A Triazolotriazine-Based Dual GSK-3β/CK-1δ Ligand as a Potential Neuroprotective Agent Presenting Two Different Mechanisms of Enzymatic Inhibition

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Glycogen synthase kinase 3β (GSK-3β) and casein kinase 1δ (CK-1δ) are emerging targets for the treatment of neuroinflammatory disorders, including Parkinson's disease. An inhibitor able to target these two kinases was developed by docking-based design. Compound **12**, 3-(7-amino-5-(cyclohexylamino)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-2-yl)-2-cyanoacrylamide, showed combined inhibitory activity against GSK-3β and CK-1δ [IC_{50}(GSK-3β) = 0.17 μM; IC_{50}(CK-1δ) = 0.68 μM]. In particular, classical ATP competition was observed against CK-1δ, and a co-crystal of compound **12** inside GSK-3β confirmed a covalent interaction between the cyanoacrylamide warhead and Cys199, which could help in the development of more potent covalent inhibitors of GSK-3β. Preliminary studies on in vitro models of Parkinson's disease revealed that compound **12** is not cytotoxic and shows neuroprotective activity. These results encourage further investigations to validate GSK-3β/CK-1δ inhibition as a possible new strategy to treat neuroinflammatory/degenerative diseases.

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease. The main pathological hallmarks of PD are the loss of dopaminergic neurons and the presence of eosinophilic inclusions, called Lewy bodies (LB), in the *substantia nigra pars compacta* of the midbrain.[1] These abnormalities are the result of a complex pathological process that includes oxidative stress, mitochondrial dysfunction, protein aggregation, and neuroinflammation. In recent years, many researchers have reported that GSK-3β is implicated in microglial-mediated inflammation.[2] Additionally, CK-1δ is also involved in the neuroinflammatory process, mainly due to its role in the Wnt and Hedgehog pathways.[3] Furthermore, GSK-3β and CK-1δ are responsible for the phosphorylation of proteins that are abundant in the LB, including *tau*, α-synuclein, and parkin.[4] The hyperphosphorylation of these proteins is responsible for their aggregation in LB.[5]

These observations suggest that dual GSK-3β/CK-1δ inhibition could be an interesting multitarget strategy to treat neuroinflammatory and neurodegenerative diseases such as PD.[6] For these reasons, we decided to use the versatile adenine-like [1,2,4]triazolo[1,5-*a*][1,3,5]triazine (TT) nucleus to begin synthesizing new inhibitors.[7] In particular, in compounds **1**–**6**, a few amino substitutions were inserted at the 7- and 5-positions.[8,9] GSK-3β inhibition was displayed by the 5,7-dicyclohexylamino derivative **4** [IC_{50}(GSK-3β) = 5.02 μM] (Table 1). Unfortunately, no affinity was detected toward the other target enzyme, CK-1δ. We therefore introduced the 3-amidophenyl moiety at the 2-position of the TT scaffold.[10] This decision was inspired by the potent CK-1δ inhibitor D4476 (**7**, Table 1), and based on previous findings (data not shown) that *meta* substitutions are preferred. Compounds **8** and **9** showed promising IC_{50} values toward CK-1δ [**8**, IC_{50}(CK-1δ) = 2.59 μM; **9**, IC_{50}(CK-1δ) = 4.28 μM] (Table 1), but they were inactive against the other kinase. We hypothesized a similar pose between ATP and TT in the binding pocket of GSK-3β. We therefore wondered if a focused substitution at the 2-position of the TT would target the noncatalytic cysteine 199 (Cys199), leading to covalent inhibition of GSK-3β, and thus to improved potency toward the target. In recent years, there has been a resurgence of interest in covalent inhibitors,[11] and the 2-cyanoacrylamide group can

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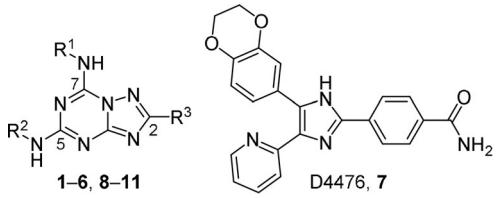
Table 1. Inhibitory activity of compounds **1**–**11** against GSK-3β and CK-1δ.



Compd	R^1	R^2	R^3	IC_{50} [μM][a] GSK-3β	CK-1δ
1[b]	H	H	H	>10	>40
2	H	*c*Hex	H	>10	>40
3	*c*Hex	H	H	>10	>40
4[c]	*c*Hex	*c*Hex	H	5.0±0.3	>40
5	H	Bn	H	>10	>40
6	H	Ph	H	>10	>40
7, D4476	–	–	–	–	0.3[d]
8	H	*c*Hex	*m*-$CONH_2$-Ph	>10	2.6±0.8
9	H	Bn	*m*-$CONH_2$-Ph	>10	4.3±1.2
10	H	*c*Hex	*m*-CN-Ph	>10	>40
11	H	Bn	*m*-CN-Ph	>10	>40

[a] Data are the mean±SD of three independent experiments performed in triplicate; >10 and >40 indicate that two independent experiments in duplicate were performed at that concentration, and the percentage of enzyme activity was more than 50% with respect to control. [b] Previously reported by Valbusa et al. and Dolzhenko et al.[8] [c] Previously reported by Akahoshi et al.[9] [d] Data from Rena et al.[10]

give a reversible thia-Michael reaction, minimizing the chance of irreversible modifications of off-target peptides.[12] For these reasons, we designed the 3-(7-amino-5-(cyclohexylamino)-[1,2,4]triazolo[1,5-*a*][1,3,5]triazin-2-yl)-2-cyanoacrylamide derivative (**12**) with an amido moiety to confer potency toward CK-1δ.

Compound **12** underwent molecular docking studies on both GSK-3β and CK-1δ (Figure 1). Compound **12** showed an ATP-competitive binding pose, interacting with residues at both the hinge and phosphate regions. Interestingly, the substituent at the 2-position was oriented toward the nucleophilic Cys199 in GSK-3β, while an unreactive isoleucine was present at the same position in CK-1δ.

Based on these observations, **12** and its derivatives (**13**–**15**) were synthesized. As reported in Table 2, introducing the cyanoacrylamide moiety substantially improved the affinity toward both GSK-3β and CK-1δ, improving IC_{50} values to the sub-micromolar range [**12**, IC_{50}(GSK-3β) = 0.17 μM; IC_{50}(CK-1δ) = 0.68 μM]. The combined GSK-3β/CK-1δ inhibitory activities suggested **12** to be one of the first examples of a dual GSK-3β/CK-1δ inhibitor. To assess the ability of compound **12** to reach the central nervous system (CNS), we conducted a PAMPA/BBB (parallel artificial membrane permeation assay/blood–brain barrier) test. This test showed a permeability of 1.34 10^{-6} cm s^{-1} (Table 2, and Supporting Information (SI) Table S3 and Figure S2), which is close to the limit of passively BBB-permeating compounds (1.29 10^{-6} cm s^{-1}). This result was expected due to the highly polar moieties in the molecule; in fact, its predicted log*P* value is 0.99. Even if in-silico-predicted pharmacokinetic properties are quite good for compound **12** (SI Table S4), the poor results in terms of BBB permeability suggest structural optimization in order to increase the chance of the compound reaching the CNS. In addition, the selectivity of compound **12** was determined against a small panel of related kinases (CDK-2, CDK-5, CK-1α1, CK-1γ1-3, CK-1ε, and CK-2α1), where it was found to be inactive at a concentration of 10 μM (SI Table S7).

To demonstrate the covalent interaction with Cys199 of GSK-3β, we used UV spectroscopy and HPLC–MS to analyze the reaction of **12** with the model thiol β-mercaptoethanol (BME; SI Figure S3, S5). As expected, the results demonstrated the formation of the **12**–BME adduct.[12a] The binding mechanism of compound **12** was confirmed through substrate competition experiments on both kinases. These experiments showed an ATP-competitive inhibition mechanism against CK-1δ, and a mixed ATP-competitive/non-ATP-competitive behavior against GSK-3β (SI Figure S6). Moreover, the percentage of GSK-3β inhibition slightly increased with increased inhibitor exposure

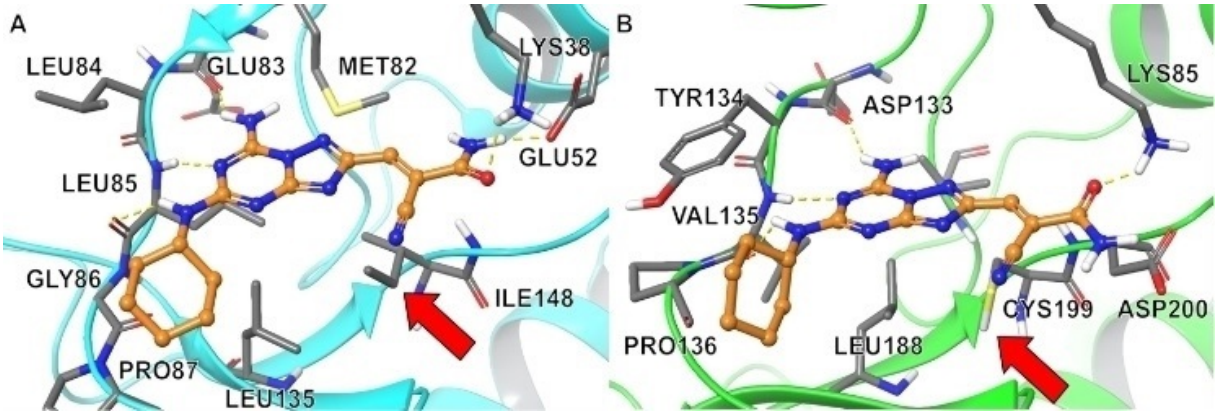
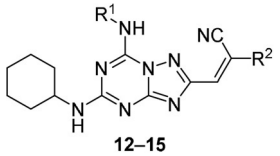


Figure 1. A) Compound **12** docked in the ATP binding pocket of CK-1δ (PDB ID: 4HNF). The red arrow indicates the residue Ile148, unsuitable to form covalent interactions. B) Compound **12** docked in the ATP binding pocket of GSK-3β (PDB ID: 1Q5K). The red arrow indicates the residue Cys199, available to form a covalent interaction. In both panels, the key residues of the binding pocket are highlighted in light-grey sticks and labeled explicitly; hydrogen bond interactions are highlighted by dotted lines.

Table 2. Inhibitory activity of compounds **12**–**15** against GSK-3β and CK-1δ and PAMPA/BBB results of compounds **12** and **15**.



12–15

Compd	R^1	R^2	IC_{50} [μM][a] GSK-3β	CK-1δ	P_e [10^{-6} cm s^{-1}][b]	Pred.[b]
12	H	$CONH_2$	0.17 ± 0.02	0.68 ± 0.03	1.34	CNS +/−
13	*c*Hex	$CONH_2$	> 10	> 10	–	–
14	H	H	> 10	> 10	–	–
15[c]	H	H	5.7 ± 0.2	> 10	2.35	CNS +/−

[a] Data are the mean ± SD of three independent experiments performed in triplicate; > 10 indicates that two independent experiments in duplicate were performed at that concentration, and the percentage of enzyme activity was more than 50% with respect to control. [b] Permeability (P_e) in the PAMPA/BBB assay of selected compounds with their predictive penetration (Pred.) in the CNS. [c] *trans* isomer.

time, confirming that a covalent interaction takes place (SI Figure S8).

X-ray crystallographic studies confirmed **12** to be a covalent inhibitor of GSK-3β (SI Figure S9). Electron density was clearly observed between the α-carbon atom of the cyanoacrylamide group and the sulfur atom of Cys199 (Figure 2). The Michael reaction leads to the formation of two new stereocenters on

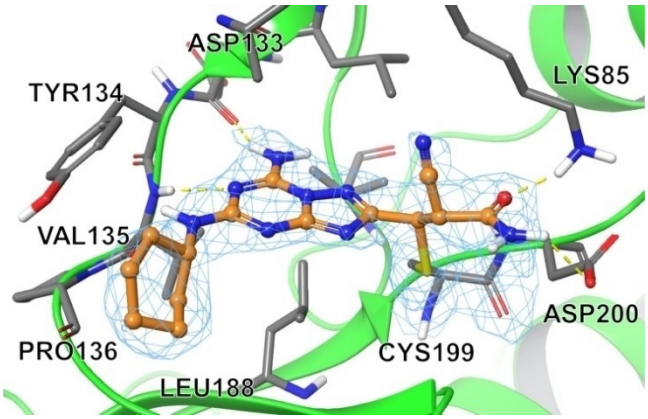


Figure 2. X-ray structure of compound **12** covalently bound in the ATP binding pocket of GSK-3β at a resolution of 2.3 Å (PDB ID: 6H0U). The electron density attributed to compound **12** is depicted as a cyan mesh map (σ level: 1.0); protein secondary structure is depicted as a green ribbon, while residues forming the binding pocket are highlighted as grey sticks. The inhibitor is represented as a ball-and-stick model.

the compound **12**–Cys199 adduct. The X-ray structure allowed us to determine the stereo configuration at both carbons (*R*,*R*) of the Michael addition. This finding allows us to state that, in our case, GSK-3β catalyzes merely the *syn* addition on the cyanoacrylamido group of compound **12**. Further investigations are needed to elucidate the catalytic mechanism that underlies this reaction.

We then assessed the neuroprotective potential of compound **12**'s GSK-3β/CK-1δ inhibition in in vitro models of PD, using rat PC12 pheochromocytoma cells in the presence of neurotoxins (4-phenyl-1-methyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA)).[13] In this cell line, **12** displayed no cytotoxicity up to a concentration of 10 μM (Figure 3A). It also prevented neurotoxin-induced cell death in a concentration-dependent manner (Figure 3C and SI Figure S11).

Recent studies reported compelling evidence for a linkage between Wnt/β-catenin signaling and inflammatory events during PD progression. Moreover, it is widely recognized that kinase upregulation, including GSK-3β, leads to β-catenin degradation.[14] We therefore analyzed the influence of 6-OHDA on

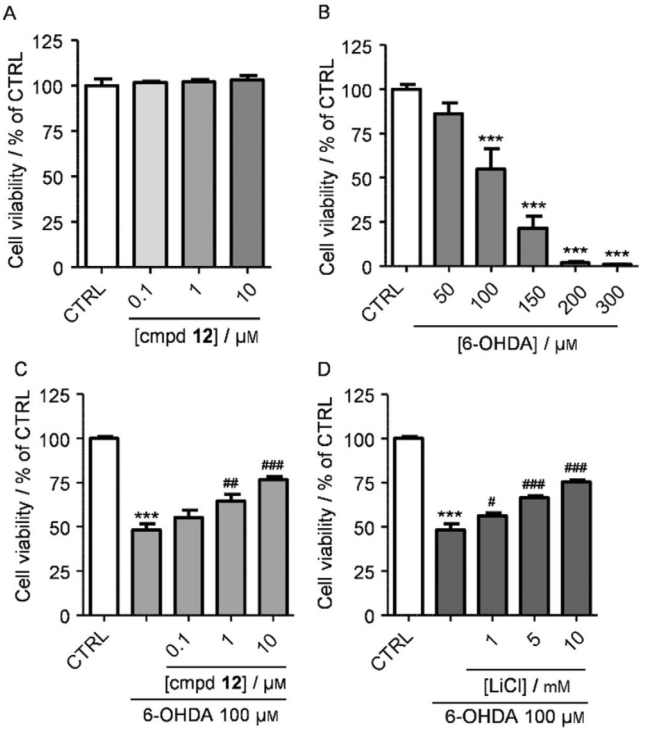


Figure 3. A) Compound **12** does not affect PC12 cell viability. B) Dose-dependency of 6-OHDA toxicity in PC12 cells. C),D) PC12 cells were pretreated with increasing concentrations of compound **12** (C) or LiCl (D), and then with 6-OHDA. Data are the mean ± SEM ($n = 3$); $^{***}p < 0.001$ vs. control (CTRL), $^{\#}p < 0.1$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. 6-OHDA. One-way ANOVA followed by Newman–Keuls test.

GSK-3β activity and β-catenin expression (Figure 4). The results showed that at 100 μM, 6-OHDA decreases the level of phospho-Ser9 GSK-3β. This suggests that 6-OHDA increases the activity of GSK-3β. Compound **12** prevented 6-OHDA-induced cell death by inhibiting GSK-3β (Figure 4 A). It also promoted β-catenin stabilization (Figure 4 B), thus restoring its neuroprotective potential.

In conclusion, herein we disclose the characterization of a new [1,2,4]triazolo[1,5-*a*][1,3,5]triazine derivative. Compound **12** is the first dual GSK-3β/CK-1δ inhibitor reported, and it behaves with two different mechanisms of action (covalent/reversible). An X-ray structure revealing a covalent interaction between a ligand and Cys199 of GSK-3β is reported here for the first time. In vitro experiments demonstrated that **12** is fairly effective in protecting neuronal-like cells from damage, which suggests further studies to evaluate the inhibition of GSK-3β and CK-1δ as a strategy for treating neurodegenerative diseases such as PD and neuroinflammatory disorders. In addition, the obtained crystal structure could help the design and development of new potent covalent inhibitors toward GSK-3β using compound **12** as a lead compound.

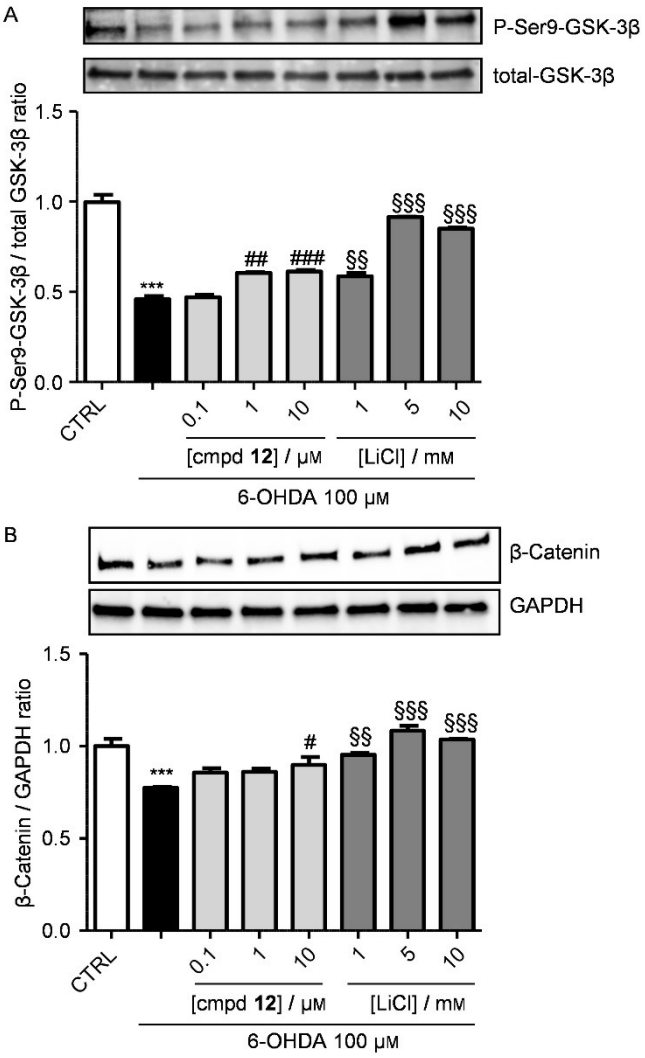


Figure 4. A) 6-OHDA decreases the level of phospho-Ser9 GSK-3β. Compound **12** and LiCl counteract this effect. P-Ser9-GSK-3β was normalized to total GSK-3β. B) Compound **12** reverses the downregulation of β-catenin induced by 6-OHDA. LiCl was used as a positive control. GAPDH protein levels were used as loading control. Data are the mean ± SEM ($n = 3$); $^{***}p < 0.001$ vs. control (CTRL), $^{\#}p < 0.1$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $p < 0.01$, §$p < 0.001$ vs. 6-OHDA. One-way ANOVA followed by Newman–Keuls test.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: casein kinase 1δ · glycogen synthase kinase 3β · neuroinflammation · Parkinson's disease · thia-Michael reaction

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