic growth stage.

Within the aPKC family, there are two isozymes, PKCζ and PKCs/ι. Although their regulatory regions differ from those of other members in the PKC family, they do share 84% sequence homology (6). aPKC isozymes are co-translationally phosphorylated by mTORC2 on the turn motif, followed by phosphorylation by phosphoinositide-dependent protein kinase-1 on the activation loop (7). Similar to other PKC family members, aPKCs maintain their phosphorylated status after maturation, which keeps it in an auto-inhibited conformation. Thereafter, activation of aPKCs requires the phosphorylation of the activation loop (8).

Unlike other PKC family members, aPKCs do not have established activators since their activation is not dependent on phospholipid hydrolysis. Instead, they are activated by binding to protein scaffolds (9). After binding to scaffold proteins, aPKCs are harbored near the plasma membrane in proximity to their substrates. In addition, this type of binding can relieve auto-inhibitory constraints by moving the pseudo-substrate domain away from the substrate-binding cavity. The interaction

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of PKCζ with the scaffolding protein p62 results in the tethering of the basic PKCζ pseudo-substrate to the acidic surface of the Phox-Bem1 domain of p62, which maintain PKCζ in an open and active conformation (10). Similarly, PKCζ can also be maintained in an open conformation when bound to the cell polarity-associated protein Par6 (11). There are several known substrates that can form complexes with aPKCs to transduce signals, including Par3, Par6, LLGL scribble cell polarity complex component 2, Rho-associated coiled-coil containing protein kinase 1, microtubule affinity regulating kinase 2 and the Hippo pathway component KIBRA (8,12-16).

Although aPKCs are better known for their function in regulating cell polarity during embryonic development, previous studies also revealed their potential role during adulthood. The N-terminal truncated PKCζ was found to be necessary for maintaining synaptic potentiation in hippocampal slices (17,18). Another previous study found that the intraventricular injection of PKCζ can activate synapses, but inhibiting aPKCs could suppress late-stage long-term potentiation (LTP) in vivo (19). Using the ζ inhibitory peptide, a non-selective aPKC inhibitory pseudo-substrate peptide, blocking aPKC function in the brain is found to reverse LTP and impair spatial memory in the rat hippocampus (20). Furthermore, it was previously demonstrated that PKCζ is involved in Alzheimer's disease (AD), such that PKCζ could regulate β-secretase 1 (BACE1) trafficking and distribution in the hippocampus neuron and therefore reduce Aβ accumulation (21).

The crystal structure of PKCζ has been resolved and widely available for a number of years. However, there remains to be a limited number of small molecules, if any, that can regulate aPKC activity. The only known aPKC inhibitor to date is the pseudo-substrate peptide (22), which has limited translational development potential due to its large molecular size.

To discover novel aPKC agonists that may have therapeutic effects on AD, the present study first performed an in silico docking screening from a kinase hit library (K66-X4436) (23). Following the virtual docking, hit compounds were synthesized and affinity selection mass spectrometry (ASMS) and in vitro kinase activity assay performed as secondary binding confirming assays. Cell based assay was also performed to evaluate compound hits protective effects in amyloid-β (Aβ) toxicity cell model and to investigate potential mechanisms such as reactive oxygen species (ROS) generation, mitochondria function, amyloid protein precursor (APP) processing and Aβ accumulation.

Materials and methods

Cell line and reagents. The Neuro-2a or N2a cell line (a mouse neuroblastoma cell line) was purchased from American Type Culture Collection (cat. no. CCL-131). The WT7 cell line, which is N2a cells stably expressing both the human APP695 Swedish mutant and wild-type human presenilin-1 (PS1), was a generously provided by Professor Sangram Sisodia, Department of Neurobiology of University of Chicago (Chicago, USA).

The Aβ 25-35 fragment was purchased from Dalian Meilun Biology Technology Co., Ltd. The CCK8 cell viability kit and RIPA cell lysis buffer (strong; cat. no. P0013B) was purchased from Beyotime Institute of Biotechnology. The mitochondrial membrane potential assay kit (cat. no. JC-1) and ROS staining dye dichloro-dihydro-fluorescein diacetate (DCFH-DA) were purchased from Shanghai Yeasen Biotechnology Co., Ltd. The PKCζ (cat. no. v9731) and PKCζ (cat. no. v3751) Kinase Enzyme Systems were purchased from Promega Corporation. Human Aβ (1-40) ELISA kit (cat. no. 298-64601) was purchased from FUJIFILM Wako Pure Chemical Corporation. Z640 was synthesized by WuXi AppTec.

Computational docking. Molecular docking aims to calculate the binding orientation of small molecules to their targets, to search for small molecules that can interact with target proteins with high affinity and selectivity. Computational docking was performed using Discovery studio 2016 (DS 2016; https://www.3ds.com/products-services/biovia/) in the present study. Briefly, the small molecule database (ID K66-X4436 KINASet), which contained 11,021 molecules, was obtained from J&K Scientific, Ltd. and used as the screening library. The small molecules in this database were prepared using DS 2016. The crystal structure of PKCζ [protein databank (PDB) code: 3A8W] was downloaded from the PDB for the present study. The PKCζ structure was prepared for molecular docking analysis using DS 2016. The active sites of PKCζ were defined using the PDB site records, where the radius of the active site is 10.3 Å. The PKCζ and the small molecule database were docked using the ‘libdock’ function in DS 2016. According to the libdock score, 5,000 small molecules were chosen for docking with PKCζ using the ‘CDOCKER’ tool in DS 2016. The strength of the interaction was evaluated based on CDOCKER energy and CDOCKER interaction energy. Finally, hit compounds from this virtual screening yielding positive interactions were synthesized.

ASMS. ASMS was performed as described previously (24-26). Briefly, an automated ligand identification system was used to detect signal. This is a dual-chromatography liquid chromatography (LC)/mass spectrometry (MS) system that can separate the unbound compounds from protein-bound compounds at the first step before using the reversed-phase of LC/MS to identify any binding compounds. A positive ionization method was used and nitrogen was used as nebulizing gas and drying gas. MS detection was accomplished using a high-resolution Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc.) scanning from 150 to 800 m/z at 100,000 resolutions with a mass accuracy of <5 ppm and a scan rate of 1 Hz. The nebulizer was set at 40 psi, drying gas temperature at 350°C with a flow rate of 1.2 l/min.

Preparation of Amyloid-β oligomer aggregates. The Aβ 25-35 fragment was dissolved in distilled water to a concentration of 500 μM (stock solution). To obtain the neurotoxic form of Aβ 25-35, the peptide solution was placed in an incubator at 37°C for 7 days and stored at -80°C until further use. Each batch of Aβ oligomers was examined by Thioflavin (THT) staining before use.

Cell viability assay. The proliferation of the cells was detected via CCK-8 assay (Beyotime Institute of Biotechnology). Briefly, prior to the treatment, N2a or WT7 cells were plated in 96-well plates at a density of 1x10⁴ cells/well in DMEM media.
with 10% FBS for 24 h. Growth medium was replaced with fresh culture medium without FBS then and cells were treated with 50 μM Aβ-oligomers with or without different concentrations of Z640 (0, 0.03, 0.1, 0.3, 1, 3, or 10 μM) at 37°C for 24 h. After incubation, cell viability assay was conducted by treating cells with CCK-8 reagents (10 μl/well) at 37°C for 2 h. Plates were read at 450 nm using a BioTek Synergy HT Multi Mode Microplate Reader. The difference in optical density (OD) relative to untreated control group or Aβ-oligomers treatment alone group were measured to draw dose-response curve. Half maximal effective concentration (EC50) was calculated. All experiments were performed in 5 replicates.

Measurement of ROS generation. ROS generation was measured using the DCFH-DA dye. Cells were incubated at 37°C for 1 h in the dark in HEPES containing DCFH-DA (200 μM). Intracellular fluorescence was measured using a spectrofluorometer (BioTek Synergy HT Multi-Mode Microplate Reader; Agilent Technologies, Inc.) at an emission wavelength of 525 nm and an excitation wavelength of 488 nm. The images were also captured using a confocal microscope (Leica TCS SP8, with 4× objective lens; Leica Microsystems GmbH). The intensity of fluorescence staining was analyzed using ImageJ software (1.50i; National Institutes of Health).

Measurement of mitochondrial membrane potential (MMP). MMP was measured by using the fluorescent probe JC-1. Cells were incubated with the JC-1 staining solution (5 mg/ml) for 20 min at 37°C. The fluorescence intensity of both mitochondrial JC-1 monomers (excitation, 514 nm; emission, 529 nm) and aggregates (excitation, 585 nm; emission, 590 nm) were detected using a microplate reader (BioTek Synergy HT Multi-Mode Microplate Reader; Agilent Technologies, Inc.) and the images were also captured using a confocal microscope (Leica TCS SP8, with 4× objective lens; Leica Microsystems GmbH). The collected Fluorescence Unit from microplate reader was shown as fluorescence ratio of red to green.

Aβ40 ELISA. The WT7 cells were plated into 96-well plates at a density of 3x10^4 cells/ml and incubated with the Aβ-oligomers and indicated concentrations of Z640 (0, 0.03, 0.1, 0.3, 1, 3, or 10 μM) at 37°C for 24 h. After incubation, the culture medium was collected, and the protein concentration was determined by BCA protein assay (Thermo Scientific, Inc.). Following protein denaturing, 50 μg total protein was loaded onto 10% SDS PAGE gel and then transferred onto PVDF membranes. Membranes were then blocked with 5% BSA (Beyotime Institute of Biotechnology) for 1 h at room temperature. Following blocking, membranes were incubated with primary antibody overnight at 4°C and then the secondary antibody 1 h at room temperature. The primary antibodies used in the present study included mouse-anti-APP (1:2,000; cat. no. 6E10; Biolegend, Inc.), rabbit anti-BACE1 (1:1,000; cat. no. 5606T, Cell Signaling Technology, Inc.), rabbit anti-pBACE1-496 (1:1,000; cat. no. PA5-10574; Invitrogen; Thermo Fisher Scientific, Inc.), rabbit anti-PS1 (1:5,000; cat. no. MAT5232; MilliporeSigma), rabbit anti-pPKCβ (1:500; cat. no. 9368T, Cell Signaling Technology, Inc.), rabbit anti-APP C-terminal fragment (1:10,000; cat. no. 18717T; CTFβ); MilliporeSigma) and β-actin (1:5,000; cat. no. sc-47778, Santa Cruz Biotechnology, Inc.). The secondary antibody used were HRP-labelled anti-Rabbit IgG (1:5,000; cat. no. 7074, Cell Signaling Technology, Inc) and HRP-labelled anti-mouse IgG (1:5,000; cat. no. 7076, Cell Signaling Technology, Inc). Bands were visualized by using enhanced ECL reagent (cat. no. WBULS0500, MilliporeSigma) and developed in dark room. The bands were semi-quantified by using ImageJ software (1.50i; National Institutes of Health).

Statistical analysis. Results were analyzed using GraphPad Prism Software Version 6.0 (GraphPad Software, Inc.). All data were presented as the mean ± standard deviation. The significance of difference was determined using unpaired Student's t-test or one-way analysis of variance followed by Tukey's test. The significance of differences among cell types were determined by using Two-way ANOVA followed with Bonferroni and Sidak's test. The EC50 was calculated automatically by using non-linearized curve regression. P<0.05 was considered to indicate a statistically significant difference.

Results

In silico library docking and screening. After in silico screening, Z640 was selected (Fig. 1A). As shown in Fig. 1B and C, Z640 was able to bind PKCζ in an allosteric binding position. The CDocker energy was calculated to be-24.075 kcal/mol whereas the CDocker interaction energy was calculated to be-38.4802 kcal/mol. Molecular studies revealed that Z640 may form conventional hydrogen bonds with the Arg339, Lys340 and Ala349 residues of PKCζ, in addition to forming carbon hydrogen bonds with Ser475, Gly440 and Arg339. Furthermore, Z640 may form pi-cation interactions with Lys340 and pi-alkyl interactions with Arg441 (Fig. 1D).

Z640 can activate aPKC activity in vitro. The ASMS results indicated that Z640 can bind to PKCζ with an affinity value of 1.37 μM (Fig. 2A), which is comparable to the binding affinity
were screened using the template of PKC. The program was used for compound library screening and >10,000 compounds were tested if such binding could regulate aPKC kinase activity.

of ATP to the majority of the kinases. The present study then tested if such binding could regulate aPKC kinase activity. By using in vitro kinase activity assays, both recombinant PKCζ and PKCπ were treated with Z640 in ascending doses. Z640 was found to increase PKCζ and PKCπ kinase activity in a dose-dependent manner, with an in vitro EC50 of 1.09 and 3.47 µM, respectively (Fig. 2B and C).

Z640 can protect neuronal cell lines against Aβ oligomer-induced toxicity. Considering that aPKC expression has been previously reported to be downregulated in AD and may therefore be involved in AD pathogenesis (27,28), the present study next considered the hypothesis that upregulating aPKC activity may be therapeutic for AD. A cell-based assay mimicking AD toxicity was first established by treating N2a or WT7 cells with Aβ oligomers. WT7 cell was generated by stably overexpress human APP Swedish-mutation and human presenilin-1 (PS1) into N2a cell. Generally, APP will get through sequential cleavages by several different enzymes and Aβ40 or 42 will be produced after the final cleavage conducted through PS1 (γ secretase complex). The human APP-Swedish mutation has long been proved having much stronger affinity to γ secretase compared with wildtype APP and thus will produce a higher level of Aβ40 or 42. In this regard, WT7 cell is an ideal cell model to mimic in vivo AD pathology due to its high Aβ generation and accumulation (29,30). Aβ oligomers were found to induce significant cell toxicity at concentrations of 50 µM and higher. In particular, at 50 µM concentration, Aβ oligomers induced higher levels of toxicity in WT7 cells compared with N2a cells (data not shown). The cells were then treated with different concentrations of Z640 alone. Z640 could not induce cell death at concentrations <100 µM (Fig. 3A). Since 10 µM is the most frequently applied concentration for testing hit compound function in cell-based assays without inducing significant off-target effects (31), 10 µM was deemed to be a safe concentration for the use of Z640 for subsequent cell experiments thereafter.

Z640 was next applied in the cell-based assays to examine the effects of Z640 against Aβ oligomer-induced toxicity. Z640 was found to significantly reduce Aβ oligomer-induced cell death at 10 µM, with more potent protective effects observed in WT7 cells compared with N2a cells (Fig. 3B). This finding suggests that the protective effects of Z640 may be specifically associated with APP processing and Aβ generation, since the only difference between these two cell lines is the level of Aβ expression. Furthermore, the EC50 of Z640 in both Aβ oligomer toxicity models was also evaluated and results indicated that Z640 has an EC50 of 4.57 µM in WT7 cells while 419.8 µM in N2a cells (Fig. 3C). This data also showed a much stronger protective effect of Z640 in WT7 compared with N2a cell.

Z640 can protect cells by correcting Aβ oligomer-induced ROS elevation and mitochondria damage. Mitochondria damage is one of the most extensively reported mechanisms underlying Aβ oligomer-induced cell death. As Z640 was found to protect Aβ oligomer-mediated cytotoxicity, the present study subsequently tested if Z640 treatment can protect against cell death by reducing mitochondria damage. JC-1 staining was used to test the effects of Z640 on MMP. In healthy cells, JC-1 exists as a monomer in the cytoplasm (green) but forms aggregates (red) in mitochondria due to the higher MMP. However, in apoptotic and necrotic cells, JC-1 only exists in its monomeric form and is therefore only stained in the cytoplasm (green). Using JC-1 live cell staining followed by both confocal microscopy and fluorescence intensity reading, the degree of mitochondria impairment was next examined after Z640 and/or Aβ oligomer treatments. The results showed that the Aβ oligomers could damage mitochondria membrane integrity, whereas 10 µM Z640 could largely restore this impairment (Fig. 4).

Since elevated intracellular ROS is frequently accompanied with mitochondria impairments and is used as a biomarker for measuring mitochondrial stress, the effects of Z640 on intracellular ROS levels were next tested. Aβ oligomers were found to significantly increase intracellular ROS levels but 10 µM Z640 could markedly reverse this (Fig. 5A-C). Furthermore, an experiment was performed to assess any potential dose-dependent effects. Z640 was able to reduce Aβ oligomer-induced
ROS elevations in a dose-dependent manner, with an EC50 of 5.11 µM (Fig. 5D).

**Z640 can regulate APP processing and reduce Aβ generation.** A previous study demonstrated that PKCζ knockdown or inhibition can promote the retrograde trafficking of BACE1, thereby increasing Aβ generation and intracellular accumulation (32). However, there is no evidence demonstrating how the overexpression of PKCζ or elevating its kinase activity can regulate Aβ generation. By using the WT7 cell line, the effects of Z640 on APP processing and Aβ generation was assessed. Consistent with previous reports (33,34), Aβ oligomers treatment promoted APP processing towards the β-cleavage pathway and therefore increased both CTF-β and Aβ generation. Following treatment with 10 µM Z640 it was found that although Z640 alone could not induce significant changes in full-length APP and APP-CTFβ, it could significantly increase the expression of APP-CTFα and the ratio of APP-CTFα/IPP-CTFβ (Fig. 6). This observation suggested that Z640 could regulate the direction of APP processing. Following co-treatment together with 50 µM Aβ-oligomers, 10 µM Z640 could almost completely reverse the effects of

**Figure 2.** Z640 binds to PKCι in vitro and increases both PKCι and PKCζ kinase activity. (A) Purified PKCι was treated with different concentrations of Z640 to detect its binding affinity by affinity selection mass spectrometry. The EC50 is 1.37 µM. (B) PKCι and (C) PKCζ in vitro kinase activity was detected and represented as luminescence units (LU). EC50 is 3.47 and 1.09 µM, respectively. n=3 for both experiments. PKC, protein kinase C; EC50, half maximal effective concentration.

**Figure 3.** Z640 can protect neuronal cell lines from Aβ oligomer-induced cell death. (A) N2a and WT7 cells were treated with the indicated concentrations of Z640 for 24 h before cell viability was measured. (B) N2a and WT7 cells were treated with 50 µM Aβ-oligomers alone or combined with 10 µM Z640 for 24 h. Cell viability was then measured. (C) N2a and WT7 cells were treated with 50 µM Aβ-oligomers together with indicated concentration of Z640 for 24 h. Cell viability was measured and dose-response curve were drawn. *P<0.05 and **P<0.01 vs. control; #P<0.05 vs. Aβ-oligomers; &P<0.05, N2a vs. WT7 cells. n=6 for each experiment. Aβ, amyloid beta.
Aβ-oligomers on APP processing to the level similar to that in Z640 alone. This finding strongly suggested that both Aβ oligomers and Z640 are competitors in the regulation of APP processing through the same signaling pathway. However, the present study also showed that although Z640 could not alter BACE1 expression levels, it could reduce PS1 expression. To the best of the authors' knowledge, there have been no reports indicating that aPKCs can regulate PS1 expression. Therefore, this finding may shed light on the possibly novel functions of aPKCs on regulating PS1 expression.

The effects of Z640 on soluble Aβ generation was next investigated by measuring Aβ40 levels in the culture medium. The results showed that Z640 could effectively reduce Aβ40 generation in a dose-dependent manner with an EC_{50} of 2.73 µM (Fig. 6i).

Discussion

The present study first performed the virtual docking screen of aPKCs with K66-X4436 KINASet database. The reason virtual docking was chosen as a starting point was because this is a much more convenient way to select a hit compound than other ways, such as screening from a real compound library by ASMS or surface plasmon resonance. K66-X4436 is a diverse kinase library, which means the chance to obtain positive hits from this library are higher than other non-organized libraries. After docking, a novel aPKCs agonist structure was screened and named as Z640. Furthermore, By using ASMS, its binding affinity to PKCι was determined to be 1.37 µM in vitro. Although aPKCs have distinct structures compared with other families of PKCs, they do have an active catalytic domain that can utilize ATP to phosphorylate their substrates. In the present study, computational docking was performed through the entire PKCι structure instead of only in the aTP adapting pocket, which is typically performed instead for searching for aPKC inhibitors. Based on the binding position, the orientation of Z640 with the lowest energy was found to be at a site far away from the aTP binding site (35,36). Therefore, it was predicted that the binding of Z640 to PKCι may cause allosteric structural changes, which increases the in vitro kinase activity of aPKCs without altering the catalytic site. However, the detailed structure-activity relationship must be determined by comparing apo and co-crystallization structures collected using X-ray crystallography or nuclear magnetic resonance in future.

Another discovery of the present study was that aPKC activation could reduce Aβ generation by shifting APP processing according to the cell-based assay. So far, there have been several hypotheses for the occurrence of AD, the Aβ hypothesis is one of the most studied among them. People have known that Aβ oligomers could damage neuron from multiple aspects, such as oxidative stress, calcium flux hyperactivation, hyper-active microglia, suppress synaptic morphology and...
function and may even induce Tauopathy (37,38). After the AD pathology is initiated, Aβ oligomers will continuously accumulate in the cortex/hippocampus microenvironment and keep attacking synapse and thus worsen the disease situation (39). In a previous study, in patients with AD, the activity of the Par3/aPKC complex was found to be reduced (40). In addition, the Par3/aPKC complex was previously found to regulate BACE1 trafficking in cultured primary hippocampal neurons (21,32,41). These previous studies demonstrated that after inhibiting aPKC function in the primary neurons, BACE1 would be translocated in a largely retrograde manner from the cell membrane into the trans-Golgi network to increase Aβ generation. However, due to the lack of effective agonists, it was not possible to test if activating the Par3/aPKC complex could reduce Aβ generation, which is a key concept for the translational development of aPKC agonists for potential AD therapeutics. In the present study, by using Z640 it was demonstrated that following aPKC activation, a PP processing can be shifted towards the α-cleavage pathway to generate cTF-α instead of cTF-β, which decreases the levels of Aβ. To the best of the authors’ knowledge, these results demonstrated for the first time that aPKC agonists have potential benefits in an AD model, which strongly supported the feasibility of developing aPKC agonists for AD therapeutics.

Aβ monomers have been observed to interact with higher order oligomers and fibrils to directly form aggregation nuclei and pathogenic dimers (42). Furthermore, Aβ oligomers have been confirmed to upregulate Aβ generation through several pathways, including activating the glutamate synapse or enhancing calcium influx (31). These previous

Figure 5. Z640 can reduce Aβ oligomer-induced reactive oxygen species elevation. (A) N2a and WT7 cells were treated with 50 µM Aβ oligomers alone or combined with 10 µM Z640 for 24 h before being stained with DCFH-DA for 20 min. Confocal imaging was captured at x4 magnification. Scale bar=300 µm. (B and C) N2a and WT7 cells were treated with 50 µM Aβ oligomers alone or combined with 10 µM Z640 for 24 h before being stained with DCFH-DA for 20 min. Fluorescence units at the green spectra was detected using a plate reader. (D) WT7 cells were treated with indicated concentrations of Z640 together with 50 µM Aβ oligomers for 24 h before being stained with DCFH-DA for 20 min. Fluorescence units at the green spectra was detected using a plate reader. n>20 fields or five experiments for confocal imaging, n=3 for fluorescence intensity detection. *P<0.05 vs. Control; #P<0.05 vs. Aβ oligomers group. Aβ, amyloid-β; DCFH-DA, dichloro-dihydro-fluorescein diacetate.
findings suggest that Aβ monomers and Aβ oligomers may function synergistically to induce neuronal cell death. Based on this Aβ oligomer theory, the hypothesis that inhibiting Aβ monomer generation by activating aPKC may reduce Aβ oligomer-induced neuronal cell toxicity was tested in the present study. The comparative data collected from n2a and WT7 cells suggested that Z640 can exert more potent protective effects against Aβ oligomers in WT7 cells, consistent with this hypothesis. However, it was also noted that Z640 could exert protective effects against Aβ oligomer-induced n2a cells toxicity, which does not express endogenous Aβ.

This result indicated activated aPKC might also protect cells from pathways other than by regulating Aβ generation. For example, some studies indicated that aPKC activation might promote cell survival and proliferation by activating β-Catenin/Wnt signal pathway (43-45). The Wnt/β-Catenin pathway is a very conserved signal pathway and exists extensively in the majority of types of tissue and cells. The activation of β-Catenin/Wnt signal pathway might increase cell resistance to undesired or toxic microenvironments and thus show protective effects in N2a cells. However, potential off-target effects of Z640 cannot be excluded in the present study. By using the DS 2016 program, other potential bio-targets of Z640 were also explored. The results indicated Z640 may bind to several enzymes, including lyases or hydrolases (data not shown). The unspecific binding may also lead to this extra protection. A comprehensive kinase crosstalk panel screening should be performed in the future for clarification.

Another key finding in the present study is that Z640 yielded similar EC50 values in all assays tested. Z640 was able to act against Aβ induced toxicity with an EC50 of 4.57 μM, reduce Aβ 40 generation with an EC50 of 2.73 μM and could reduce Aβ oligomer-mediated ROS production with an EC50 of 5.11 μM. Furthermore, Z640 could activate aPKCs kinase activity with a EC50 of 3.46 and 1.08 μM. Considering the similar activation performance of Z640 on above bio-events, it was hypothesized that these bio-events might occur in a
cascade way and the common upstream modulator is Z640. Z640 could activate aPKCs in the cell and the activated aPKC will then reduce Aβ generation and thus reduce Aβ oligomer-mediated cell toxicity.

The present study had several limitations that should be addressed. The optimization of the dose of Z640 is required. In the present study, Z640 showed an EC50 value ≤5 µM for the majority of the experiments, which would serve as a good starting point for translational development in the future. In addition, future studies should be focused on modifying Z640 to optimize its binding affinity. A co-crystallization or soaking structure will assist in this type of investigation. Another limitation is that the selectivity was not optimized. A panel kinase activity screening for Z640 is required to determine its selectivity. Finally, more hits libraries should be screened in future work to find better starting structures.

In summary, by using a computational docking/modeling program, an in silico screening of a commercial hits library was performed, which revealed the non-selective aPKC agonist Z640. Its ability to bind to aPKCs was verified in vitro using ASMS and kinase activity assays. Z640 was next applied in a cell-based assay panel and it was found that Z640 could alleviate Aβ oligomer-induced cell apoptosis by reducing ROS generation to preserve mitochondria function. Finally, Z640 could regulate APP processing to reduce Aβ generation to preserve mitochondria function.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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