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Research Paper

Soluble Gamma-secretase Modulators Attenuate Alzheimer's β-amyloid Pathology and Induce Conformational Changes in Presenilin 1

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ABSTRACT

A central pathogenic event of Alzheimer's disease (AD) is the accumulation of the Aβ42 peptide, which is generated from amyloid-β precursor protein (APP) via cleavages by β- and γ-secretase. We have developed a class of soluble 2-aminothiazole γ-secretase modulators (SGSMs) that preferentially decreases Aβ42 levels. However, the effects of SGSMs in AD animals and cells expressing familial AD mutations, as well as the mechanism of γ-secretase modulation remain largely unknown. Here, a representative of this SGSM scaffold, SGSM-36, was investigated using animals and cells expressing FAD mutations. SGSM-36 preferentially reduced Aβ42 levels without affecting either α- and β-secretase processing of APP nor Notch processing. Furthermore, an allosteric site was identified within the γ-secretase complex that allowed access of SGSM-36 using cell-based, fluorescence lifetime imaging microscopy analysis. Collectively, these studies provide mechanistic insights regarding SGSMs of this class and reinforce their therapeutic potential in AD.

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disorder and the leading cause of dementia. There is currently no treatment available to slow or halt disease progression. The underlying mechanisms of AD on the cell and molecular levels are still not completely elucidated. Considerable genetic, pathological, biochemical, and molecular biological evidence supports the amyloid-cascade hypothesis, stating that the production and excessive accumulation of a small peptide, amyloid-β (Aβ), is the primary pathological event leading to AD (Gandy, 2005; Hardy and Selkoe, 2002; Tanzi and Bertram, 2005). Specifically, the accumulation of Aβ, particularly the neurotoxic Aβ42 peptide, affects neuronal synaptic functions and triggers an inflammatory response which is followed by neuritic injury and the generation of pathological tau proteins, ultimately leading to neuronal dysfunction and cell death.

AD is a genetically complex disease. Four AD genes (*APP*, *PSEN1*, *PSEN2* and *APOE*) have been identified which primarily serve to increase the ratio of Aβ42 to Aβ40 or in the case of the Swedish mutation, absolute Aβ peptide levels. The imbalance triggered by these genetic aberrations enhances the oligomerization of Aβ into neurotoxic assemblies and ultimately leads to dementia (Bertram and Tanzi, 2008; Choi et al., 2014; Tanzi and Bertram, 2005). In the amyloidogenic pathway, Aβ is produced via sequential proteolytic cleavage of the type I transmembrane protein, amyloid-β (A4) precursor protein (APP) by β- and γ-secretase, respectively (Bertram and Tanzi, 2008; Zhang and Saunders, 2007). γ-Secretase is a heterogeneous protein complex, formed by at least four transmembrane proteins: presenilin (PS1), presenilin enhancer 2 (PEN2), nicastrin, and anterior pharynx-defective 1(APH-1) (Bertram and Tanzi, 2008; Edbauer et al., 2003; Sisodia and St George-Hyslop, 2002). Over 200 mutations in the PS1-encoding gene (*PSEN1*) have been identified to cause early-onset familial AD (EOFAD), underscoring the relevance of the enzyme with respect to the disease. γ-Secretase regulates the intramembrane proteolysis of APP

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and numerous other substrates that have previously undergone ectodomain shedding, including Notch (Gu et al., 2004; Kopan and Ilagan, 2004; Sisodia and St George-Hyslop, 2002). The processing of Notch at the ε-site (or s3) represents a critical function of γ-secretase which yields a large cytoplasmic peptide, the Notch intracellular domain (NICD), which can translocate to the nucleus and is essential for cellular differentiation and development (Herreman et al., 1999; Kopan et al., 1994).

One essential strategy for AD therapeutics has focused specifically on APP processing and attenuating Aβ production (Bertram and Tanzi, 2008; Selkoe, 2001; Zhang, 2012, 2017). Initially, a class of drugs known as γ-secretase inhibitors (GSIs), were developed which potently inhibit γ-secretase activity. Despite the ability to preclude Aβ production, GSIs exhibit unfavorable activities that result in cell toxicity through increasing the levels of APP carboxy-terminal fragments (CTFs; CTFα and CTFβ), and side effects potentially elicited through down-regulation of Notch processing (Mitani et al., 2012). One of the well-characterized GSIs is semagacestat (or LY450139) (Doody et al., 2013; Mitani et al., 2012). Although it decreased the levels of all Aβ species (Potter et al., 2013), semagacestat recently failed in the pivotal phase 3 clinical trial for AD (Doody et al., 2013). Nevertheless, the results provide useful knowledge toward the features of a therapeutic for AD, which should avoid unfavorable adverse events associated with inhibition strategies in targeting either γ-, or β-secretease (De Strooper, 2014; Ward et al., 2017; Willem et al., 2015). Furthermore, this result provides evidence for a general need of improved understanding with respect to the critical biological roles of γ-secretase especially within the context of therapeutic development (De Strooper, 2014; Zhang, 2017).

A group of small molecule with a more promising therapeutic mechanism are known as γ-secretase modulators (GSMs), which modulate the cleavage activity of γ-secretase (and likely a host of other substrates) and specifically reduce the levels of the fibrillogenic Aβ42 peptide without altering the ε-site cleavage of APP or numerous other γ-secretase substrates, including Notch (Brendel et al., 2015; Imbimbo et al., 2007; Kounnas et al., 2010; Rogers et al., 2012). Since GSMs spare the ε-site processing of Notch, these compounds are considered likely to be safer and better tolerated than GSIs.

Previously, we reported the development and characterization of a series of GSMs with promising biological activities (Kounnas et al., 2010). The initial aminothiazole class of compounds displayed high potency for inhibiting the secretion of the Aβ42 peptide, however these compounds also suffered from poor aqueous solubility (Kounnas et al., 2010). Utilizing rational medicinal chemistry design, a class of aminothiazole GSMs was developed with improved physicochemical properties which include increased aqueous solubility and thus were referred to as soluble GSMs (or SGSMs)(Wagner et al., 2014). Importantly, these SGSMs were effective at reducing the levels of Aβ using cell-based models (D'Avanzo et al., 2015; Wagner et al., 2014). Further pharmacokinetic evaluation of this aminothiazole class of SGSMs in mice identified a lead compound, SGSM-36, which showed good brain penetration, as well as good clearance, half-life, and volume of distribution (Rynearson et al., 2016). These results collectively support the continued development of this class of compounds as a potential therapy for AD (Rynearson et al., 2016).

To advance our understanding of this aminothiazole class of compounds, further investigation using AD animal-based models and mechanistic studies were required to establish proof of concept in future clinical applications. Thus, in this study, we further characterized the representative aminothiazole SGSM with improved physicochemical properties for the potential effects on γ-secretase processing of APP in vivo using a well-studied AD mouse model, in addition to cell models of the disease. Furthermore, we investigated the effects of our representative aminothiazole SGSM on PS1 conformation using cell-based, fluorescence lifetime imaging microscopy (FLIM) analysis.

2. Materials and Methods

2.1. Animal Studies

All procedures conducted on animals were approved by MGH IACUC and conform to current animal welfare guidelines. The design and protocol followed our previous report (Kounnas et al., 2010). Briefly, Tg2576 mice which express human APP695 gene harboring the Swedish double mutation (KM670/671NL) were used (Hsiao et al., 1996; Kounnas et al., 2010). 3-Month old female Tg2576 mice were sourced from Taconic Biosciences (APPSWE; Model 1349) and used for short-term efficacy studies (n = 7 or n = 8 per group). Daily dosing was performed for three consecutive days by oral gavage in an 80% PEG 400 (v/v) vehicle. Three hours post drug administration on the last day, the animals were transcardially perfused under isoflurane anesthesia, blood was collected from mice via cardiac puncture into heparinized tubes. Whole blood was centrifuged (10,000 *g* for 10 min) to isolate plasma, aliquoted and stored at − 80 °C for analysis. After brain dissection, tissue was processed for postmortem analyses, including biochemical studies.

2.2. Brain Extraction

Mouse brains were processed using previously reported methods (Kounnas et al., 2010; Veeraraghavalu et al., 2013, 2014). Briefly, brain hemispheres were homogenized in Tris-buffered saline (TBS) containing 1× Halt protease inhibitor cocktail (Thermoscientific) via polytron and applied to the following steps. Samples from the TBS homogenates were extracted with M-PER Mammalian Protein Extraction Reagent with 1× Halt protease inhibitor cocktail (Thermoscientific) and spun at 10,000 *g* for 20 min at 4 °C. The supernatant (MTBS) was used to detect total proteins, including membrane-bound proteins. In addition, TBS homogenates were spun at 100,000 *g* for 60 min at 4 °C. The supernatant was used to detect TBS soluble Aβ levels; and the pellets of TBS homogenates were further homogenized in 70% formic acid via polytron and spun at 100,000 *g* for 60 min. Finally, the supernatant of the formic acid extracts after centrifugation were used to detect TBS-insoluble Aβ levels. All samples were neutralized in 1 M Tris base buffer and then diluted before performing ELISA or Meso Scale analysis.

2.3. Cell Culture and Mouse Primary Cortical Neuron Culture

The Chinese hamster ovary CHO cell line stably expressing *Indiana* mutation in APP, also known as 7PA2 cells, has been previously reported (Walsh et al., 2002; Welzel et al., 2014). These cells were cultured and maintained on regular tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units per ml penicillin, 100 μg per ml streptomycin, and 200 μg/ml G418. Serum-free medium was utilized for experiments using 7PA2 cells. CHO cells stably expressing wild type APP751 and transiently transfected with the GFP-PS1-RFP FRET reporter has been previously reported (Uemura et al., 2009) and were used for the FLIM assay of PS1 conformational changes. Mouse primary neuronal cultures were prepared from cerebral cortex of CD-1 wild type mouse embryos at gestation day 16–18, as described previously (Berezovska et al., 1999). Briefly, the brain tissue was dissociated with Papain Dissociation Kit (Worthington Biochemical Corporation, Lakewood, NJ). The dissociated cells were plated on poly-D-lysine coated dishes and maintained in Neurobasal Medium supplemented with 2% B27, 1% Glutamax and 1% penicillin and streptomycin (ThermoScientific, Waltham, MA) in a 5% CO_2 incubator at 37 °C for 8–11 days in vitro (DIV).

The three-dimensional (3D) human neural cell culture and treatment were performed using the previously published protocol (Choi et al., 2014). Briefly, the HReN-mGAP cells expressing APP Swedish and London mutations and PSEN1 δE9 mutation were utilized. They were plated at a cell density of 20,000,000 cells per ml in a mixture of Matrigel and then 3D-differentiated for five weeks. Subsequently, cells

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