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6-Amino-4-(pyrimidin-4-yl)pyridones: Novel glycogen synthase kinase-3β inhibitors

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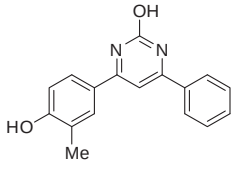
ABSTRACT

The synthesis and structure–activity relationships for a novel series of 6-amino-4-(pyrimidin-4-yl)pyridones derived from a high throughput screening hit are discussed. Optimization of lead matter afforded compounds with good potency, selectivity and central nervous system (CNS) exposure.

Glycogen synthase kinase-3 (GSK-3) is a serine and threonine kinase that regulates a diverse group of physiological functions ranging from differentiation and development, to metabolism, cell cycle regulation, and neuroprotection.[1] A considerable body of literature implicates GSK-3 dysregulation in Alzheimer's disease (AD).[2,3] A histopathological hallmark of AD, neurofibrillary tangles (NFTs) are composed of a highly phosphorylated form of the tau protein. Glycogen synthase kinase-3β (GSK-3β) has been shown to phosphorylate tau at AD relevant epitopes, implicating it as a potential mediator of NFT formation and suggesting that a GSK-3β inhibitor could represent a promising therapeutic approach for the treatment of AD and other neurodegenerative disorders.[4] Additionally, GSK-3 inhibition of the activity of glycogen synthase and insulin receptor substrate-1, key targets in the insulin signaling pathway, offers support to the hypothesis that an inhibitor may be an effective treatment in type 2 diabetes and insulin resistance.[5] Evidence has also suggested GSK-3 inhibition as a potential therapeutic approach for several other conditions, including bipolar disorder, schizophrenia, Huntington's disease, stroke, cardiac ischemia, age-related loss of bone and muscle, chronic inflammatory conditions, and as an adjunct to facilitate cancer chemotherapy.[6–9] Although, modulation of GSK-3 clearly has the potential to be an interesting pharmaceutical target, the likelihood that inhibition of GSK-3 may affect multiple systems raises concerns that the range of its functions may be too broad to safely and selectively modulate a particular disease state.

While it is widely appreciated that kinase dysregulation may underlie a variety of pathologic conditions, kinase inhibitors have traditionally represented challenging pharmaceutical targets due to the difficulties involved in designing agents that are selective for an individual kinase. Historically, the use of kinase inhibitors has largely been limited to the field of oncology, where there is a greater tolerance for off-target actions and cytotoxicity is a specific objective of treatment. As more selective kinase inhibitors have been identified, interest in their use in additional therapeutic areas has grown. To explore their suitability as therapeutic agents for CNS disorders, we sought to develop a series of potent, selective and brain penetrant GSK-3β inhibitors.

High throughput screening of the Pfizer compound collection identified compound **1** (Fig. 1) as a potent and ligand efficient (LE = 0.52)[10] GSK-3β inhibitor with an IC_{50} of 17.2 nM in a cell free enzyme inhibition assay.[11] A co-crystal structure of compound **1** with GSK-3β shows the compound bound in the active-site cleft between the N- and C-lobes of the kinase (Fig. 2).[12] The phenol oxygen makes a pair of hydrogen bonds with a backbone amide



1

GSK-3β CFA IC_{50}	17.2 nM
CLK1 IC_{50}	210 nM
CK2 IC_{50}	27.0 nM
MDR BA/AB	0.83
CL_{int}	24.7 mL/min/kg

Figure 1. HTS hit, compound **1**.

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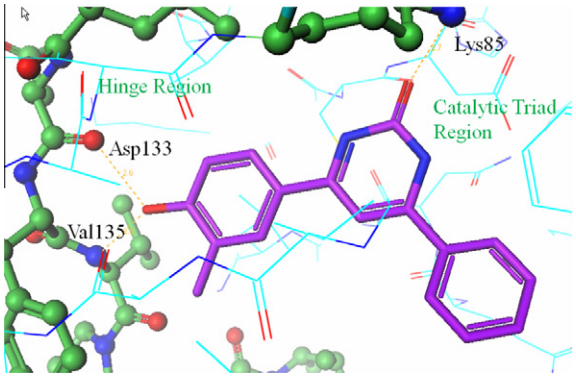


Figure 2. X-ray crystal structure structure of HTS hit, compound **1** bound to GSK-3β.[12] A co-crystal structure of compound **1** with GSK3-β shows the compound bound in the active-site cleft between the N- and C-lobes of the kinase.

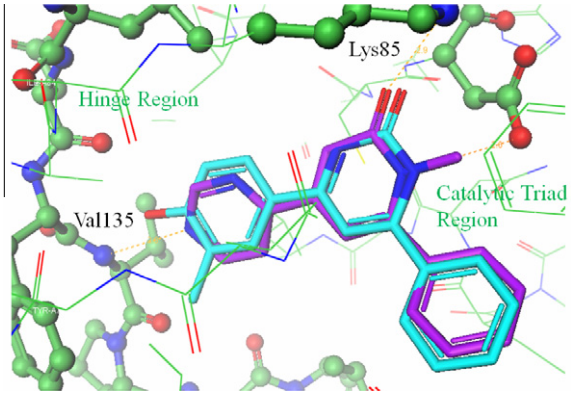
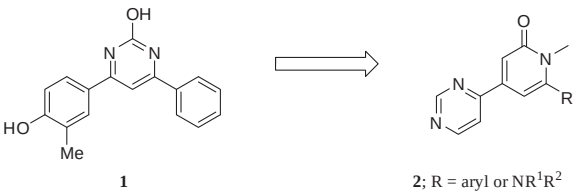


Figure 4. Overlap of X-ray crystal structure of HTS lead, compound **1** (in blue) with proposed template **2** (in purple).

and the carbonyl groups of residues Val135 and Asp133 in the hinge region. Another hydrogen bond is formed between Lys85, which is part of the catalytic triad, and the keto-oxygen on the pyrimidone ring. Although, its *c* log *P* is high (4.5), the lead compound demonstrated reasonable human microsomal clearance in vitro[13] (CL_{int} 24.7 mL/min/kg). Despite bearing two hydrogen bond donors, compound **1** was permeable, as demonstrated by an apical to basal measurement in an MDCK MDR cell line (MDR AB 13.9×10^{-6} cm/sec) and showed no P-gp mediated efflux liability (MDR BA/AB 0.83).[14] In order to evaluate kinase selectivity, compound **1** was screened against a panel of 50 kinases.[15] Notable among the kinases screened, compound **1** inhibited CDC-like kinase-1 (CLK1) and casein kinase 2 (CK2) with IC_{50}'s of 210 nM and 27 nM, respectively.

Structural modifications were sought to address two potential concerns with lead compound **1**: the metabolic liabilities associated with a phenol moiety[16] and the number of hydrogen bond donors in the HTS hit. Analysis of the kinase literature suggested that 4-substituted pyridines and pyrimidines might serve as viable phenol replacements, interacting as single point hinge binders to the active-site cleft.[17] Furthermore, the X-ray structure of **1** showed that the pyrimidone nitrogens were not forming direct hydrogen bond interactions to the protein back-bone suggesting modifications would be tolerated in this region and could potentially lead to improved kinase selectivity. Therefore, several replacements of the pyrimidone ring that lacked additional hydrogen bond donors or acceptors while maintaining the carbonyl group interaction with Lys85 were proposed.

Docking studies of a series of N-Me pyridones, **2** (R = aryl) suggested good overlap with compound **1** (Figs. 3 and 4) and confirmed the potential of the series as inhibitors of GSK-3β. The carbonyl group of the pyridone ring maintains a reasonable hydrogen bonding distance (2.9 Å) to Lys85 and, in addition, the pyrimidine ring of **2** can accept a hydrogen bond from Val135. The R group occupies the solvent exposed region of the kinase suggesting that a range of substituents would likely be tolerated in the region. The proposed series has properties associated with an increased probability of CNS penetration as evidenced by favorable CNS Multi Parameter Optimization (MPO) scores.[18]

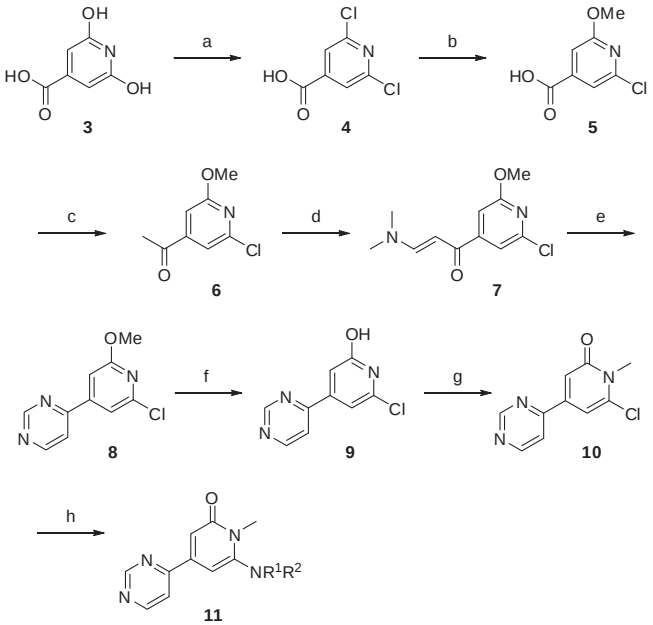


1 2; R = aryl or NR^1R^2

Figure 3. Proposed template, compound **2**.

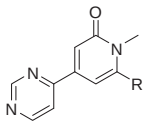
The synthesis of a diversity set of N-Me pyridones was accomplished using intermediate **10** (Scheme 1). Starting material 2,6-dihydroxyisonicotinic acid was converted to 2,6-dichloroisonicotinic acid by treatment with $POCl_3$. Displacement with NaOMe afforded compound **5** and subsequent treatment with methyl magnesium chloride yielded methyl ketone **6**. Reaction with Bredereck's reagent[19] afforded vinylogous ketone **7**, which was *cyclo*-condensed with formamidine acetate to generate **8**. Treatment with concd HCl followed by methylation with methyl iodide afforded convergent intermediate **10**. Synthesis of the final analogs was accomplished by treatment of **10** with 1–5 equiv of the appropriate amine in the presence of 2–3 equiv of Hunig's base at temperatures ranging from 0 to 100 °C in DMF or NMP.[20]

Initially we investigated analogs where R = aryl. These compounds exhibited reduced potency (see example **2**, Table 1), perhaps as a result of the increased distance between the hinge



Scheme 1. Reagents and conditions: (a) $POCl_3$, $(CH_3)_4$ NCl, 130 °C, 48 h; (b) NaOMe/MeOH, 80 °C, 48 h; (c) (1) MeMgCl/THF, −45 °C to 0 °C, 1 h, (2) methyl formate, −25 °C, 30 min; (d) Bredereck's reagent, 130 °C, 1 h; (e) K_2CO_3, formamidine acetate/DMF, 100 °C, 5 h; (f) concd HCl, 80 °C, 4 h; (g) LiO*t*-Bu, MeI/acetone, 80 °C, 24 h; (h) HNR_1R_2, Hunig's base/NMP, 0–150 °C, 1–48 h.

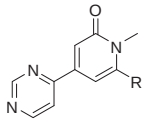
Table 1
GSK-3β enzyme inhibition, human liver microsomal stability and CNS MPO scores

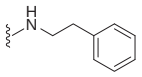
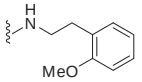
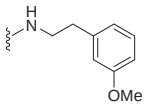
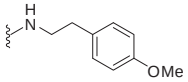
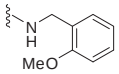
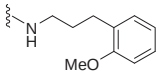
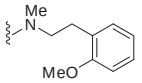


Compd	R	CFA[a] IC_{50}, (nM) (LE)	WCA[b] IC_{50}, (nM)	CL_{int}[c] (mL/min/kg)	CNS MPO score[d]
1	HTS Hit	17.2 (.52)	>5280	24.7	4.7
2		>894	>10,000	<7.80	6.0
12		50.1 (.55)	2210	15.2	6.0
13		62.0 (.50)	>910	32.0	6.0
14		233 (.46)	>5040	<7.60	6.0

[a] GSK-3β cell free enzyme inhibition assay;[11] IC_{50} values are the mean of at least three experiments. LE = ligand efficiency.[10]
[b] GSK-3β whole cell enzyme inhibition assay;[21] IC_{50} values are the mean of at least three experiments.
[c] Human Liver Microsome Incubation assay.[13]
[d] CNS Multi Parameter Optimization score.[18] Provides a holistic assessment of a compound's attributes with respect to PK, safety and drug-likeness. Score >4.5 indicates an increased probability of CNS penetration.

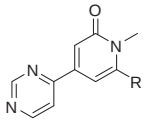
Table 2
GSK-3β enzyme inhibition, human liver microsomal stability and CNS MPO scores

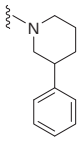
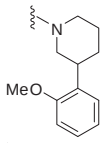
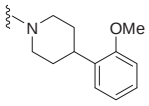
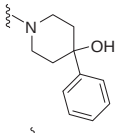
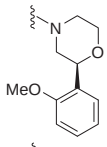
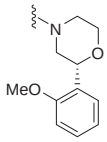


Compd	R	CFA[a] IC_{50} (nM) (LE)	WCA[b] IC_{50}, (nM)	CL_{int}[c] (mL/min/kg)	CNS MPO score[d]
15		>592	>10,000	nt	5.8
16		17.4 (.44)	800	119	5.8
17		>401	>9000	nt	5.8
18		>731	>10,000	60.7	5.8
19		>252	>9000	nt	5.8
20		>260	>10,000	211	5.8
21		133 (.38)	1690	>304	6.0

[a] GSK-3β cell free enzyme inhibition assay;[11] IC_{50} values are the mean of at least three experiments. LE = ligand efficiency.[10]
[b] GSK-3β whole cell enzyme inhibition assay;[21] IC_{50} values are the mean of at least three experiments.
[c] Human Liver Microsome Incubation assay.[13]
[d] CNS Multi Parameter Optimization score.[18] Provides a holistic assessment of a compound's attributes with respect to PK, safety and drug-likeness. Score >4.5 indicates an increased probability of CNS penetration.

Table 3
GSK-3β enzyme inhibition, human liver microsomal stability and P-gp efflux ratio



Compd	R	CFA[a] IC_{50} (nM) (LE)	WCA[b] IC_{50} (nM)	CL_{int}[c] (mL/min/kg)	MDR BA/AB[d]	CNS MPO score[e]
21		115 (.38)	4640	149	1.23	5.8
22		84.1 (.39)	2320	61.4	1.35	5.8
23		51.0 (.37)	3210	116	1.56	5.8
24		8.50 (.42)	610	<7.60	1.01	5.8
25		6.10 (.39)	840	16.3	1.25	5.8
26		62.8 (.37)	1530	27.6	nt	5.8

[a] GSK-3βcell free enzyme inhibition assay;[11] IC_{50} values are the mean of at least three experiments. LE = ligand efficiency.[10]
[b] GSK-3β whole cell enzyme inhibition assay;[21] IC_{50} values are the mean of at least three experiments.
[c] Human Liver Microsome Incubation assay.[13]
[d] MDR-MDCK assay.[14]
[e] CNS Multi Parameter Optimization score.[18] Provides a holistic assessment of a compound's attributes with respect to PK, safety and drug-likeness. Score >4.5 indicates an increased probability of CNS penetration.

binding nitrogen of the pyrimidine ring and Val135 (3.8 Å) as compared to the 2.8 Å hinge interaction with the phenol moiety in the original hit, compound **1**. Additionally, the phenol in compound **1** makes two hydrogen bonds with the hinge, acting as both hydrogen bond acceptor and donor, whereas the pyrimidine in compound **2** maintains only hydrogen bond acceptor capabilities. Replacement of the aryl moiety on the new core with a variety of simple amine substituents, however, afforded a group of analogs which inhibit GSK-3β activity in the cell free enzyme assay while maintaining good ligand efficiency (see Table 1, compounds **12**–**14**).

A cohort of secondary amine substituted analogs (Table 2) was prepared as part of a larger library of compounds. Preliminary SAR revealed an *ortho*-methoxy substituent on an appended phenyl ring affords at least a 30-fold increase in activity in the cell free enzyme inhibition assay compared to the *meta* or *para* methoxy substituted analogs (see example **16** vs examples **17** and **18**). Additionally, this set of compounds illustrated the importance of chain

length to an appended phenyl group; a two carbon linker is optimal for activity in the cell free assay (see example **16** vs examples **19** and **20**). Example **21** confirmed that removal of the hydrogen bond donor and formation of a tertiary amine is tolerated. CNS MPO scores, a holistic assessment of a compound's attributes with respect to pharmacokinetics, safety and drug-likeness, were favorable (>5) for each analog indicating the compounds have an increased probability of CNS penetration.[18] Compound **16** demonstrated potent activity (17.4 nM) in the cell free assay. Further in vitro evaluation showed no potential for P-gp liability, but a CL_{int} of 119 mL/min/kg in the in vitro human liver microsome assay indicated that rapid clearance would be a liability for this analog.

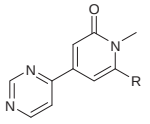
The low clearance values for examples **12** and **13** suggested that constraining example **16** into a piperidine or morpholine ring had the potential to reduce clearance and maintain potency. In an attempt to exploit this SAR, morpholine and piperidine analogs substituted with an aryl group in the 2 or 3 position were generated according to the procedure in Scheme 1 (Table 3). The most potent of this group, examples **24** and **25**, maintained reasonable ligand efficiency, exhibited improved in vitro human liver microsome intrinsic clearance values, demonstrated inhibition at <1 μM in a GSK-3β whole cell enzyme inhibition assay, and showed no P-gp liability in an in vitro assay.

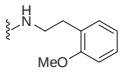
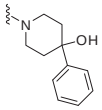
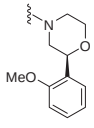
Compounds **16**, **24** and **25** were selected for further study which included screening against a broad panel of kinases. Unlike the original hit, Compound **1**, compounds **16** and **25** were devoid of activity at CK1 and CLK2, while compound **24**, although inactive at CK1, still inhibited CLK2.

Compounds **16**, **24** and **25** were also submitted to an in vivo assay to assess CNS penetration.[22] Compounds **24** and **25** demonstrated good free brain to free plasma ratios in the in vivo assay, however the study indicated a somewhat less favorable ratio for compound **16**.

Additionally, the compounds of interest were screened in an in vitro micronucleus assay for an early risk assessment of potential genetic toxicology hazards within the series.[23] Compounds **16**, **24** and **25** were negative in this preliminary genetic toxicology assay. These data, along with CK2 and CLK1 selectivity data are summarized in Table 4.

Table 4
GSK-3β enzyme activity, selectivity, human liver microsomal stability and in vivo brain: plasma ratio for **16**, **24** and **25**



Compd	R	CFA[a] IC_{50} (nM) (LE)	CK2% inhib @ 10 μM	CLK1% inhib @ 10 μM	CL_{int}[b] (mL/min/kg)	b/p[c]	IVMN pre-screen[d]
16		17.4 (.44)	24%	28%	119	0.35	Neg
24		8.50 (.42)	3%	100%	<7.60	0.96	Neg
25		16.1 (.39)	2%	15%	16.3	1.86	Neg

[a] GSK-3β cell free enzyme inhibition assay;[11] IC_{50} values are the mean of at least three experiments. LE = ligand efficiency.[10]

[b] Human Liver Microsome Incubation assay.[13]

[c] In vivo brain to plasma measurements (rat).[22] Calculations were based on the average concentrations in brain and plasma at the 1 h time point.

[d] In vitro micronucleus assay, high throughput screen in CHO cells, no activation.[23]

In conclusion, a series of 6-amino-4-(pyrimidin-4-yl)pyridines has been developed as novel glycogen synthase kinase-3β inhibitors. A single point hinge binding motif for the series helped to provide improved selectivity over a range of kinases. Removal of hydrogen bond donors from the core increased the probability of CNS penetration and reduced the potential for metabolic liability. Although none of the compounds demonstrates activity in the whole cell enzyme inhibition assay of better than 500 nM, several analogs exhibit good pharmacokinetic properties and offer improvements in potency and selectivity over the original HTS lead compound.

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11. *GSK-3β cell free enzyme assay*:
Recombinant human GSK-3β was expressed in SF9/Baculo virus cells. His-tag protein was purified by affinity chromatography to a Ni-NTA Superflow column. Enzyme activity was assayed as the incorporation of [^{33}P] from the gamma phosphate of [^{33}P]ATP (PerkinElmer) into biotinylated peptide substrate bio-LC-S-R-H-S-S-P-H-Q-pS-E-D-E-E-E-OH (Anaspec). Reactions were carried out in a buffer containing 8 mM MOPS (pH 7.0), 10 mM Magnesium acetate, 0.2 mM EDTA, 1 mM DTT and 2 μM cold ATP. The ^{33}P-ATP was added for 0.025 μCi/well (120 μL) and the final concentration of substrate was 1.0 μM. Enzyme was pre-incubated with test agent for 30 min at room temperature followed by initiation of the reaction by the addition of substrate mix. Incubations were carried out at RT for 60 min. Reactions were stopped by addition of 0.66 volume of buffer containing: 12.5 mM EDTA, 0.25%Triton-X 100, 125 μM ATP, and 6.2 mg/ml streptavidin coated SPA beads (Amersham) in PBS without Ca or Mg. Radioactivity associated with the beads was quantified by scintillation counting of CPM in a Trilux counter (PerkinElmer).
12. PDB Id. 3Q3B. This structure was originally solved by Dr. Jay Bertrand and his team at the Pharmacia Discovery Research Labs in Nerviano, Italy.
13. Assay method adapted from published protocols: (a) Riley, R. J.; McGinnity, D. F.; Austin, R. P. *Drug Metab. Dispos.* **2005**, *33*, 1304; (b) Obach, R. S. *Drug Metab. Dispos.* **1999**, *27*, 1350.
14. Assay method adapted from published protocol: Permeability is assessed as an apical to basal measurement in the MDCK MDR cell line. Venkatakrishnan, K.; Tseng, E.; Nelson, F.; Rollema, H.; French, J. L.; Horner, W.; Gibbs, M. A. *Drug Metab. Dispos.* **2007**, *35*, 1341.
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20. *Synthetic examples and analytical data*:
Compound **24** was synthesized by dissolving intermediate **13** (100 mg, 0.45 mmol) in 1 ml DMF and adding Hunig's base (196 μL, 1.13 mmol) and 4-(4-phenyl)-4-hydroxypiperidine (200 mg, 1.13 mmol). The resulting mixture was heated at 100 °C for 19 h. After cooling to room temperature, water was added and the reaction mixture was extracted three times with ethyl acetate. The combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified on a silica gel flash column eluting with 4% MeOH in CH_2Cl_2 to afford 50 mg of the desired product as a yellow oil which crystallizes on standing (30% yield).
^{1}H NMR (400 MHz, chloroform-*d*) d ppm 1.90–2.00 (m, 2H) 2.26 (td, *J* = 12.98, 4.30 Hz, 2H) 3.08–3.18 (m, 2H) 3.32 (td, *J* = 12.01, 1.95 Hz, 2H) 3.59 (s, 3H) 6.67 (d, *J* = 1.76 Hz, 1H) 6.84 (d, *J* = 1.95 Hz, 1H) 7.26–7.32 (m, 1H) 7.33–7.41 (m, 2H) 7.49–7.56 (m, 2H) 7.64 (dd, *J* = 5.27, 1.37 Hz, 1H) 8.79 (d, *J* = 5.47 Hz, 1H) 9.25 (d, *J* = 1.17 Hz, 1H). ^{13}C NMR ($CDCl_3$) 31.76, 38.40, 47.89, 70.91, 93.63, 112.14,

117.87, 124.70, 127.68, 128.78, 146.99, 157.23, 158.17, 159.24, 161.98, 164.58. MS 363 (m+1).

Compound **16** was prepared in a similar manner affording the desired product in a 42% yield. ^{1}H NMR (400 MHz, chloroform-*d*) d ppm 3.01–3.10 (m, 2H) 3.05 (t, *J* = 6.34 Hz, 2H) 3.41 (s, 3H) 3.48 (td, *J* = 6.34, 4.49 Hz, 2H) 3.89 (s, 3H) 5.03 (br s, 1H) 6.14 (d, *J* = 1.76 Hz, 1H) 6.44 (d, *J* = 1.95 Hz, 1H) 6.90–6.97 (m, 2H) 7.16 (dd, *J* = 7.42, 1.56 Hz, 1H) 7.22–7.27 (m, 2H) 7.64 (dd, *J* = 5.27, 1.37 Hz, 1H) 8.78 (d, *J* = 5.27 Hz, 1H) 9.26 (d, *J* = 1.37 Hz, 1H). ^{13}C NMR ($CDCl_3$) 27.49, 29.54, 44.95, 55.92, 82.88, 102.97, 111.00, 117.92, 121.64, 127.25, 128.73, 130.94, 147.67, 151.54, 157.44, 158.00, 159.13, 162.82, 163.07. MS 337 (m+1).

21. *GSK-3β whole cell assay*:
A tetracycline-regulated expression system was used to produce a stable CHO cell line (Clontech CHO AA8 #630904) carrying inducible expression of human GSK3β and human Tau driven from modified pRevTRE Tet-Off vectors. The human Tau gene encoding the 441aa full length protein (Accession #NM_005910) was subcloned into vector pRevTre. GSK3β (Accession #NM_002093) was subcloned into a modified pRevTre vector carrying zeocin antibiotic resistance. A stable inducible cell line was created expressing both the Tau gene and the GSK3β gene in the CHO Tet-Off cell line (CHO AA8). This cell line was maintained in the uninduced state in MEM Alpha medium (Invitrogen 12571-063) containing 10% FBS (Clontech # 8630-1), doxycycline (5 ng/mL Sigma D9891) zeocin (300 μg/mL Invitrogen R250-01), Hygromycin (300 μg/mL Roche 10843555001), and Geneticin (300 μg/mL Invitrogen 10131-035). Transgene induction was initiated by removal of doxycycline from the medium for 24 h. Following full induction of GSK3β and τ, cells were incubated for 2 h with test compounds before lysis (lysis buffer: 250 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 0.5% NP40, protease inhibitors (Roche 11873580001), Phosphorylated τ was measured in cellular lysates using AlphaLISA (Perkin-Elmer) in 384 well Packard Optiplate (Perkin Elmer Cat.# 6007299). A capture antibody to total tau (HT7, Pierce) was conjugated to Acceptor beads and added to lysate at 10 μg/mL final concentration in the assay. Following a 60 min incubation at room temperature, biotinylated phospho-epitope specific detection antibody (AT8, Pierce) was used at 5 nM final concentration and incubated for 30 min. Streptavidin donor beads (Perkin-Elmer) were added at 40 μg/mL final concentration and incubated for 60 min in the dark. The plate was read on an Envision plate reader (Perkin Elmer) using AlphaScreen settings.
22. B/P Measurements:
Male Sprague–Dawley rats (*n* = 3/time point) were administered 5 mg/kg of PF-X via subcutaneous administration. Blood samples were collected via cardiac puncture after euthanization with CO_2 at 0.5, 1, 2, and 4 h post-dose. Samples were placed in to EDTA tubes and placed on ice. CSF was collected via the cisterna magna and whole brain was collected via decapitation. CSF and brain samples were immediately stored in dry ice. Blood samples were spun down to collect plasma. Plasma, brain and CSF samples were stored at −20 °C until analysis. LC/MS/MS was used to measure plasma, brain and CSF drug levels. Brain to plasma ratios were calculated using the average concentrations in brain and plasma measured at the 1 h time point.
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