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Substituted dithiazole piperazine benzamides as novel amyloid beta peptide reducing agents

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Abstract

Alzheimer's disease is a persistent neurodegenerative disorder of elderly characterized clinically by irreversible loss of memory due to accumulation of amyloid beta peptides within the amyloid plaques. We report the parallel synthesis and screening results of diverse substituted di-thiazole piperazine benzamides. A new compound **TPI-1917-49** was identified as a promising amyloid reducing agent by lowering the levels of A β at least in two cell types and *in vivo*.

Alzheimer's disease (AD) is a devastating and persistent neurodegenerative disorder of elderly characterized by cognitive decline and memory loss resulting in personality changes and ultimately leading to a total dependence on nursing care. It is now estimated that nearly 35.6 million patients are affected by AD worldwide and that about 4.6 million new cases are added up each year causing enormous social and economic burden.¹ Accumulation of amyloid plaques made up of amyloid β peptide (A β), derived from amyloid precursor protein (APP) by consecutive actions of β - and γ -secretases is a major hallmark of AD. Current therapy that are based on either AChE inhibition or NMDA receptor antagonism can neither slow nor reverse the disease progression as they do not treat the underlying cause of AD. About 1064 clinical trials have been attempted throughout the world to bring an effective therapy for AD based on all possible mechanisms of action including in most recent years both β - and γ -secretase inhibitors. However, all these attempts to develop disease-modifying therapy have so far failed. It is also highly significant that A β has been implicated to play a vital role in the pathogenesis of not only Alzheimer's disease but also traumatic brain injury (TBI),² cerebral amyloid angiopathy (CAA)³ and Glaucoma.⁴ Like Alzheimer's disease, there are no effective prevention or treatment strategies for CAA and TBI. Therefore, invention of any anti-amyloid drug would have wider clinical applications especially for disease modifying therapies.

Small molecules containing thiazole moiety has been demonstrated to possess drug like properties against varieties of diseases⁵ resulting in so far 17 FDA-approved drugs

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containing the thiazole ring.⁶ The indications to which thiazole derivatives are prescribed include asthma (Cinalukast), bacterial infections (Ceftizoxime), diarrhea (Nitazoxanide), myelogenous leukemia (Dasatinib), pain (Meloxicam), duodenal ulcers (famotidine), anthelmintics (thiabendazole) and as vitamin (thiamine). Among these riluzole is an important and the only thiazole derivative approved by the FDA for CNS disorders and it is both neuroprotective and anticonvulsant.⁷ Thus, although the thiazole ring is an important and highly reactive scaffold it is not well exploited for CNS disorders. More recent investigations have developed potent γ -secretase inhibitors⁸ and cdk5/p25 inhibitors⁹ as a potential treatment for AD based on the fact that cdk5/p25 hyperphosphorylate tau. Additionally, Thioflavin T is a benzothiazole dye that binds amyloid beta peptide of AD and therefore used in the diagnosis of amyloid fibrils both *ex vivo* and *in vitro*.¹⁰ In the absence of an effective disease modifying therapy for AD, and also because the thiazole ring is highly reactive and has successfully been derived to make many drugs, we decided to synthesize and characterize a variety of novel derivatives of thiazole and screened them for their effect in lowering A β levels in a cell-based assay and identified thiazole compound (**TPI-1917-49**) as potential A β lowering small molecule compound with novel mechanism of action.

We performed a diversity oriented synthesis of different amino thiazoles namely substituted di-thiazole piperazine benzamides (Scheme 1). 25 compounds were prepared (Scheme 2). The synthesis and characterization of the thiazole library were previously reported by our group.¹¹

To screen for A β reducing agents, instead of an ELISA method which is fast but sometimes gives ambiguous results, we directly used Western blots to quantify A β and other APP metabolites. An advantage of Western blots is that it is less likely to give false positive results, though it consumes more time and involve more labor than ELISA assays.

Following the screening of different thiazole compounds, we successfully identified substituted aryl-thiazole compound **TPI-1917-49** which showed promising results in lowering the levels of A β at least in two cell types and *in vivo*. Interestingly, **TPI-1917-49** appears to decrease A β generation by increasing α -secretase mediated APP processing so that little substrate APP is available for amyloidogenic processing and A β generation by β -secretase. This implies that a plethora of side effects attributed to β - or γ -secretase inhibition, which is responsible for the withdrawal of many candidate drugs from clinical trials, can be completely avoided.

The identified active compound **TPI-1917-49** was prepared following the strategy outlined in Scheme 3. Starting from *p*-methylbenzhydrylamine hydrochloride (MBHA-HCl) resin bound 3-nitro-4-fluoro benzamide, the fluoro group was displaced with Boc-piperazine. Following reduction of the nitro group in the presence of tin chloride and deprotection of the Boc group from the piperazine, the generated amines were treated with Fmoc isothiocyanate. The Fmoc was deprotected with a solution of piperidine in DMF and the resin-bound aryl di-thiourea was treated overnight with 2-bromo-1-(3-hydroxyphenyl)ethanone in DMF at 85 °C. Following HF cleavage the desired 3-((4-(3-

hydroxyphenyl)thiazol-2-yl)amino)-4-(4-(4-(3-hydroxyphenyl)thiazol-2-yl)piperazin-1-yl)benzamide (**TPI-1917-49**) was obtained in good yield and high purity.

The generation and characterization of CHO cells stably expressing APP751wt (7WD10 cells) for the secretion of A β in to the conditioned medium (CM) have been described previously.^{12, 13} For the immunoprecipitation of A β , 7WD10 cells were grown in 6-well plates and treated with thiazole derivatives at 1.0 μ M final concentration in duplicate wells. After 48 hours of drug exposure, the CM was collected, centrifuged to remove cell debris and immunoprecipitated overnight using a monoclonal Ab9 antibody (recognizes 1–16 amino acids of A β) to pull-down A β . A β was detected by immunoblots using a mixture of 6E10/82E1 antibodies as described in our published papers.^{12, 13} The CM was also immunoblotted to detect sAPP α (6E10), sAPP β (anti-sAPP β -wt Rabbit IgG from IBL America Ltd) and sAPPtotal (63G) using indicated antibodies. To detect APP and c-terminal fragments CTFs (CTFs, CT15 antibody), the cells were lysed using lysis buffer with complete protease inhibitor mix (Sigma). Samples were subjected to SDS-PAGE, transferred and immunoblotted with their respective antibodies and detected by enhanced chemiluminescence method. Quantification of Western blot signals was done using imageJ software.¹⁹

Results from the initial screen revealed that the novel thiazole derivatives tested showed differential effects on A β levels and some compounds actually robustly increased A β . However, N, 4-disubstituted amino thiazole compound (**TPI-1917-49**, C₂₉H₂₆N₆O₃S₂) visibly reduced A β levels at 1.0 mM concentration compared to DMSO controls (Fig. 1). In dose-response experiments, we confirmed **TPI-1917-49**-induced significant reduction of A β levels starting at 500.0 nM (31%, p<0.05), 1.0 μ M (54%, p<0.01) and 10.0 μ M (63%, p<0.01), however the 50.0 nM and 100.0 nM concentrations of **TPI-1917-49** reduced A β levels by 21% and 20%, respectively but were statistically insignificant (Fig. 2A). Reduced A β levels were accompanied by increase in CTFs in a dose dependent manner starting from 50 nM to 10.0 μ M (Fig. 2B, upper panel). However, visible alterations in CTF levels were noted from 100 nM to 10.0 μ M. Thus, there is a rough correlation between decreased A β levels with increased CTF levels. Interestingly, secreted APP α levels, but not sAPP β levels appear to be increased dose dependently up to 40% in **TPI-1917-49** treated 7WD10 cells compared to DMSO controls (Fig. 2B, panel 3 and 4). Additionally, there is a dose-dependent increase in the levels of APP full length (APP-FL) as judged from the shorter exposure blot (Fig. 2B, panel 2, labeled shorter exposure). Careful observations also revealed that it is the α -CTF which is increased by **TPI-1917-49** dose-dependently (up to 80%), not β -CTF. Taken together, increased levels of APP-FL, sAPP- α and CTF- α but not sAPP- β and CTF- β clearly suggests that **TPI-1917-49** increases not only the turnover of APP but also its cleavage towards non-amyloidogenic processing of APP. This clearly explains the reason for dose-dependent reduction in the levels of A β by **TPI-1917-49**. This has tremendous advantage for safe therapeutic drug development for AD. Apart from increased A β levels, sAPP α levels are reduced in the cerebrospinal fluids (CSF) of AD patients and that sAPP α plays an essential role in neurite outgrowth.^{14, 15} Thus, it is not only 'too much of A β ' but also 'too little of functional sAPP α ' might be of potential pathogenic significance in AD. Further, There is evidence that exogenous administration of sAPP α in

both mouse and rat brains increases retention of memory^{16, 17} and that experimentally increasing sAPP α levels by crossing transgenic mice overexpressing ADAM-10 (α -secretase) and APPV717I mice led to alleviation of LTP and cognitive deficits most likely because of increased sAPP α levels.¹⁷ Thus, **TPI-1917-49** mediated reduction in A β levels and increased sAPP α levels are both favorable to AD therapy.

To rule out any cell-specific effects of **TPI-1917-49** on A β levels, and also to test whether the effect of **TPI-1917-49** is restricted to APPwt only, we next treated Neuro-2a (N2a) cells stably expressing APP695swe¹² with **TPI-1917-49** at 1.0 μ M concentration. **TPI-1917-49** decreased A β levels in N2a cells also (Fig. 1B) clearly suggesting that **TPI-1917-49** reduces amyloidogenic processing not only of APPwt but also APP with Swedish mutation and that this activity of **TPI-1917-49** does not appear to be restricted to either cell type or genotype of APP.

Since any new compound might be cytotoxic, we next identified the minimum concentration of **TPI-1917-49** required for its cytotoxicity by two independent assays, the lactate dehydrogenase (LDH) release assay and dimethylthiazolyl diphenyl tetrazolium bromide (MTT) based cell proliferation assay. Fig. 3 shows that **TPI-1917-49** does not markedly alter LDH release or MTT reduction up to 1.0 μ M but induces significant amount of cell death only at 10.0 μ M. Thus, **TPI-1917-49** decreases A β levels at non-toxic concentrations (500 nM and 1.0 μ M). Therefore, the toxic nature of **TPI-1917-49** in general at higher doses should not be an impediment for testing it in preclinical trials in the mouse models of AD. The trend in cytotoxicity as deduced from both LDH and MTT assays was similar attesting to the consistency of the results. Taken together, our preliminary study provided clear evidence that **TPI-1917-49** may reduce A β generation and increase sAPP α levels at non-toxic concentrations and therefore has the potential to modify the pathological course of AD.

Next, to see the effect of **TPI-1917-49** *in vivo*, we preliminarily treated APP/PS1 double transgenic mice (AP E9) with **TPI-1917-49** by i.p. injections daily at a dose of 0.5 mg/kg body wt. starting from 4- to 6-months of age for 60 days. The dose was calculated based on its pharmacokinetics in mice and also based on dose-response data from cell cultures. The molecular weight of **TPI-1917-49** is 436.23 g/mole. From the figure 4A, a 4 mg/kg body weight administration of **TPI-1917-49** by intravenous route led to accumulation of about 40 ng/mL in the brain by 10 minutes which was gradually eliminated almost completely by 20 minutes. Exposure of **TPI-1917-49** at 0.5 mg/kg dose even just for 2 months, resulted in 39% ($p<0.05$) reduction in amyloid plaque numbers. A representative brain section for each of untreated and mice treated with **TPI-1917-49** is shown in Fig. 4C & D in which the visibly reduced plaques is shown as white bright spots in **TPI-1917-49** treated mice.

In conclusion, we showed strong evidence that the identified compound **TPI-1917-49** can reduce A β generation *in vivo* and therefore has the potential to modify the pathological course of AD.

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Figure 1.

Screening of about 100 novel thiazole derivatives identified di-substituted amino thiazole DSAT-49 (**TPI-1917-49**) as Aβ reducing agent in both CHO and N2A cells stably expressing APP751wt and APP695swe, respectively. A representative Immunoblot showing detection of Aβ after exposure to **TPI-1917-49** or other derivatives labeled 1 to 4 compared to DMSO treated cells, all in duplicates.

Figure 2.

TPI-1917-49 dose-response experiments showing levels of A β , CTFs and sAPPs. **A**, a representative immunoblot showing reduced A β in CHO cells stably expressing APP751wt by **TPI-1917-49**. Quantitation of A β signals by image J revealed significant reduction in the levels of A β at 500 nM (31%), 1.0 μ M (54%) and 10.0 μ M (63%) compared to DMSO controls (all n=4). **B**, However, CTF levels were increased from 100.0 nM to 10.0 μ M concentrations (n=4), compared to controls (n=4). *p<0.05, **p<0.01 by ANOVA followed by Dunnett's post-hoc test.

Figure 3.

Effect of **TPI-1917-49** on cytotoxicity in CHO cells. Both LDH and MTT assays revealed that DSAT is cytotoxic only at concentrations higher than 10.0 μM ($n=4$ in all groups).

** $p<0.01$ by ANOVA followed by Dunnett's post-hoc test.

Figure 4.

In vivo evidence for brain penetration and decreased amyloid plaques by **TPI-1917-49**. A), Brain levels of **TPI-1917-49** after i.v. injection shows rapid clearance within 20 minutes. B), Plaque numbers decreased significantly by 39% ($p < 0.01$) after chronic i.p. injection of **TPI-1917-49** for 60 days starting from 4-months of age. C), A representative brain section showing cortex and hippocampus with many plaques in the vehicle treated mice. D), A representative brain section showing reduced plaque numbers after **TPI-1917-49** administration.

TPI-1917

substituted di-thiazole piperazine
benzamides

Scheme 1.

Thiazole library screened against AD.

Scheme 2.

Structures of the substituted di-thiazole piperazine benzamides screened against AD.

Scheme 3.

a) Boc-piperazine, DMF; b) 55% TFA in DCM; c) 5% DIEA in DCM; d) 6 eq. Fmoc-NCS in DMF (0.3 M), RT, overnight; e) 20% piperidine/DMF; f) 10 eq. 2-haloketone in DMF (0.3 M), 70 °C, overnight; g) 10 eq SnCl₂·2H₂O in DMF (2 M), RT, overnight; h) HF/anisole (95:5), 0 °C, 90 min.