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Structure-Guided Design of a Highly Selective Glycogen Synthase Kinase-3 β Inhibitor: a Superior Neuroprotective Pyrazolone Showing Antimania Effects

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Keywords

bipolar disorders; GSK-3; kinase selectivity; mania; neuroprotection

Glycogen synthase kinase 3 (GSK-3), initially identified in the late 1970s as a regulator of glycogen metabolism,^[1, 2] is now well known to be involved in a variety of intracellular signaling events.^[3] GSK-3 is a serine/threonine protein kinase, which is encoded in mammals by two highly homologous isoforms, GSK-3 α and GSK-3 β .^[4] Since mood stabilizers, such as lithium, likely owe their effects to the inhibition of GSK-3 β , considerable effort has gone into the discovery and design of small, organic molecules that serve as GSK-3-selective lithium mimetics. Properly designed inhibitors could possibly overcome the shortcomings of this drug with considerable side effects,^[5] thereby finding applications in the treatment of central nervous system (CNS) disorders including Alzheimer's disease (AD),^[6] Parkinson's disease, stroke, traumatic brain injury, and bipolar disorders.^[7, 8]

A host of both natural and unnatural products have been investigated as ATP-competitive GSK-3 inhibitors over the past few decades. Natural products, such as staurosporine,^[9] indirubins,^[10] hymenialdisines,^[11] and paullones,^[12] have been extensively studied in this connection (Figure 1). In addition, a number of synthetic, small-molecule GSK-3 inhibitors have been reported.^[13] All of these compounds are ATP-competitive GSK-3 β inhibitors, most of which show nonselective protein kinase inhibition profiles including activity against the cyclin-dependent kinases (CDKs) and PKCs (protein kinase C) as shown in Figure 1. Poor kinase selectivity is a common problem for most ATP-competitive GSK-3 β inhibitors. Thiadiazolidinones (TDZDs) are the first reported non-ATP-competitive compounds.^[14] The lead TDZD, NP-12 (also named tideglusib), is now in phase II clinical trials for the treatment of AD and progressive supranuclear palsy (PSP). NP-12 is the only GSK-3 inhibitor under clinical development for both PSP and AD. However, none of the ATP-competitive compounds have made it into clinical trials. Improved compounds demonstrating higher selectivity for GSK-3 β are likely needed in order to properly validate the ability of this compound class to function as lithium mimetics.

Previously, we described the identification of 3-indolyl-4-indazolylmaleimides (**1**) and benzofuran-3-yl-(indol-3-yl)maleimides (**2**) as reasonable GSK-3 β inhibitor candidates showing some elements of kinase selectivity and behavioral activity in the mouse mania model.^[15, 16] Both of these compounds are derived from the natural product staurosporine (Figure 1). In further investigations aimed at improving largely target selectivity, we decided to explore the possibility of replacing the 5-membered maleimide core with other heterocyclic systems capable of maintaining the required hydrogen-bonding patterns between the small molecule and enzyme. Pyrazolones appeared to dock reasonably well in the X-ray co-crystal structure complexes [minus the original ligand] obtained previously for some of our GSK-3 β inhibitors. Herein, we present the synthesis and activity of members of a pyrazolone family. In particular, one of the lead pyrazolones showed excellent selectivity for GSK-3 β when tested against a panel of 40 other kinases. Moreover, this compound was found to be superior as a neuroprotective agent when tested in the homocysteic acid (HCA) model of oxidative stress.

In order to better understand the interactions between human GSK-3 β and our maleimide-based inhibitors, we obtained a co-crystal structure of our previously identified lead compound **2b** in complex with human GSK-3 β .^[17] Since only the NH group and one of the two carbonyl groups are involved in the tight binding to GSK-3 β with a water molecule acting as a bridge between the other carbonyl group of maleimide and the backbone, we assumed that it may be possible to further enhance the kinase selectivity of the inhibitors by removing one of the maleimide carbonyl groups. By altering one point of interaction that is not required for complexation with GSK-3, we believed that we might reduce some of the undesirable off-target interactions with other kinases. Taking into consideration both the drug-like properties of new analogues, as well as their synthetic accessibility, we now sought to replace the GSK-3 β noninteracting carbonyl group of the maleimide group by a potential hydrogen-bond donor NH group. Following this logic, a series of compounds, the 5-membered-ring pyrazolones, was considered as displayed in Figure 2. Employing the original X-ray co-crystal structures, we docked^[16] the new pyrazolones to the active site of GSK-3 β and observed that the pyrazolone portion of compound **3a** provides key hydrogen-bonding interactions with the backbone carbonyl group of Asp 133 (1.90 Å) and the backbone amide of Val 135 (1.65 Å) as shown in Figure 3. The characteristic hydrogen-bonding interaction patterns were maintained for these new compounds. Compound **3a** was previously reported as a PKC inhibitor,^[18] however no GSK-3 data has previously been shown.

With the aforementioned hypothesis in hand, a series of pyrazolones were synthesized by the general method shown in Scheme 1. The synthesis of pyrazolone **3** is straightforward and based on the cyclization of the corresponding β -keto esters **9** and hydrazine. Compound **9** was prepared by the addition of the appropriately substituted ethyl-3-indoleacetates and indole-3-carbonyl chlorides or benzofuran-3-carbonyl chlorides (Scheme 1).^[18] Preparation of indolyl-based acetates commences with alkylation of the indole **5** to afford compound **6**. The other required heterocycle-3-carbonyl chlorides **8** were prepared from indole or benzofuran-based carboxylic acid **7**. Some of the indole-3-carboxylic acids were prepared by acylation of indole **10** with trichloroacetyl chloride to form intermediate **11**, followed by basic hydrolysis. The enolates generated from indole-3-acetic acid ester **6** and lithium diisopropylamide (LDA) reacted with acyl chloride **8** to form β -ketoester **9**. Treatment of compound **9** with hydrazine in the presence of *p*-toluenesulfonic acid (*p*-TsOH) yielded the pyrazolones **3**. For the synthesis of pyrazolones **4**, heterocyclic-3-acetic acid esters **12** were used in place of the indole-3-acetic acid esters to form intermediate **13**.

The newly synthesized pyrazolones were then examined for their inhibitory activity against GSK-3 β (Table 1). Pyrazolones containing both an indole and a benzofuran group displayed micromolar potency against GSK-3 β (IC₅₀=2270 nM for **4a**; IC₅₀=2950 nM for **3b**). To our

delight, the bisindolyl-pyrazolone **3a** displayed very good GSK-3 β inhibitory activity in the *in vitro* assay ($IC_{50}=34$ nM), as was anticipated from the modeling studies. To ascertain the importance of substitution on either of the indole rings of this pyrazolone series, a 5-bromo substituent was introduced as in compounds **3c** and **3d**. The kinase assays revealed that one isomer was about six-fold more active than the other, with compound **3c** exhibiting an IC_{50} value of 535 nM. Further variations of the substituent located at the 5-position of compound **3c**, as in the 5-fluoro compound **3f** and 5-chloro compound **3g**, led to further decreases in potency ($IC_{50}=574$ nM for **3f**; $IC_{50}=2470$ nM for **3g**), while the 5-benzyloxy compound **3j** was inactive against GSK-3 β at the maximum test concentration of 10 μ M. In addition, we found that the introduction of a halogen in the 6-position of the indole ring as in compound **3e** was also unfavorable. Replacement of one of the indoles by a pyridine ring as in **4b** also led to poorer activity ($IC_{50}=4280$ nM) in comparison with **3a**. From these results, we chose compound **3a** as the lead compound for further biological studies.

A number of GSK-3 β inhibitors have been reported, however, many of these are poorly selective drugs, as they show cross-reactivity to PKC and CDK, a consequence of high homology among the catalytic domains of these enzymes. To determine whether the selectivity of pyrazolone **3a** is, in fact, better than the previously reported compounds, it was tested for inhibition at a single concentration (10 μ M) against a panel of 40 related kinases. In the presence of ATP (10 μ M), compound **3a** exhibited low inhibitory activity against 37 of the other kinases, with the exception of showing moderate inhibitory activity against the CDKs and GSK-3 α (Figure 4). A second round of kinase screening in which IC_{50} values were determined, revealed that compound **3a** is, in fact, spectacularly selective for GSK-3 (Table 2). Given the nearly identical sequences of the α and β isoforms of GSK-3, we did not anticipate any selectivity for these two kinases.

Compound **3a** was also tested in a panel of 44 cloned human and rodent CNS receptors, channels and transporters through the US National Institute of Mental Health's (NIMH) Psychoactive Drug Screening Program (PDSP). No significant activity was found in these studies, with the exception of some activity at the 5-HT $_{1a}$ receptor ($IC_{50}=704$ nM; further data can be found in the Supporting Information).

Next, lead pyrazolone **3a** was examined in a model of oxidative stress induced by homocysteic acid (HCA), where neurons were treated with the test compound alone or in the presence of HCA. Cell viability was assessed using the MTT assay, and the results are shown in Figure 5. In the preliminary assays, pyrazolone **3a** was tested at concentrations ranging from 1 μ M to 20 μ M. Full neuroprotective activity was seen at 1 μ M and up to 20 μ M without any observable toxicity at the higher doses. In a follow-up expanded dose-finding study starting at 50 nM, **3a** was found to afford nearly complete protection at 150 nM. The cell micrographs provide a more exact depiction of the live versus dead cells (Figure 6). In comparison to the known GSK-3 inhibitor SB-216763, pyrazolone **3a** showed superior neuroprotective activity.

As our interest in developing GSK-3 inhibitors relates largely to their possible use in treating bipolar disorders, we next chose to examine **3a** in an animal model of mania/hyperactivity induced by the combination of the psychostimulant amphetamine (Amph) and the anxiolytic agent chlordiazepoxide (CDP). In this model, the combination produces an increase in locomotor activity, which is blocked by known mood stabilizers, such as lithium and valproate.^[16, 19] Following pretreatment with vehicle, valproate or compound **3a** (150 mg kg $^{-1}$), eight-week old C57BL/6J male mice were injected with water, Amph (4 mg kg $^{-1}$) or a mixture of Amph (4 mg kg $^{-1}$) and CDP (2.5 mg kg $^{-1}$) and placed in open field chambers where locomotor activity was monitored for 60 min. Data were analyzed with analysis of variance followed by Fisher's PLSD post-hoc tests. Pretreatment with compound **3a** (5 min) or valproate (30 min) attenuated the locomotor activity induced by the mixture of Amph + CDP, without

affecting the activity induced by Amph alone. Decreasing the pretreatment time of valproate (5 min) to match that of compound **3a**, resulted in a decrease in both Amph and mixture-induced activity (Figure 7). The results demonstrate that inhibitor **3a** selectively reduces locomotor activity induced by a combination of amphetamine and CDP, similar to known mood stabilizers such as valproate.

In summary, with the aid of molecular modeling and X-ray studies, we have examined a new chemical scaffold to replace the heterocyclic core of the nonselective kinase inhibitor staurosporine. We designed, synthesized, and tested a small series of pyrazolones specifically for their ability to function as GSK-3 β inhibitors. The best of these analogues was found to be selective for GSK-3 over 40 other homologous kinases. In particular, compound **3a** gave an impressive dose–response curve for neuroprotection in the homocysteic acid model of oxidative stress. Additionally, compound **3a** was able to attenuate locomotor activity in the chlordiazepoxide/amphetamine-induced hyperactivity model in vivo. This new lead compound represents one of the best all-around GSK-3 inhibitors we have identified to date.

Experimental Section

Full experimental protocols can be found in the Supporting Information. All experiments were conducted in accordance with the US National Institutes of Health guidelines and using an approved protocol from the Institutional Animal Care and Use Committee of PsychoGenics, Inc. (Tarrytown, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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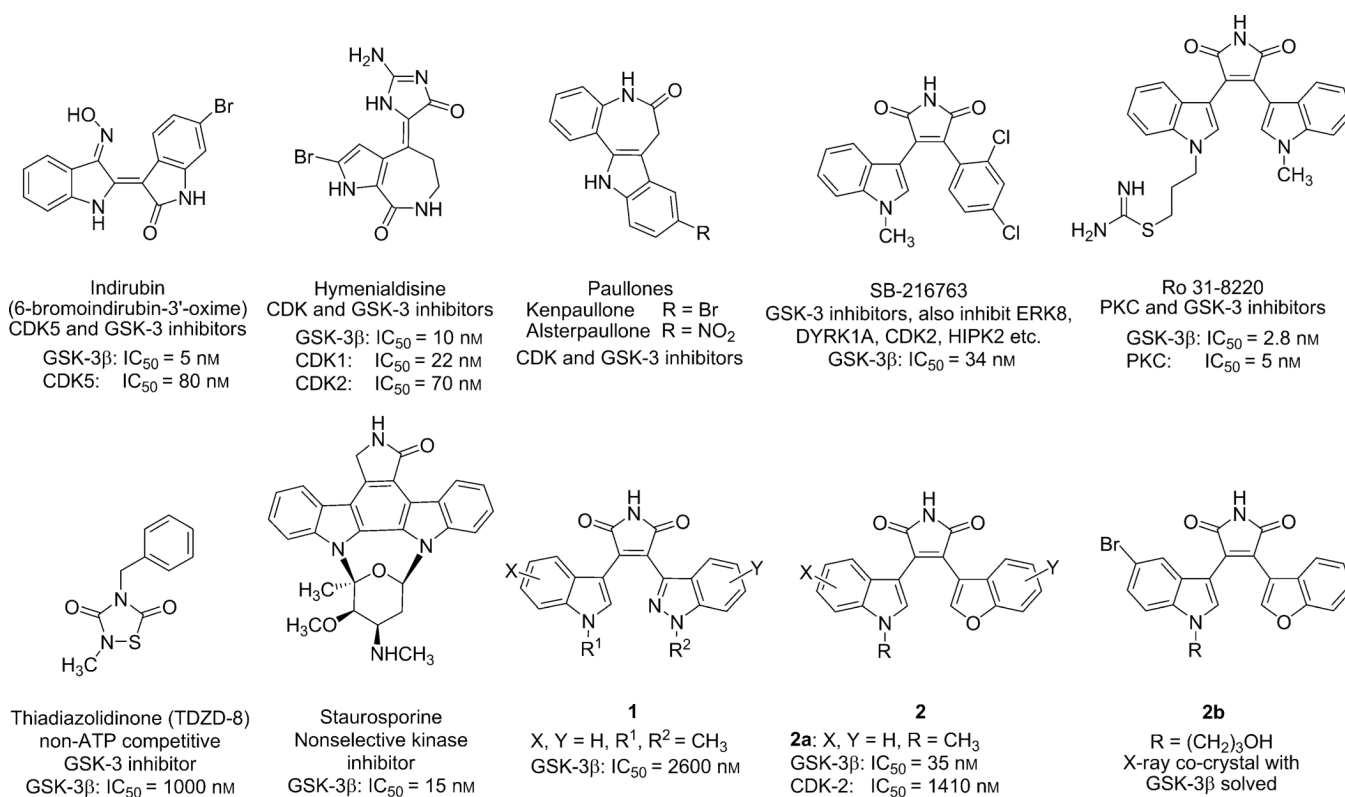


Figure 1. Selected examples of GSK-3 β inhibitors from the literature.^[9–14]

Involved in hydrogen-bonding interactions with GSK-3

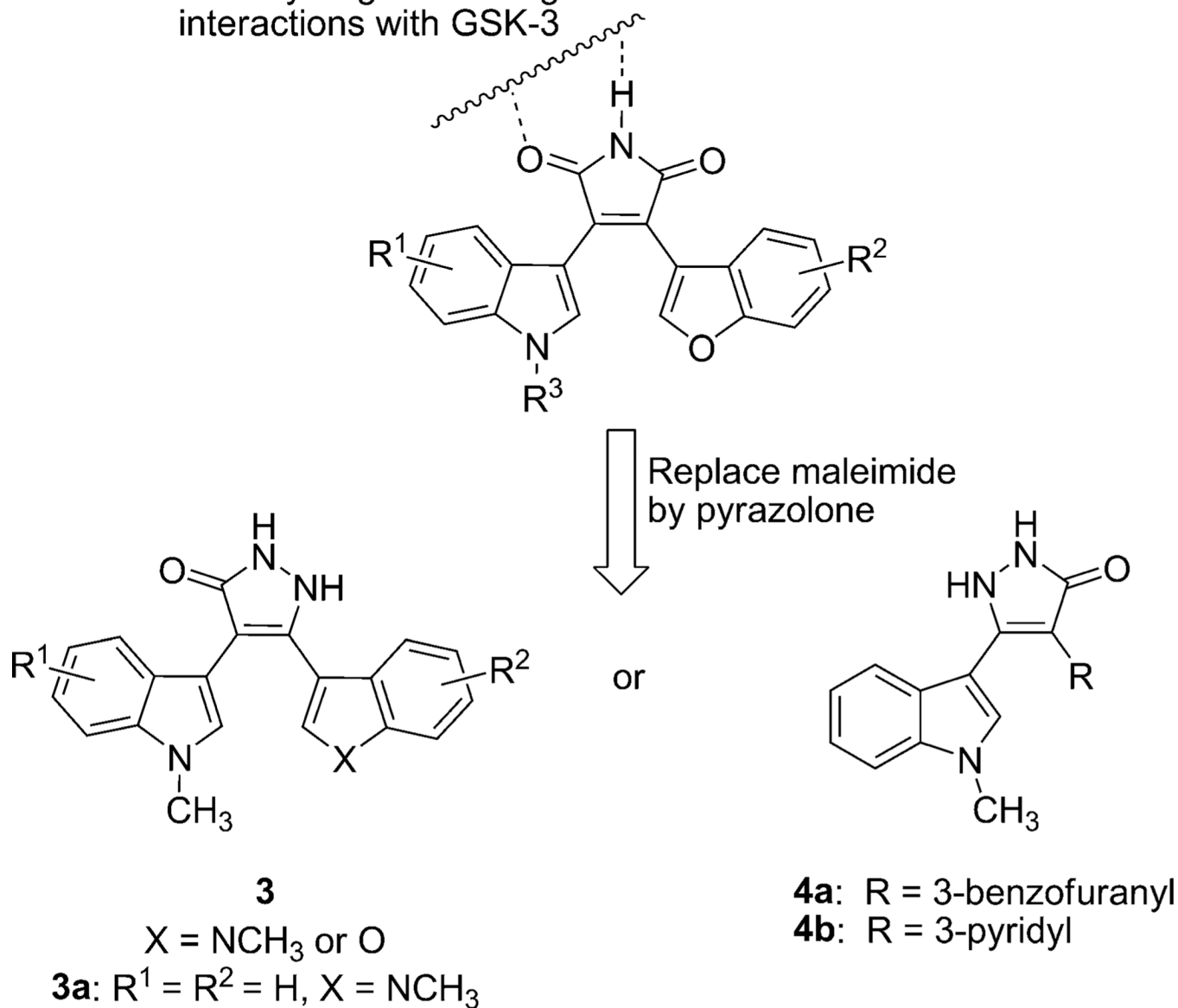


Figure 2.

Alternative GSK-3 inhibitor scaffolds with a pyrazolone (5-membered heterocycle) as a replacement for the maleimide core.

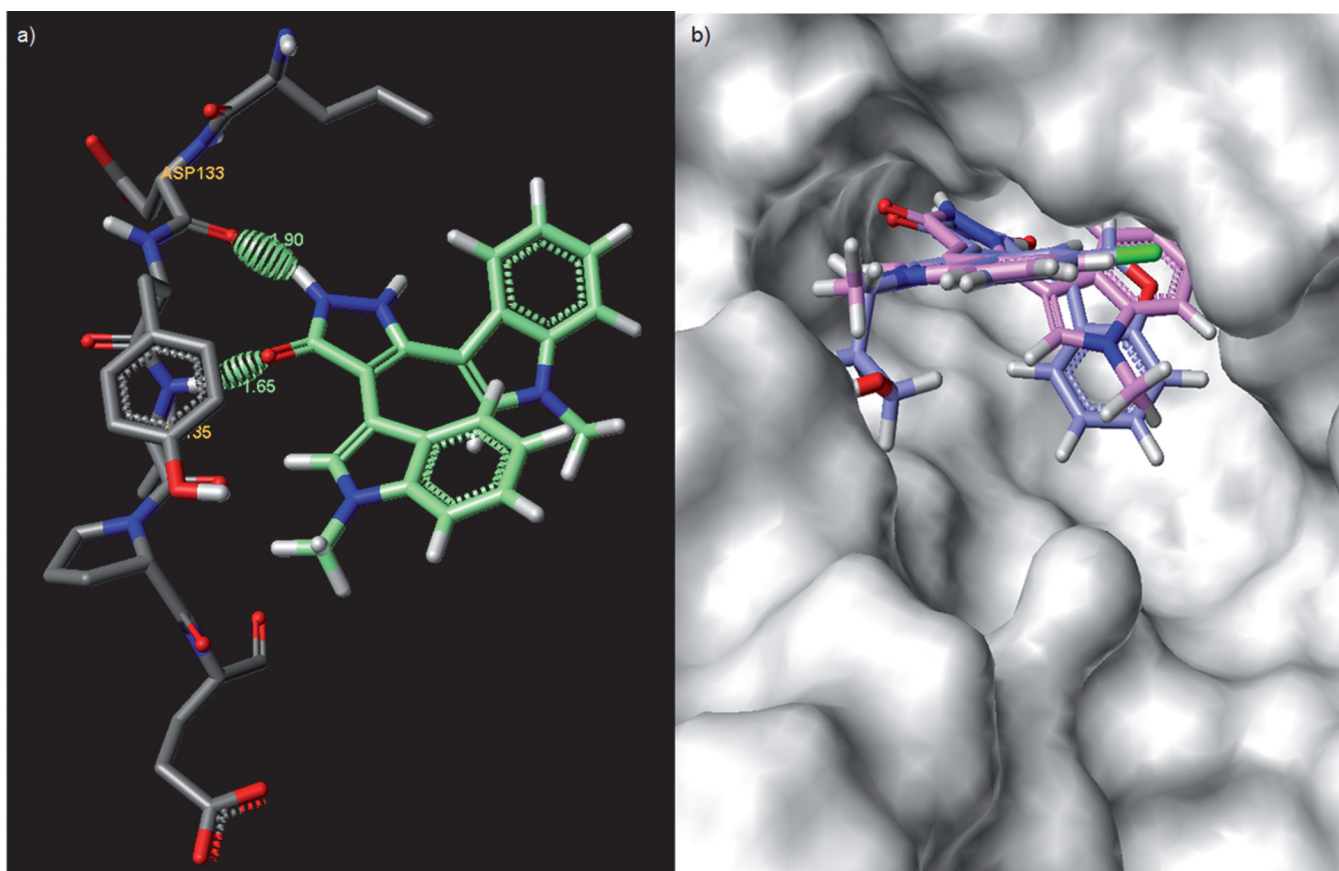


Figure 3. Docking of the pyrazolone to the X-ray structure of GSK-3 β . a) Binding orientation of **3a** in the active site showing key hydrogen-bonding interactions. b) Solvent-exposed surface map of GSK-3 β (grey) showing the binding pocket for **2b** (blue) and **3a** (pink).

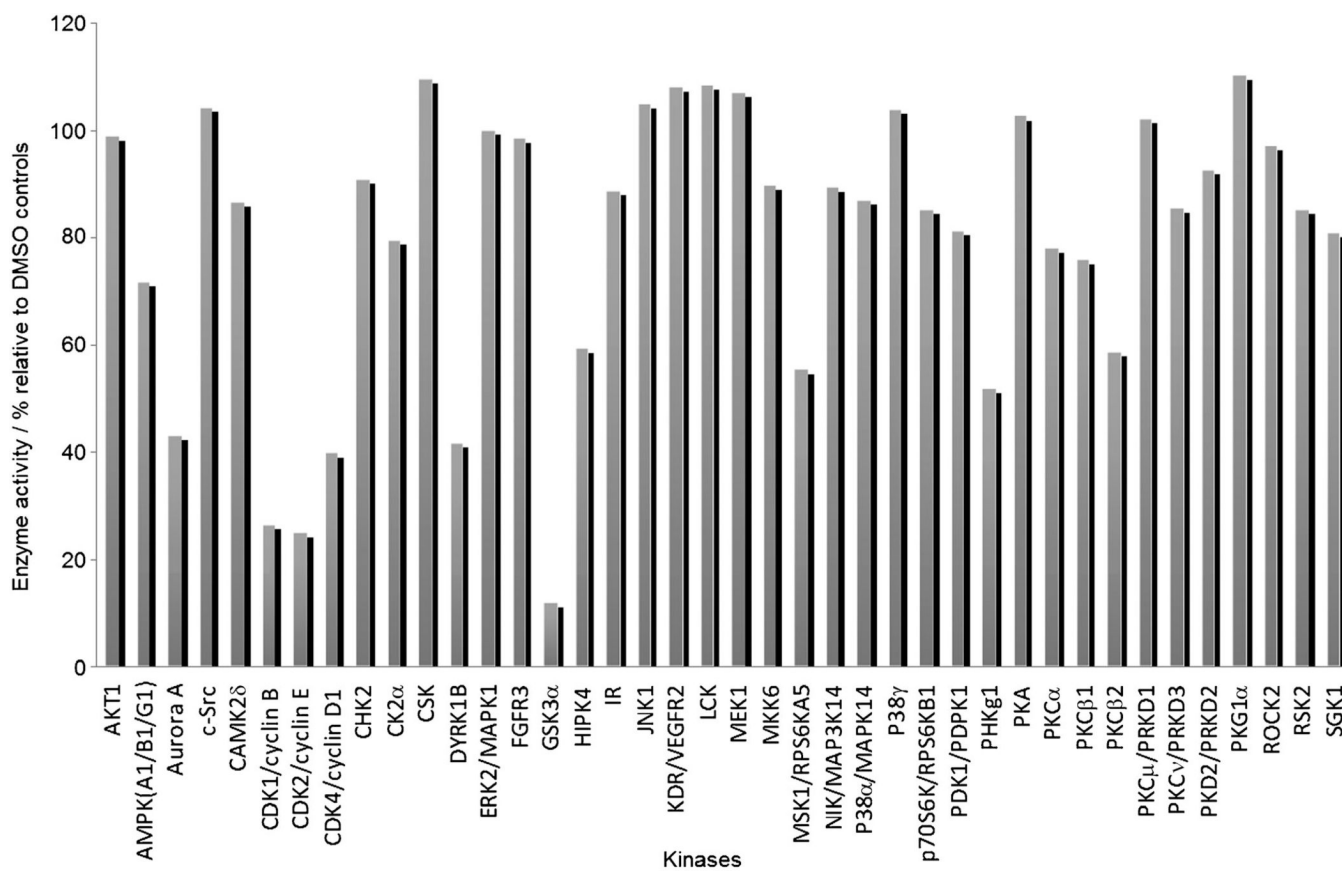


Figure 4. First-pass kinase screening of **3a** at 10 μM concentration against a panel of 40 kinases.

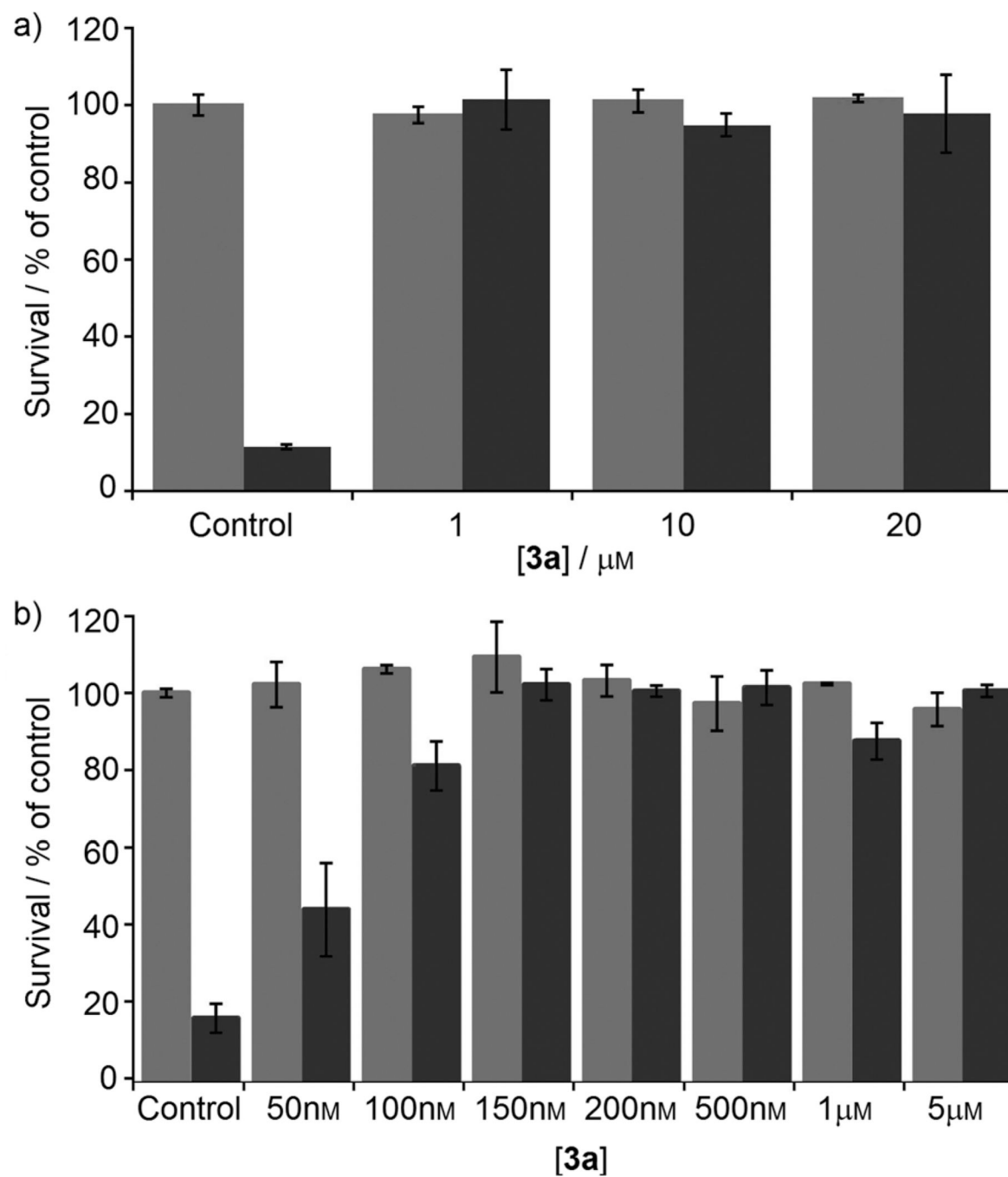


Figure 5.
 HCA oxidative stress assay: neurons were treated with pyrazolone **3a**, with (■) or without (□) addition of HCA. Viability was assessed after 48 h by an MTT assay.

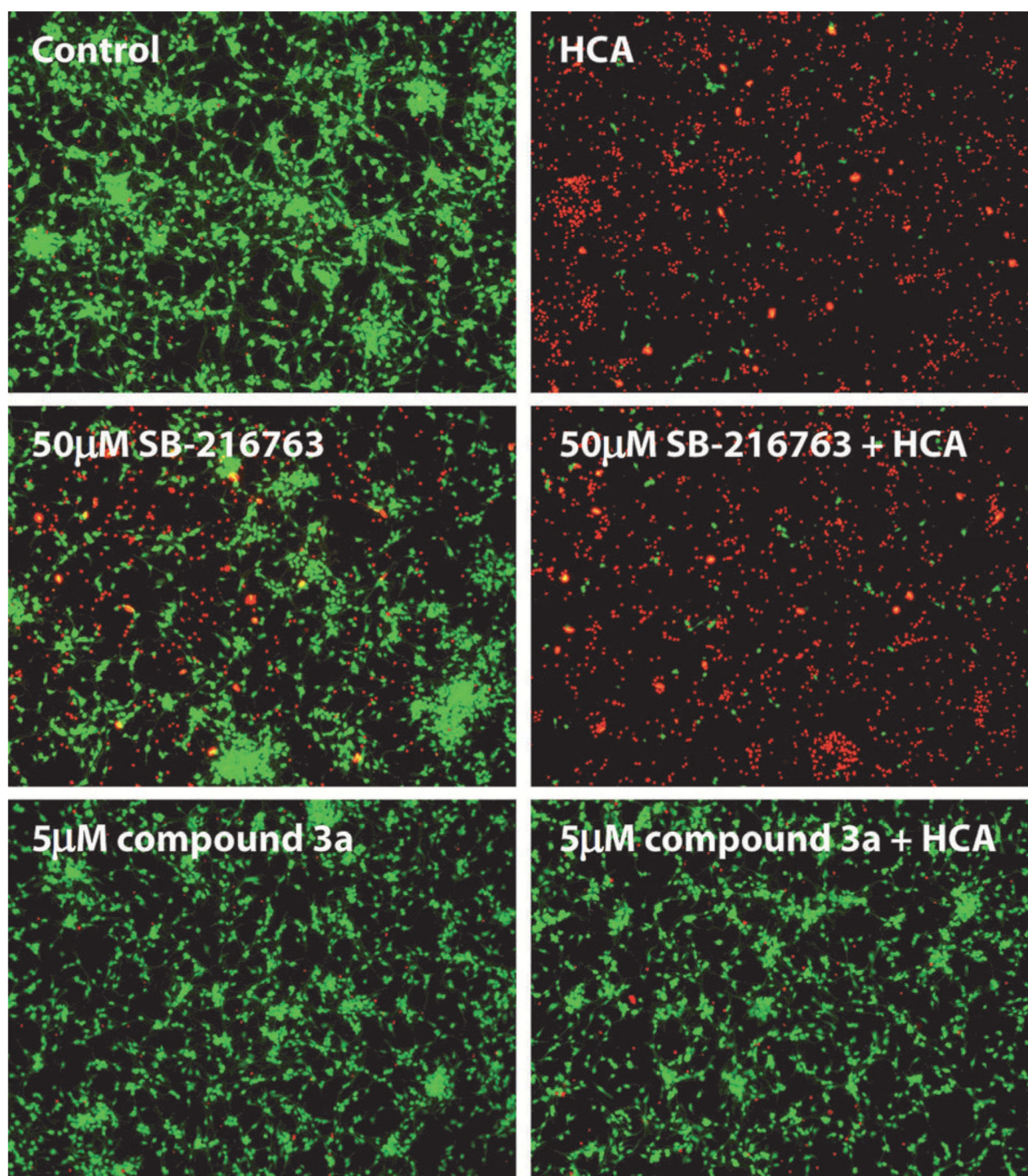


Figure 6.
Oxidative stress model: neuronal cell micrographs of live (■) and dead (■) cells in the presence of the GSK-3 inhibitors alone or in combination with HCA.

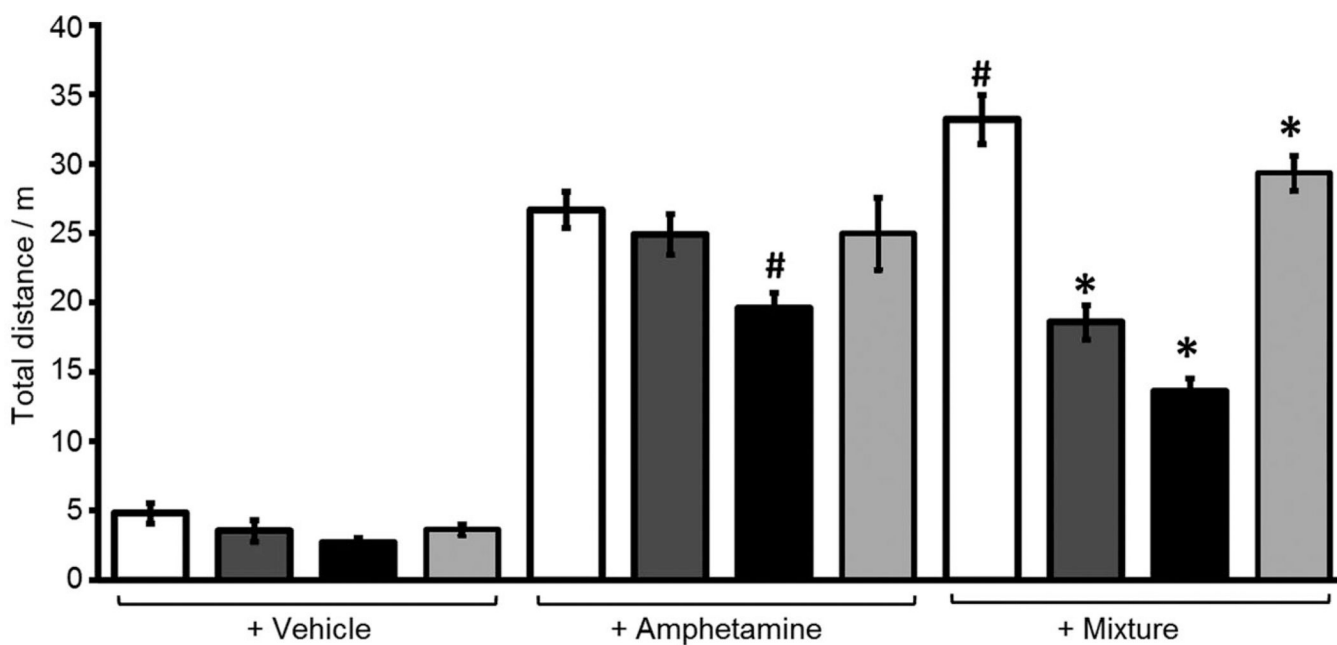


Figure 7.

Compound **3a** or valproate reduced the locomotor hyperactivity induced by chlordiazepoxide (CDP) + amphetamine (Amph) mixture in C57BL/6J mice. Locomotor activity was quantified for a period of 1 h. Each bar represents the mean (\pm SEM) of the total distance traveled (m) for at least eleven mice. The mixture of Amph + CDP produced greater locomotor activity than Amph alone (#, $p < 0.05$ Veh–Mix vs Veh–Amph; Fisher's PLSD). Pretreatment with **3a** or valproate reduced the locomotor activity produced by the Amph + CDP mixture (*, $p < 0.05$ 3a–Mix or Val–Mix vs Veh–Mix; Fisher's PLSD). Vehicle control:

□

; valproate (400 mg kg⁻¹; 30 min):

■

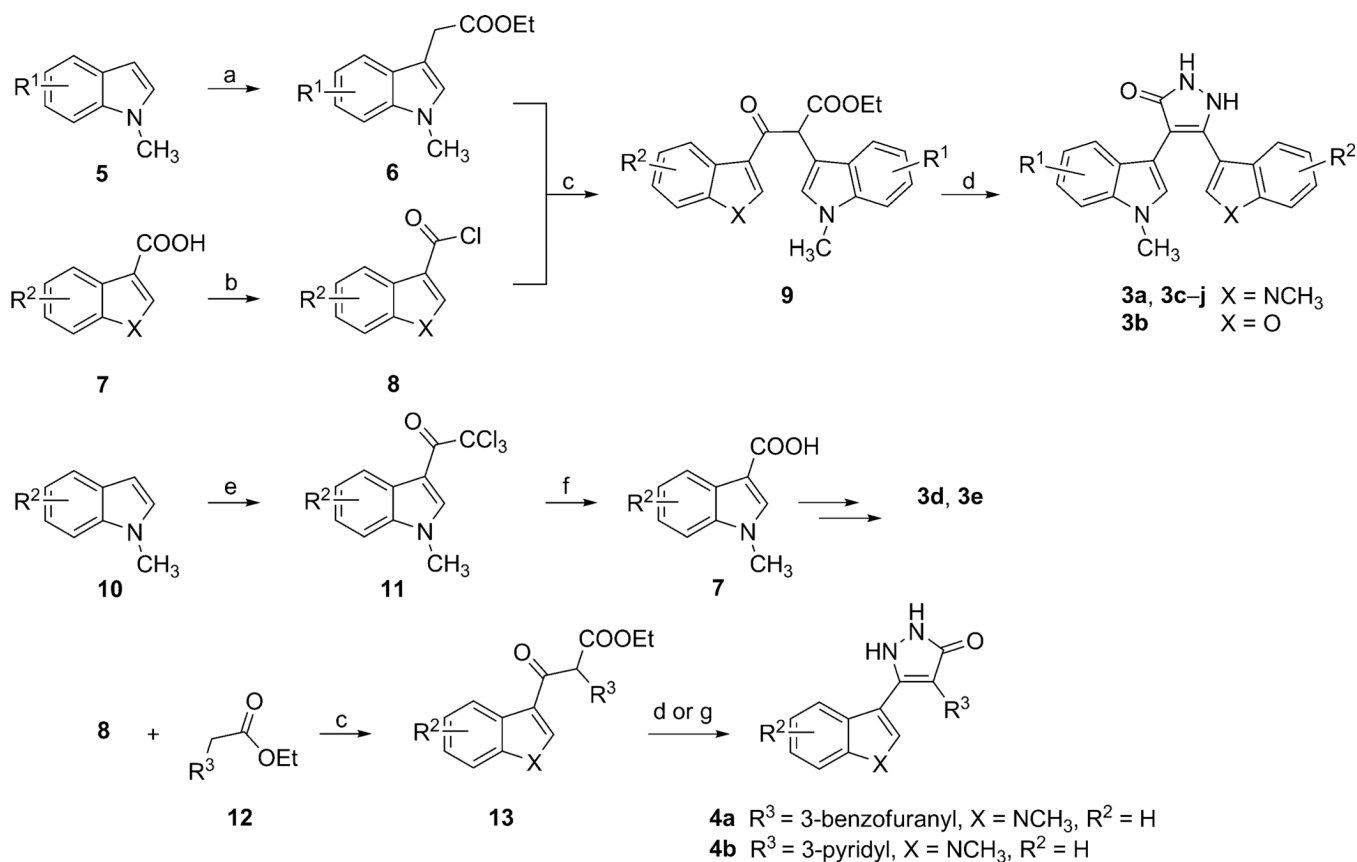
; valproate (400 mg kg⁻¹; 5 min):

■

; **3a** (150 mg kg⁻¹; 5 min):

■

.

**Scheme 1.**

Reagents and conditions: a) 5% Cu(OTf)₂, ethyl diazoacetate, CH₂Cl₂, 12 h, 45–55 %; b) SOCl₂, cat. DMF, RT, 16 h; c) LDA, THF, –78 °C, 16 h, 28–66 %; d) NH₂NH₂·XH₂O, EtOH, *p*-TsOH, MW, 5 h, 15–40 %; e) Trichloroacetyl chloride, pyridine, THF, 0 °C → RT, 3 h, 80–90 %; f) 2M NaOH, 1,4-dioxane, H₂O, reflux, 16 h, then H⁺, 60–70 %; g) NH₂NH₂·XH₂O, 1,1-dioxane, AcOH, reflux, 16 h, 58%.

Table 1GSK-3 β inhibition by pyrazolones and staurosporine.

Compd	R ¹	R ²	IC ₅₀ ^[a] [nM]
Staurosporine	–	–	11.2
3a	H	H	34.0
4a	–	H	2270
3b	H	H	2950
3c	5-Br	H	535
3d	H	5-Br	3090
3e	H	6-F	2060
3f	5-F	H	574
3g	5-Cl	H	2470
3h	4-F	H	1490
3i	7-F	H	2070
3j	5-OBn	H	NA ^[b]
4b	–	H	4280

^[a] The synthesized pyrazolones were evaluated for their ability to inhibit phosphorylation of primed substrate (YRRAAVPPSPSLSRHSSPHQ (pS) E DEEE; 20 μ M) by human GSK-3 β in the presence of 10 μ M ATP. These compounds were tested by Reaction Biology, Inc. (<http://www.reactionbiology.com>).

^[b] Not active (maximum test concentration = 10 μ M).

Table 2Further in vitro kinase selectivity summary of lead compound **3a**.^[a]

Kinases	IC ₅₀ ^[nM]		SI ^[b]
	3a	Staurosporine	
ALK5/TGFBR1	>100 000	10 100	>2941
Aurora A	7900	<1.0	232
CDK1 cyclin B	3250	<1.0	96
CDK2 cyclin E	1800	1.3	53
CDK3 cyclin E	7090	5.4	209
CDK4 cyclin D1	2970	6.1	87
CDK5 p25	4220	2.4	124
CDK6 cyclin D3	8180	17.2	241
CDK7 cyclin H	–	155	–
CDK9 cyclin T1	8800	7.4	259
DYRK1 B	5970	6.3	176
GSK3 α	37.6	2.8	1
HIPK4	10 000	408	294
MSK1	14 000	<1.0	412
PHK γ 1	14 100	1.2	415
PKC α	45 700	1.6	1344
PKC β 2	23 000	<1.0	677

^[a]The compound was tested in ten-dose IC₅₀ mode with three-fold serial dilution starting at 100 μ M. Control compound staurosporine was tested in ten-dose IC₅₀ mode with three-fold serial dilution starting at 20 μ M. Reactions were carried out at 10 μ M ATP. These compounds were tested by Reaction Biology, Inc. (<http://www.reactionbiology.com>).

^[b]Kinase selectivity index (SI) of compound **3a** for GSK-3 β over other kinases tested.