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Discovery and structural insight of a highly selective protein kinase inhibitor hit through click chemistry†

Guoxian Gu,‡ Huihui Wang,‡ Pi Liu,‡ Chenzeng Fu, Zhonghua Li, Xuefeng Cao, Yunping Li, Qinghong Fang, Feng Xu, Jie Shen* and Peng George Wang*

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Novel bisaryl maleimide derivatives to mimic natural kinase inhibitors were prepared through click chemistry. A highly selective hit was discovered in a 124-kinase-assay, and docking studies revealed a π–π stacking interaction with the Phe67 at the P-loop of GSK-3β kinase.

Protein kinases constitute a large family of more than 500 enzymes to control a plethora of signal transduction processes within cells.[1,2] Many human diseases are associated with kinase dysfunction, and therefore selective protein kinase inhibitors are of great potential for therapeutic applications.[3] Indolocarbazole and structurally related natural products (Fig. 1) are a family of non-selective ATP competitive inhibitors.[4] Extensive efforts have been made to derivatize these natural indolocarbazole products to afford potent inhibitors with improved selectivity. Some bisaryl maleimide derivatives (Fig. 2), which can be considered as the "open" form of natural indolocarbazole scaffold, demonstrated better selectivity.[5–7] For example, Enzastaurin and Ruboxistaurin are two PKC selective kinase inhibitors under clinical trials.[8]

Being highly efficient and convenient, the Cu(I)-mediated Huisgen cycloaddition reaction, the premier example of "click chemistry",[9] became a powerful tool for hit discovery in the past decade.[10–12] Herein, it was employed to accomplish the goal of finding a novel selective hit against human kinases.

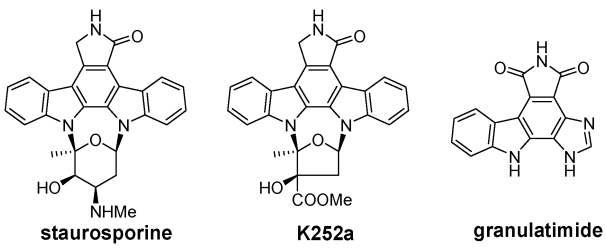

Fig. 1 Natural indolocarbazole derivatives as kinase inhibitors.

College of Pharmacy and Tianjin Key Laboratory of Molecular Research, State Key Laboratory of Medicinal Chemical Biology, Nankai University, Tianjin300071, P. R. China. E-mail: jieshen@nankai.edu.cn

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‡ These authors contributed equally to this work.

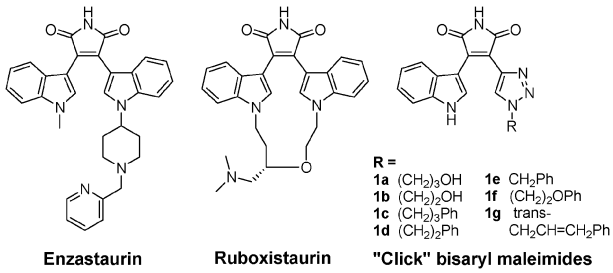
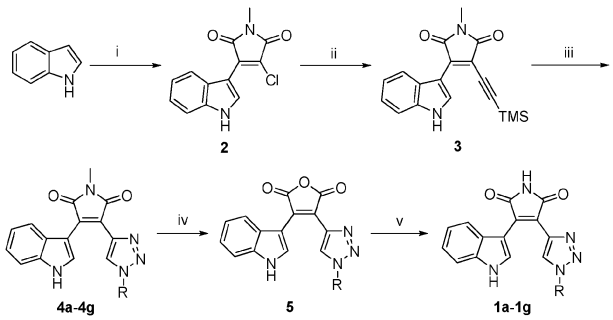


Fig. 2 Bisaryl maleimides: open form of the indolocarbazole scaffold.

Moreover, the quickly constructed and structurally diversified novel bisaryl maleimides (Fig. 2) would provide useful molecular probes for elucidating the structural features of kinases and for highlighting the future designs of inhibitors thereof.

In the initial stage, seven click bisaryl maleimides were prepared (Scheme 1). Compound **2** was obtained by coupling indole magnesium bromide with *N*-methyl-dichloromomaleimide in tetrahydrofuran (THF) solution. *N*-Methyl-dichloromomaleimide was obtained from maleic anhydride through two steps according to the procedure reported by Relles.[13] Then, the Sonogashira reaction was employed to give alkyne precursor **3** in 75% separation yield. After de-silylation with one equivalent tetrabutylammonium fluoride (TBAF), alkyne precursor **3** reacted with azides to give the middle compound **4a–4g** in the presence



Scheme 1 Synthesis of **1a–1g**. *Reagents and conditions*: (i) (a) EtMgBr, THF, 60 °C, 1 hour; (b) 2,3-dichloro-*N*-methylmaleimide, 65 °C, 1 hour, 52%; (ii) $Pd(PPh_3)_4$, CuI, trimethylsilylacetylene, THF, Et_3N, room temperature, 75%; (iii) (a) TBAF, methanol, THF; (b) R–N_3, sodium ascorbate, $CuSO_4(H_2O)_5$, room temperature, 22%–71%; (iv) NaOH, room temperature, 2 hours; HCl, *p*H = 1, room temperature, overnight. (v) NH_4OAc, DMF, 140 °C, 1 hour, 28%–81% (two steps).

Table 1 MTT assays of "click" bisaryl maleimides

Compounds	*In vitro* cytotoxicity IC_{50}/μM			
	SH-SY5Y[a]	A549[b]	NCI-H460[c]	K562[d]
1a	> 50	> 50	> 50	> 50
1b	> 50	> 50	> 50	> 50
1c	1.83	2.43	13.31	9.82
1d	> 50	> 50	> 50	> 50
1e	5.42	3.95	13.48	10.54
1f	1.77	0.82	3.60	2.22
1g	> 5	> 50	> 50	> 50
Doxorubicin	0.06	0.17	0.02	0.08

[a] Human derived neuroblastoma. [b] Carcinomic human alveolar basal epithelial cell. [c] Human non-small cell lung cancer cells. [d] Human immortalised myelogenous leukaemia.

of copper sulfate pentahydrate and sodium ascorbate. The *N*-methylated compound **4** was treated with aqueous NaOH followed by acidification with hydrochloric acid affording anhydride **5**. Then the anhydride **5** was heated with ammonium acetate to afford **1a–1g** in moderate yields.

To quickly verify the physiological activities of the newly prepared click bisaryl maleimides **1a–1g**, MTT assays were performed utilizing four cancer cell lines (Table 1). Three compounds **1c**, **1f** and **1g** could effectively inhibit *in vitro* tumor cell growth at low micromolar level. These three compounds shared similar molecular structures which all had a three-atom chain between the maleimide ring and phenyl ring. Therefore, only compound **1c** was selected as a representative to enter into kinase inhibition assay (Fig. 3). In the initial one-dose screening, only four kinases of the 124-kinase panel were significantly inhibited by **1c** at 500 nM. IC_{50} values of these four kinases were then determined (Table 2). ZAP70 was identified as a false positive since its IC_{50} value was only 1.66 μM.[15] Taken together, a highly selective hit compound **1c** was identified

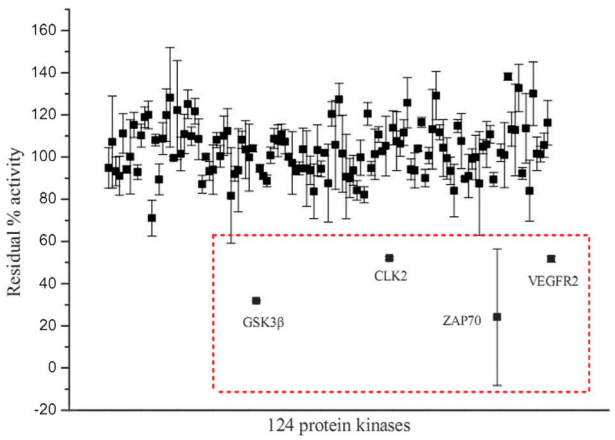


Fig. 3 A radioactive ^{33}P-ATP filter-binding assay containing 124 kinases against **1c** at 500 nM.[14]

Table 2 Kinase inhibition activity of **1c**[a]

	GSK-3β	CLK2	ZAP70	VEGFR2
1c	100 ± 20	350 ± 0	1660 ± 610	200 ± 50

[a] IC_{50} data in nM. Curves obtained by measuring 10 concentrations in duplicate (3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM). The full 8 curves are included in ESI.

which only showed high potency against three kinases in a 124-kinase panel.

To the best of our knowledge, there is no bisaryl maleimide derivative in publications to show such surprisingly high selectivity in an assay panel of more than 100 kinases. Some "selective" inhibitors, which had a bisaryl maleimide scaffold, turned out not so selective when the number of assayed kinases was increased.[16] It was therefore of great interest to understand the structural origin for the high selectivity of compound **1c**. Prior to the computational studies, more assay experiments were carried out. The ATP-competition experiment confirmed compound **1c** as an ATP competitive inhibitor (Fig. 4). Compounds **1d**, **1e** and **1g** were also assayed against GSK-3β, and these three compounds were all one to two magnitude less potent than the hit compound **1c** (Table 3).

Docking studies of compounds **1c**, **1d**, **1e** and **1g** to GSK-3β crystal structure (PDB code 1Q41) were performed, and consistent computational results to the assay data were obtained. As shown in Fig. 5, the active binding site of GSK-3β is bound by the hinge loop between the N-lobe and C-lobe, the P-loop which would contact with the phosphate moiety of ATP, the C-helix at the N-lobe, and the A-loop which would take a key role to activate ATP.[17] Compound **1c**, when docked with GSK-3β, forms three hydrogen-bonds with the protein: the –NH of the indole ring with the backbone of Ile62, the –NH of the maleimide with the backbone of Tyr134, and the carbonyl group of maleimide with Val135. A distinct feature of this protein structure (PDB code 1Q41) is that Phe67 flips inwards the active site, and therefore Phe67 is around 3 to 4 Å away from the phenyl moiety of **1c**. Such a distance is suitable for the π–π interaction. Obviously, either shortening the chain length between the phenyl ring and the triazole ring (*e.g.* compounds **1d** and **1e**), or improperly freezing the chain (*e.g.* compound **1g**) would attenuate such a π–π interaction. It should be addressed that Phe67 completely extends outwards the active site when GSK-3β binds with ATP derivatives (PDB code 1PYX). This means the inwards flipped Phe67 might cause a dramatic conformation change of P-loop,

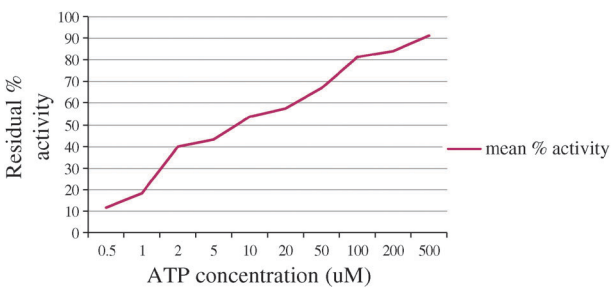


Fig. 4 ATP-competition assay of compound **1c** against GSK-3β.[14]

Table 3 GSK-3β inhibition activity of selected "click" bisaryl maleimides[a]

	1c	**1d**	**1e**	**1g**
GSK-3β	0.10 ± 0.02	34.89 ± 6.50	1.73 ± 0.38	1.49 ± 0.34

[a] IC_{50} data in μM. Curves obtained by measuring 10 concentrations in duplicate (3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM). The full 6 curves are included in ESI.

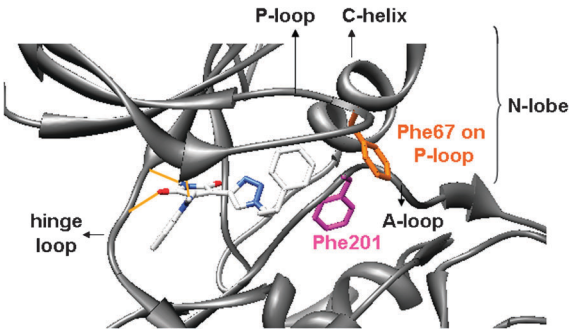


Fig. 5 Docking mode of compound **1c** into the ATP-site of GSK-3β. Autodock4.2 was applied to conduct the docking studies of GSK3b (PDB code: 1Q41) with hit compound **1c**. Experiment parameters: grid spacing = 0.375, box size = 22.5 × 22.5 × 22.5, grid center = 38 : 6 : 32, GA runs = 50, population size = 200, quaternion = 30.0, torsion = 30.0. Other parameters are set to default.

and in turn the dramatic topological change of the ATP binding site. Such a mechanism of deformed P-loop was recently utilized for computational design of a selective kinase inhibitor.[18] This mechanism might also account for the observed high selectivity of compound **1c** in 124-kinase assay. Nevertheless, at this stage, other possible mechanisms to explain the selectivity could not be ruled out. For example, the Phe201 residue on the flexible A-loop might take an inactive "Phe-out" conformation[19] to contact with the phenyl ring of **1c**. Dynamic simulation studies and hit-to-lead optimization work are currently ongoing to further elucidate these questions.

In summary, a novel kinase inhibitor **1c**, which targeted only three out of 124 human kinases, was identified. This is the second hit-discovery project from our research laboratory utilizing click chemistry. In both cases, the triazole ring does not take a simple role of linker fragment. In the histone deacetylase inhibitor project, the triazole ring forms a π–π stacking interaction within the narrow "tube-like" binding pocket;[20] and in this work, the triazole ring is well designed to construct a novel scaffold to mimic natural indolocarbazole derivatives. The success of these two examples implicates that the combination of click chemistry with rational design would be a powerful approach for hit discovery in medicinal chemistry.

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