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Structure-activity relationship study of 2,4-diaminothiazoles as cdk5/p25 kinase inhibitors

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Abstract

Cdk5/p25 has emerged as a principle therapeutic target for numerous acute and chronic neurodegenerative diseases, including Alzheimer's disease. A structure-activity relationship study of 2,4-diaminothiazole inhibitors revealed that increased Cdk5/p25 inhibitory activity could be accomplished by incorporating pyridines on the 2-amino group and addition of substituents to the 2- or 3-position of the phenyl ketone moiety. Interpretation of the SAR results for many of the analogs was aided through in silico docking with Cdk5/p25 and calculating protein hydrations sites using WaterMap. Finally, improved in vitro mouse microsomal stability was also achieved.

Cyclin-dependent kinase 5 (Cdk5) is a member of the serine/threonine cyclin-dependent kinase (Cdk) family.[1] Many Cdks have emerged as potential therapeutic targets for a variety of diseases, particularly in oncology.2 Unlike other Cdks that are activated upon binding to ubiquitously expressed cyclin proteins, Cdk5 activity is predominantly in postmitotic neurons due to the restricted distribution of its activator protein p35.3 Furthermore, membrane-associated p35 can be proteolyticly processed by cysteine proteases, such as calpains, to generate p25 that similarly activates Cdk5 by repositioning the activation loop.4 Even though the catalytic efficiencies of Cdk5/p35 and Cdk5/p25 are similar, it is thought that the altered subcellular compartmentalization of p25 to the cytosol and nucleus leads to neuronal toxicity.[5]

Various neuronal insults can initiate a cascade of events (i.e. increased intracellular [Ca^{+2}] followed by calpain activation) leading to increased phosphorylation of various protein substrates, such as tau.[6] The hyperphosphorylation of tau (and several other proteins) in many acute and chronic neurodegenerative diseases has highlighted the potential role of

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Supplementary data
Supplementary data associated with this article can be found, in the online version, at ().

Cdk5/p25 in a number of these conditions, especially Alzheimer's disease.[7, 8] In particular, transgenic animals producing elevated levels of p25 have increased amounts of phosphorylated tau and demonstrate Alzheimer's-like neuronal lesions.9 In addition to Alzheimer's disease, Cdk5/p25 has been implicated in cerebral ischemia,10 multiple sclerosis,11 Huntington's disease,12 Parkinson's disease[13] and amyotrophic lateral sclerosis (ALS).[14] In addition, Cdk5 has been shown to mediate the phosphorylation of PPAR-γ at specific sites, which leads to insulin resistance. Although the mechanism of Cdk5 activation in adipocytes is unknown, this study potentially extends the therapeutic scope of Cdk5/p25 inhibition beyond neurological disorder.15

Due to the potential role of Cdk5/p25 in various pathological conditions, considerable efforts have been expended to identify potent (and ideally selective) inhibitors. A variety of inhibitor structure classes have been described, including roscovitine (**1**),[16] aloisine-A (**2**)[17] and indirubin-3'-oxime (**3**),18 which are all ATP-competitive and have also been co-crystallized with Cdk5/p25.19, [20] Previously, we reported a colorimetric enzyme-linked immunosorbent assay (ELISA) based high throughput screening protocol for Cdk5 that utilizes full-length tau as substrate.[21] Using this procedure the natural product bellidin (**4**, IC_{50} = 0.2 μM) and the 2,4-diaminothiazole **5** (IC_{50} = 2.0 μM) were discovered as Cdk5/p25 inhibitors (Figure 1). Both compounds were also co-crystallized with Cdk5/p25 (PDB code: 3O0G) and found to bind at the ATP-site in a similar manner to **1** – **3**, except that **5** caused significant movement of two side chain residues (Asn144 and Lys33) in Cdk5 compared to the other four inhibitors.[22] Herein, we report a structure-activity relationship (SAR) study of the 2,4-diaminothiazole inhibitors with significant improvement in Cdk5/p25 inhibitory activity.23, 24

The 2,4-diaminothiazoles were prepared according to the route outlined in Scheme 1.[25] An amine **6** was first allowed to react with thiocarbonyl diimidazole, **7**, at room temperature over 1 h to form the desired isothiocyanate **8**, which was generally not isolated. Several isothiocyanates **8** were commercially available (for example, 3-pyridylisothiocyanate) and used directly in the next step. 1-Amidino-3,5-dimethylpyrazole nitrate, **9**, and DIPEA were added to the isothiocyanates and the resulting reaction mixture was heated at 50 °C for 2 – 16 h to give **10** in 10 – 60% overall yield. Next, cyclization of **10** in the presence of alpha-bromoketones in DMF at 50 – 70 °C for 2 – 16 h gave the 2,4-diaminothiazoles **11**. In certain cases where R^2 contains Boc-protected amine removal of the protecting group was achieved by treatment with TFA in DCM at room temperature followed by salt formation with 4 N HCl in 1,4-dioxane. In addition, intermediate **10** could be treated with $MeNH_2$ in methanol to generate **12**, which was subsequently cyclized to give **11** (R^3 = Me), albeit in only ~ 10% yield. The remaining material was **11** (R^3 = H).

Compounds were evaluated for Cdk5/p25 inhibition using a radiometric assay.[5, 26] The goals of the SAR study were to increase potency, to replace the aromatic nitro, to increase *in vitro* metabolic stability, and to increase aqueous solubility.

The aromatic nitro in **5** was not necessary for activity. For example, it was initially replaced with an electron withdrawing fluorine (**13**) without diminishing activity, whereas replacement with an electron donating methoxy (**14**) resulted in reduced activity. In an attempt to introduce substituents in place of the aniline that would impart increased aqueous solubility, several piperidines were examined. For example, introduction of a 3- or 4-piperidine (**15** and **16**) did not increase potency suggesting that retaining the aryl group may be optimal. Introduction of amino substituents onto the aryl (**17** – **19**) resulted in improved inhibitory activity with IC_{50} values < 100 nM. However, phenylenediamines raised oxidative liability concerns. Therefore, the phenyl ring was replaced with a 2-pyridine (**20**),

which demonstrated an IC_{50} of 33 nM and lowered the $cLogD_{7.4}$ to 3.35 compared to 4.89 for **5**.[27]

Given the results obtained with **20**, the substituents on the aryl ketone were more closely examined. The fluorine could be transposed to the 2-position (**21**) retaining potent inhibitory activity, but not the 4-position (**22**). An electron donating methoxy substituent at the 2-position (**23**) only resulted in a slight decrease in activity, but introduction at the 3- or 4-positions (**24** and **25**) was much more detrimental. Next, the 2-pyridine was replaced with a 3-pyridine and various substituents on the arylketone were examined. Again 2- and 3-F (**26** and **27**), but not 4-F (**28**), resulted in potent inhibition. Removal of the fluorine (**29**) or replacement with a 3-nitro (**30**) was detrimental. Replacement of the fluorine with a methoxy was only tolerated at the 3-position (**31**). When the 2-pyridine was replaced with a 4-pyridine, only the 3-F (**35**) or 2-OMe (**37**) analogs gave potent inhibition. Interestingly, replacing the aryl of the ketone with 2-, 3- or 4-pyridine (**40** – **42**) resulted in significant loss of activity. Finally, introduction of a methyl onto the thiazole amine (**43**) abolished inhibitory activity as might be expected due to the steric clash of the N-methyl group with the H-bond acceptor carbonyl of GLU81 (Figures 2a and b).[28] Disruption of the apparent internal H-bond between the 4-amino hydrogen and the ketone oxygen would require >8 kcal/mol based on a quantum mechanical coordinate scan.[29]

During the course of the SAR study it was noted that the analog subset of R^1 as 2- or 3-pyridyl, and the R^2 as 2- or 4-substituted phenyl exhibits a trend of the 4-substitued phenyl analogs being less active compared to the 2-substituents phenyl analogs. In order to probe these observations further an analysis of the protein hydration sites was conducted. The compounds in Table 1 were docked using Glide[30a] in XP mode with a core constraint based on the crystallized analog **5**. Using WaterMap30b, c to calculate the protein hydration sites in the vicinity of the ATP binding pocket reveals site 16 as having a stable $\Delta G = -3.2$ kcal/mol as shown in Figure 3. In contrast, site 48 has an unstable $\Delta G = 6.2$ kcal/mol.

Using the listed activities, a binding $\Delta\Delta G = -2.6$ kcal/mol was calculated for the exemplar pair of **26** and **28**. Both ligands displace site 48 and the unlabeled sites with a favorable effect on binding affinity. In addition, the 4-fluorophenyl of **28** also displaces site 16. The displacement of a stable hydration site results in a predicted $\Delta\Delta G$ in agreement with the observed value within experimental and computational error. Similar $\Delta\Delta G$ values were observed for the other ortho/para pairs in the subset. For compounds in Table 1 where R^2 is 4-pyridyl and for **15** – **19** the SAR is possibly due to a different binding orientation. Further studies will be necessary to address this possibility.

The selectivity of **26** was briefly examined against Cdk2 and GSK-3β two structurally related serine/threonine kinases belonging to the CMGC kinase subgroup. Compound **26** displayed potent activity against both kinases with IC_{50} values of 25 nM and 45 nM ([ATP] = 60 μM) for Cdk2 and GSK-3β, respectively.

Next, the inhibitory effect of **26** was studied in primary neurons. Rat brain cells were incubated with compound at five different concentrations for 4 h at 37 °C, lysed and immunoblotted with antibodies against Cdk5 and phosphorylated tau at Serine 235 (Ptau235), a predominant phosphorylation site.[31] Roscovitine, **1**, was used as a positive control. As shown in Figure 4, **26** displayed a dose-dependent inhibition of tau phosphorylation with an EC_{50} value of 5.5 μM.

In vitro mouse microsomal stability of **26** was also assessed at 10 μM and a $t_{1/2}$ = 29 min was determined. Based on computational models predicting the intrinsic oxidative stability to CYP450 3A4 and 2D6, the 2-position of the pyridine was predicted to be a site of oxidation. Since this area of the molecule is also predicted not to make close contacts with

Cdk5 when bound, assuming a similar binding mode as **5**, an increase in metabolic stability was anticipated with additional of a trifluoromethyl at this position (**44**). Although the Cdk5 inhibitory IC_{50} increased to 160 nM the mouse microsomal half-life increased to 66 min.

In summary, a series of potent 2,4-diaminothiazoles were designed and synthesized based upon the previously identified Cdk5/25 inhibitor **5**. Incorporation of pyridines in place of the 4-chlorophenyl and addition of substituents to the 2- or 3-position of the phenyl ketone moiety resulted in increased inhibitory potency, such as **26** (LDN-193594). Interpretation of the SAR results for many of the analogs was aided through in silico docking with Cdk5/p25 and calculating protein hydrations sites with WaterMap.30b, c Furthermore, addition of a substituent at the 2-position of the incorporated 3-pyridine resulted in increased in vitro mouse microsomal stability. The results of this study provide probes to study Cdk5/p25-mediated biology and should prove useful in the further optimization of this series of Cdk5 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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26. The phosphorylation of H1P (Histone H1-derived peptide PKTPKKAKKL) by ATP was conducted in buffer containing 20 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, BSA 0.5 mg/ml, 60 μM ATP, 40 μM H1P, and [γ-^{33}P]-ATP. The reactions were conducted in duplicate, initiated by the addition of Cdk5/p25 (6.4 nM), and incubated at room temperature for 45 min. The reaction was stopped by the addition of 20 mM EDTA, the mixture was then transferred to a multiscreen PH filtration plate (Millipore, Billerica, MA) and washed six times with 75 mM H_3PO_4. The plate was dried, filters were removed and samples were counted with a scintillation counter. The background reaction was conducted in the absence of Cdk5/p25. Roscovitine, **1**, was used as a positive control and demonstrated an IC_{50} value of 270 nM.
27. Calculations were made using Molinspiration. http://www.molinspiration.com/.
28. X-ray coordinates (file number: 3O0G) have been deposited with the RCSB Protein Data Bank. These coordinates were used for molecular modeling.
29. QM relaxed coordinate scans were done with Jaguar. Schrödinger, Inc.; using a 6–31G** basis set
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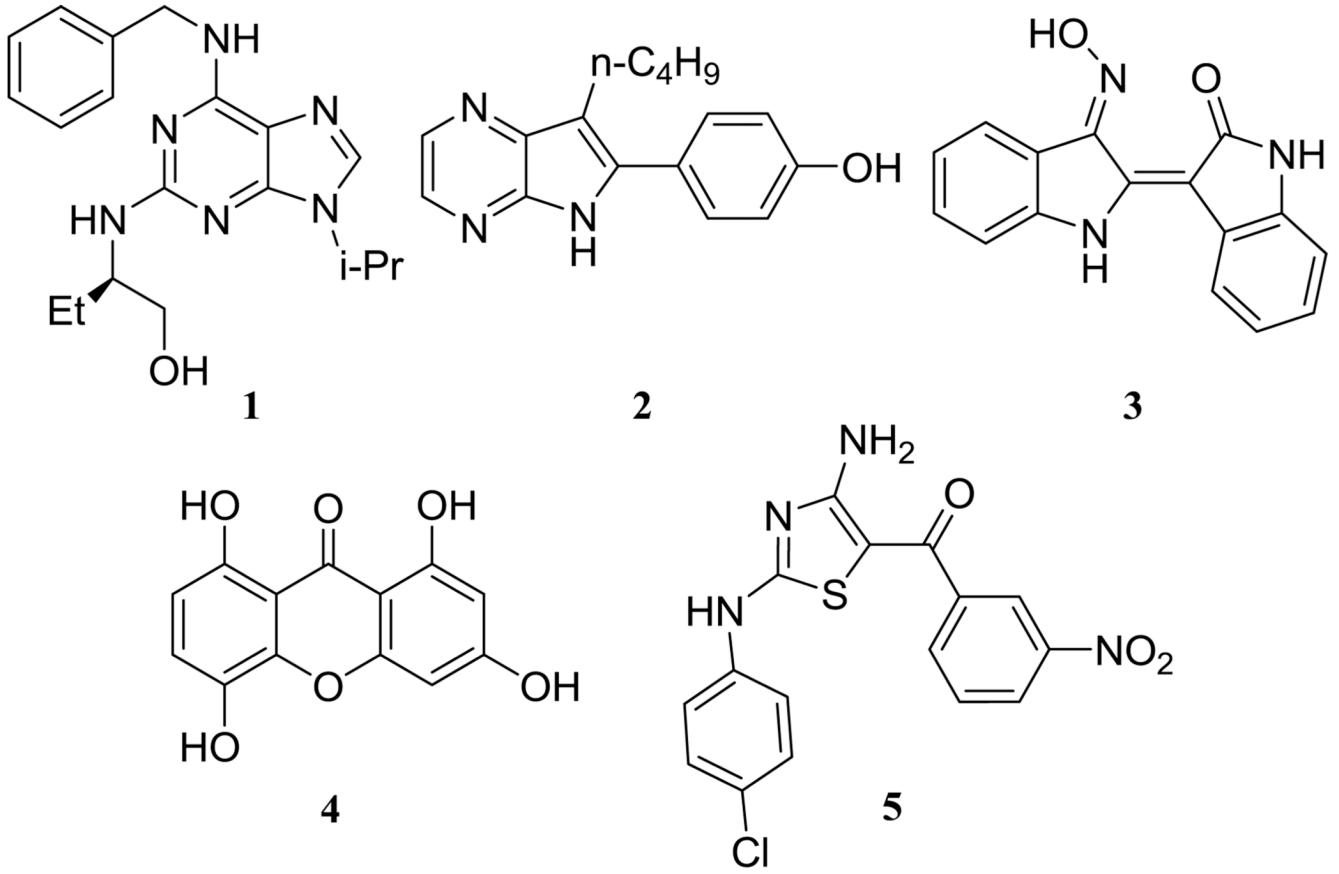
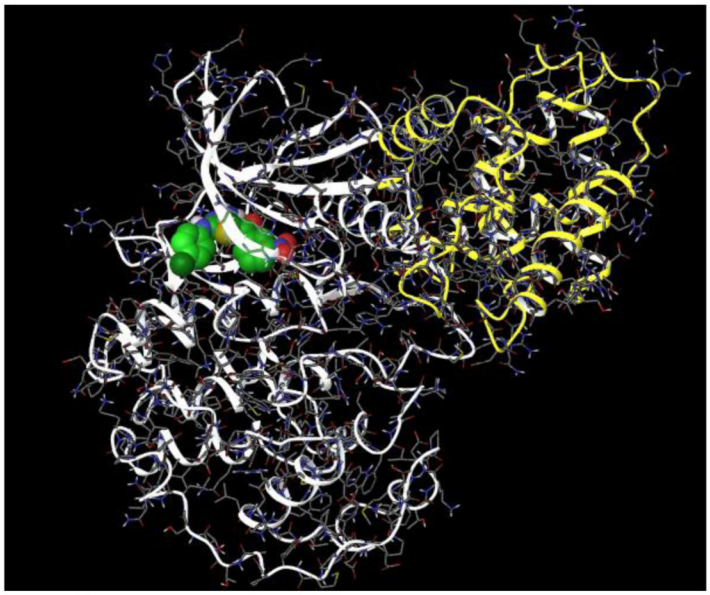
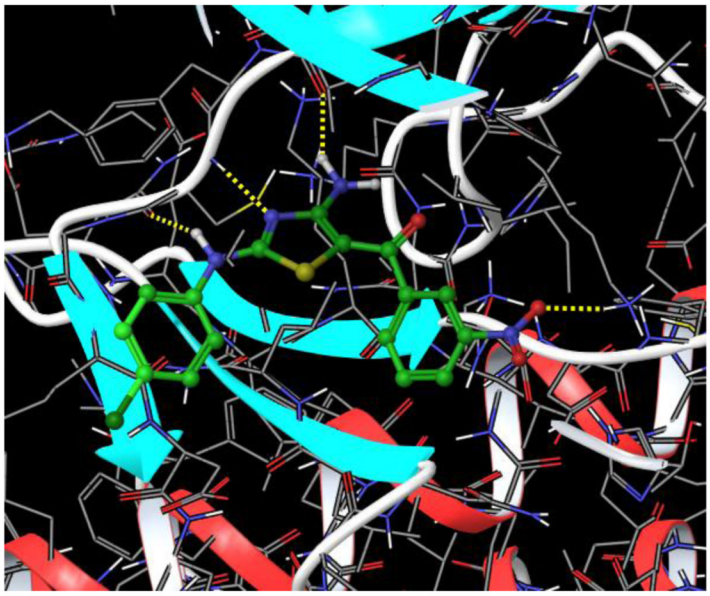


Figure 1.
Examples of Cdk5/p25 inhibitors.



2a



2b

Figure 2.

2a. A view of Cdk5/p25 complexed with **5** (in CPK rendering with green C, red O, dark green Cl, blue N and yellow S). Cdk5 is shown with white ribbons and p25 in yellow ribbons with **5** occupying the ATP binding site.

2b. A close view of **5** (ball and stick rendering) showing the H-bonds (yellow dashed lines) to the hinge residues in Cdk5 and of the NO_2 group with the catalytic Lys33.

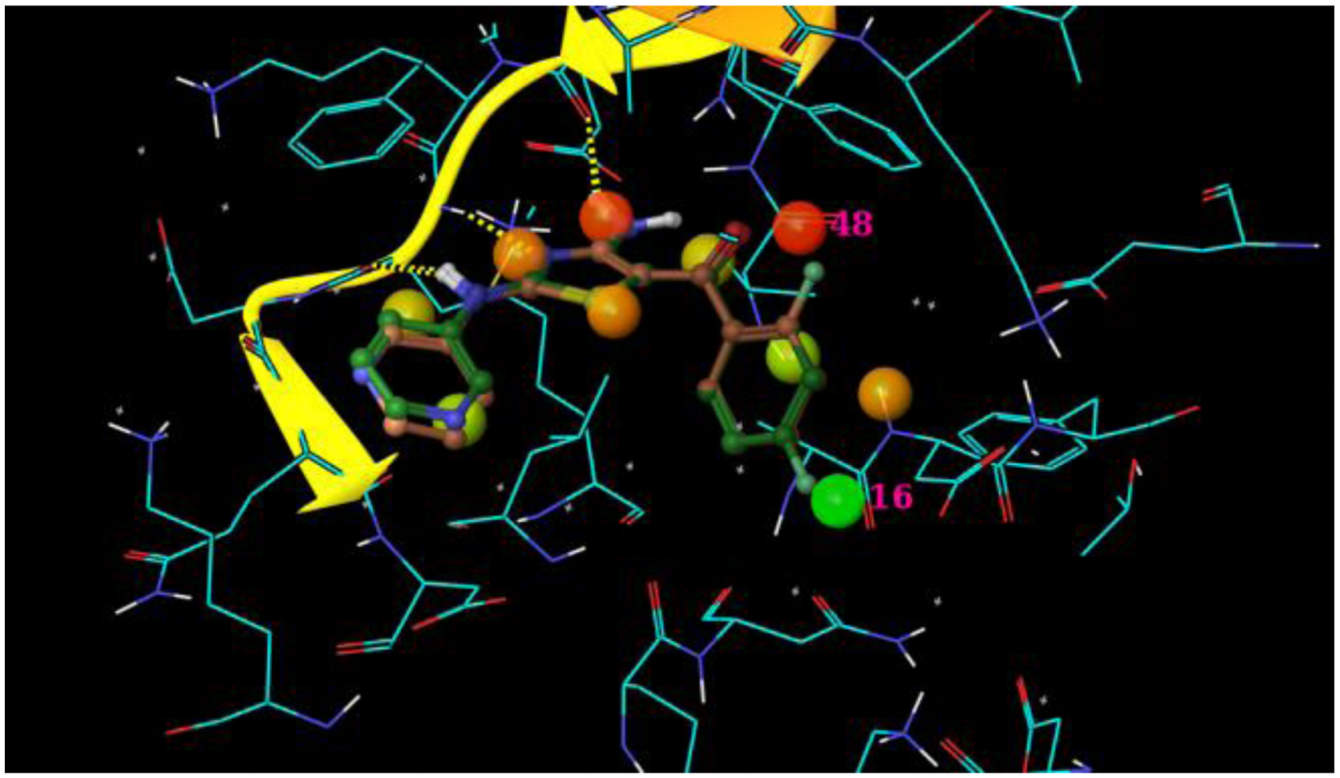


Figure 3.
A view showing **26** (brown carbons) and **28** (green carbons) docked into the Cdk5/p25 binding site. The spheres represent protein hydration sites as computed by WaterMap with a ΔG: −3.2 (bright green) to 6.2 kcal/mol (bright red). Only overlapped sites are shown for clarity.

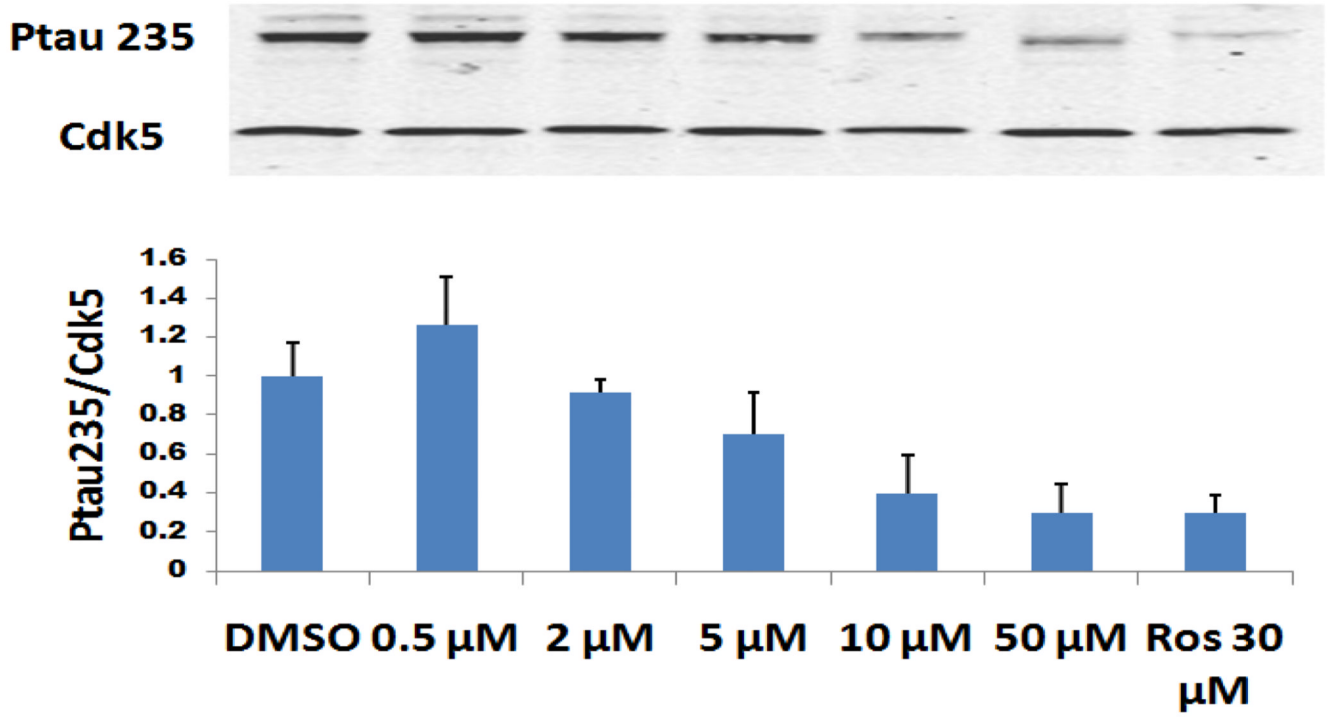
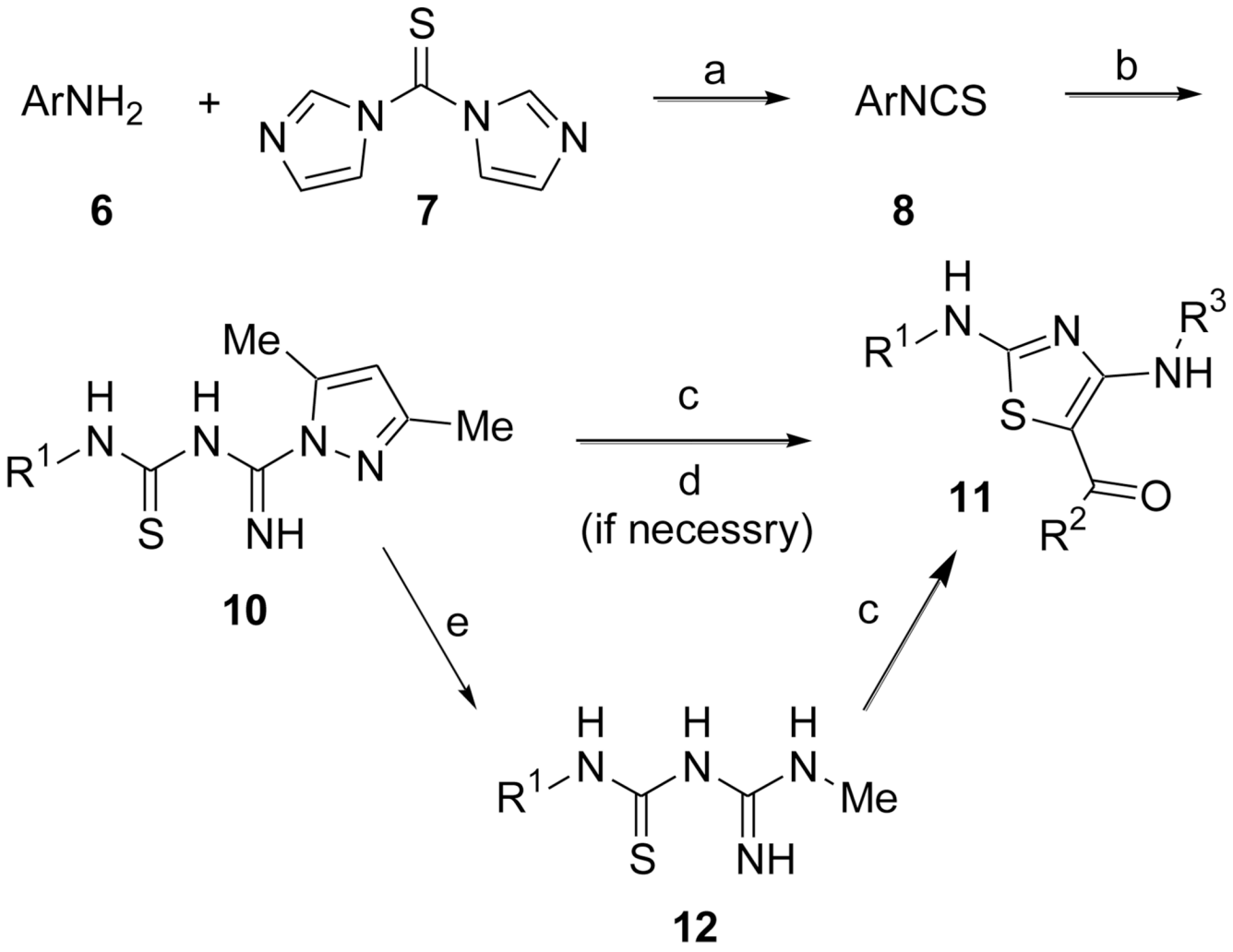


Figure 4.
The inhibition effect of **26** on tau phosphrylation in primary neurons. Western blot is presented where tau phosphorylation was probed by antibody Ptau235 and Cdk5 was used as the control. Relative band intensity is shown in the bar graph. Roscovitine, **1**, at 30 µM was used as a positive control.

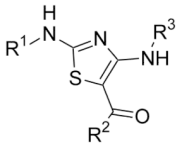


Scheme 1.

General synthetic approach to 2,4-diaminothiazoles. (a) DIPEA, DMF, rt, 1–6 h; (b) 1-amidino-3,5-dimethylpyrazole•HNO_3 (**9**), DIPEA or KOH, DMF, 50 °C, 2–16 h; (c) $R^2C(=O)CH_2Br$, TEA or DIPEA, DMF, 50 °C -70 °C, 2–16 h; (d) TFA, DCM, rt and then 4N HCl in 1,4-dioxane; (e) $MeNH_2$, MeOH, rt.

Table 1

Cdk5/p25 inhibitory result for **5** and **13** – **44**.



compound	R^1	R^2	R^3	IC_{50}, nM
5	4-ClPh	3-NO_2Ph	H	750
13	4-ClPh	3-FPh	H	700
14	4-ClPh	3-OMePh	H	4000
15	3-Piperidyl	3-FPh	H	500
16	4-Piperidyl	3-FPh	H	6200
17	3-NH_2Ph	3-FPh	H	31
18	3-NHMePh	3-FPh	H	44
19	4-NMe_2Ph	3-FPh	H	68
20	2-Py	3-FPh	H	33
21	2-Py	2-FPh	H	38
22	2-Py	4-FPh	H	1200
23	2-Py	2-OMePh	H	80
24	2-Py	3-OMePh	H	400
25	2-Py	4-OMePh	H	1100
26	3-Py	2-FPh	H	30
27	3-Py	3-FPh	H	62
28	3-Py	4-FPh	H	4100
29	3-Py	Ph	H	341
30	3-Py	3-NO_2Ph	H	130
31	3-Py	2-OMePh	H	418
32	3-Py	3-OMePh	H	11
33	3-Py	4-OMePh	H	3500
34	4-Py	2-FPh	H	360
35	4-Py	3-FPh	H	18
36	4-Py	4-FPh	H	400
37	4-Py	2-OMePh	H	60
38	4-Py	3-OMePh	H	200
39	4-Py	4-OMePh	H	600
40	3-Py	2-Py	H	800
41	3-Py	3-Py	H	100
42	3-Py	4-Py	H	1710
43	3-Py	2-FPh	Me	> 10000
44	3-(6-CF_3)Py	2-FPh	H	160