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Identification of 3,5,6-substituted indolin-2-one's inhibitors of Aurora B by development of a luminescent kinase assay

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ABSTRACT

Aurora B kinase plays an important role in the cell normal mitosis and overexpresses in a variety of tumors. Inhibition of Aurora B kinase resulted in an apoptosis of cancer cells, which prevented tumor growth in xenograft models. In this Letter, we developed a luminescent kinase assay to perform high-throughput screening for identification of small molecule Aurora B inhibitors. Two 3,5,6-substituted indolin-2-one derivatives were identified within an in-house compound library. Their new derivatives were then designed and synthesized that resulting two new inhibitors of Aurora B kinase with improved potency. Docking simulation further demonstrated the proposed binding modes between indolin-2-one inhibitor and Aurora B.

Mitosis is a key step in ensuring the genetic integrity of daughter cells. Any aberration in this process could lead to genomic instability and the production of aneuploidy, which has long been recognized as a frequent characteristic of cancer cells and a possible cause of tumorigenesis.[1] The Aurora kinase family is a collection of highly related serine/threonine kinases playing an important role in maintaining normal cell process,[2] which was classified three subsets of Aurora A, B and C in mammals, respectively. Among them, Aurora B, known as the chromosomal passenger protein, is involved in accurate chromosome segregation, spindle-checkpoint, and cytokinesis.[3] Importance of Aurora B in cell mitosis has driven interest in development of new lead compounds to develop a potential drug.

Overexpression of Aurora B at the mRNA and protein levels was reported in different types of cancer including breast, colorectal, kidney, lung and prostate carcinoma.[4] A number of small molecules inhibiting Aurora B are currently under intense clinic-pharmacological studies, such as AZD1152, GSK1070916, AT9283, PHA-739358, ENMD-2076,[5–9] etc. In this study, we developed a luminescent kinase assay and performed a screening of our in-house small molecular library. Then, structure optimization based on active compounds was conducted, and 4 new Aurora B inhibitors of 3,5,6-substituted indolin-2-one derivatives were eventually identified.

There are different assay technologies available for identification of aurora kinase inhibitors, such as radiolabeled methods,[10] ELISA[11] (enzyme-linked immunosorbent assay), DELFIA immunoassays[12] and FRET technology,[13] etc. Bioluminescent methods offer several advantages over other methods such as no isotope contamination, antibody-free and no fluorescent tag.[14] In addition, it is automation friendly with low background and is free from fluorescent compound interference. In this study, we developed a luminescent assay to identify small molecule inhibitors of Aurora B.

Three employed Aurora B peptidic substrates (Table 1)[15–17] were synthesized via a standard Fmoc peptide chemistry. All obtained crude peptides were subsequently purified by a preparative chromatography and analyzed with a fast liquid chromatographic–mass spectrometry (LC–MS) system, which purities were greater than 82% (see in the Supplementary data).

The peptidic substrates (100 μM) were preliminarily incubated with Aurora B at 0, 100 and 200 ng/well, respectively, in a 50 μL final volume containing 25 μM ATP. After certain reaction times, the phosphorylation of peptides was measured using the Kinase-Glo reagent, which can determine kinase activity by quantitating the amount of ATP remaining in solution following a kinase assay.[14] Figure 1 demonstrated that all three peptides showed strikingly less luminescence, indicating that all three peptides were able to be phosphorylated in the course of the Aurora B

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0960-894X/

Table 1
Aurora B peptidic substrates

No.	Sequences	Purity (%)	Calcd (MS, $[M+nH]^{n+}$)	Found (MS, $[M+nH]^{n+}$)
SB1	ARTKQTARKSTGGKAPRKQLAGCG[15]	82	824.5 ($n = 3$)	824.8 ($n = 3$)
SB2	LRRLSLG LRRLSLGLRRLSLGLRRLSLG[16]	97	1067.7 ($n = 3$)	1068.0 ($n = 3$)
SB3	LRRASLG[17]	99	772.5 ($n = 1$)	772.4 ($n = 1$)

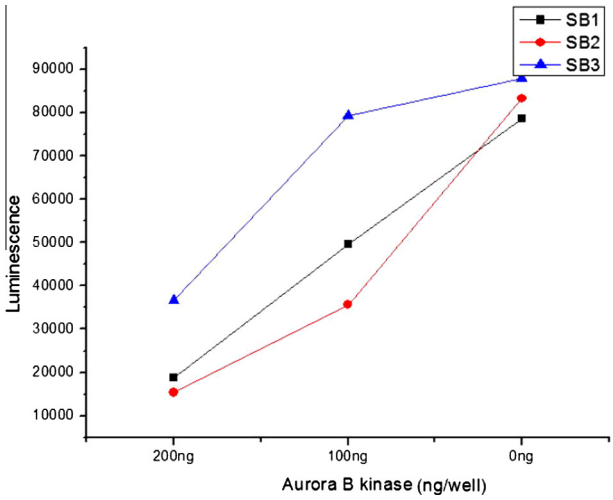


Figure 1. Peptide substrates were phosphorylated by kinase Aurora B in a dose-dependent manner.

reaction and the ATP was consumed. At the highest concentration (200 ng/well), Aurora B phosphorylated peptide SB1 was a similar extent to peptide SB2, but phosphorylated peptide SB3 was to a lesser extent. SB2 was finally selected to use as the peptide substrate in further screen of inhibitors against Aurora B, because SB2 was more sensitive to phosphorylation than SB1.

The luminescent kinase assay conditions were optimized with respects to the amount of ATP, kinase, and peptidic substrate. Figure 2A illustrated that serial twofold dilutions of ATP resulted in a linear correlation of luminescence intensity with the amount of ATP depletion. Thus, 25 μM ATP, the middle in the linear range of concentrations, and excess peptide SB2 (200 μM) were set up in 50 μL reaction final volume to determine the optimal Aurora B concentration. With the observation in Figure 2B, decreasing RLU readings of Aurora B in a concentration dependent way led to determine 300 ng/well of Aurora B as the optimal concentration for inhibitor screening assay. Additionally, decreased ATP's amount was also observed as consumption of SB2 increased in a 50 μL reactions with 25 μM ATP and 300 ng/well of Aurora B (Fig. 2C). The luminescence changed most dramatically when SB2 was at 25 μM. Collectively, the optimal conditions were finally set up

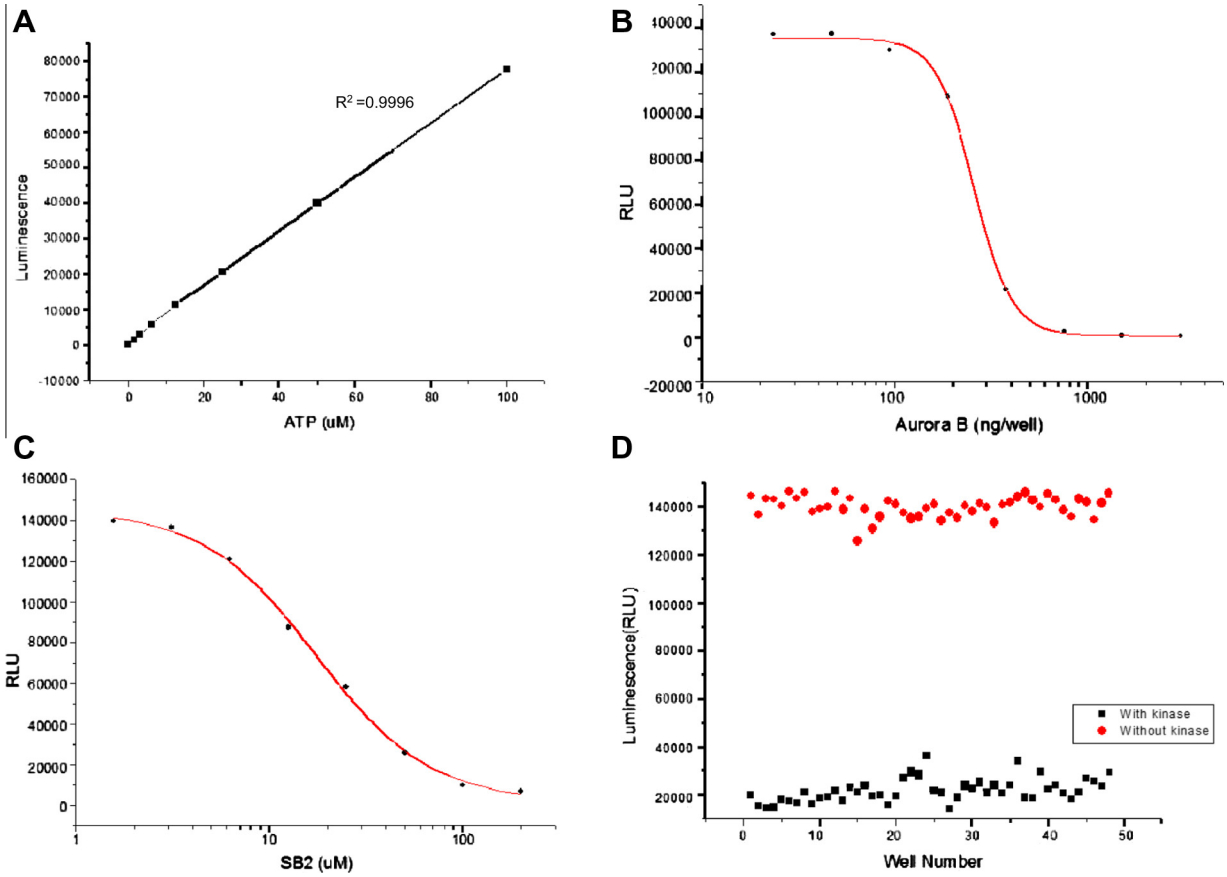


Figure 2. Optimization of luminescent kinase assay. (A) RLU readings linearly correlated with ATP concentrations. (B) Aurora B affects the change of luminescence in a concentration dependent manner in kinase reactions. (C) ATP depletion increased as SB2 increased. (D) Z' values for kinase assay, Z' values higher than 0.5 indicate that the assay is good.

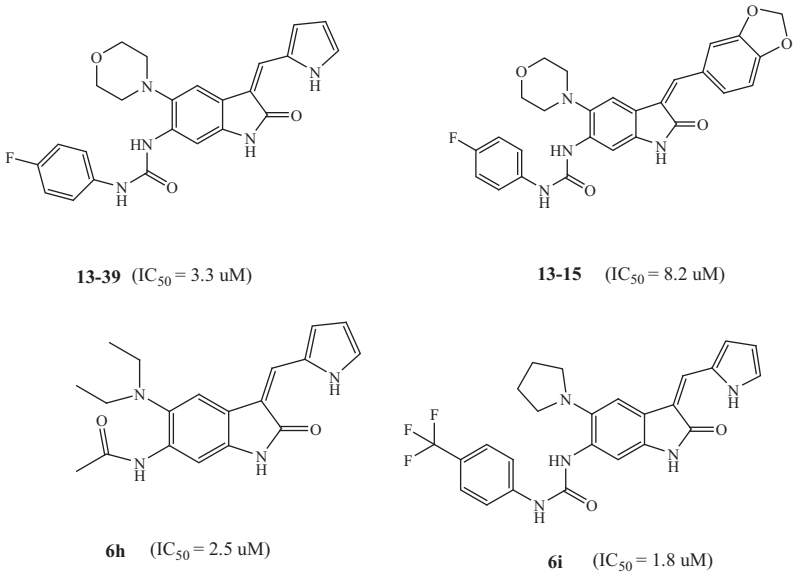
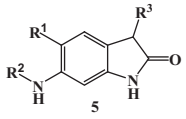
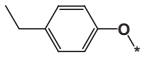
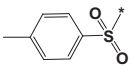
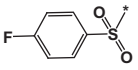
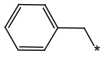
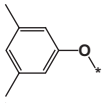
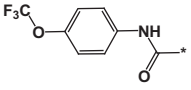
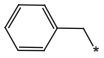
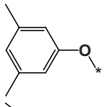
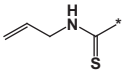
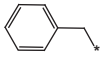
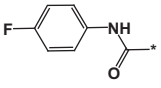
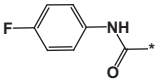
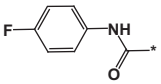
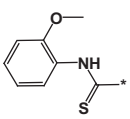


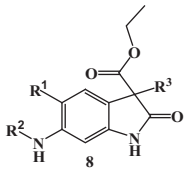
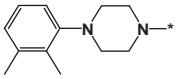
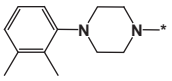
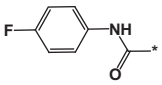
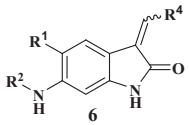
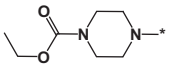
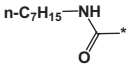
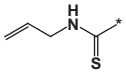
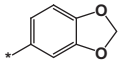
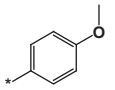
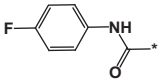
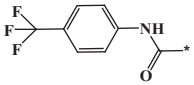
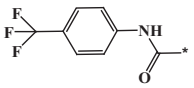
Figure 3. 3,5,6-Substituted indolin-2-ones that inhibited Aurora B kinase activity.

Table 2
Information of 3,5,6-substituted indolin-2-one derivatives

Entry	R^1	R^2	R^3/R^4	% inhibition*
 5				
5a			**H**	NA#
5b				NA
5c				NA
5d				NA
5e				NA
5f			H	NA
5g				NA
5h				35

(*continued on next page*)

Table 2 (*continued*)

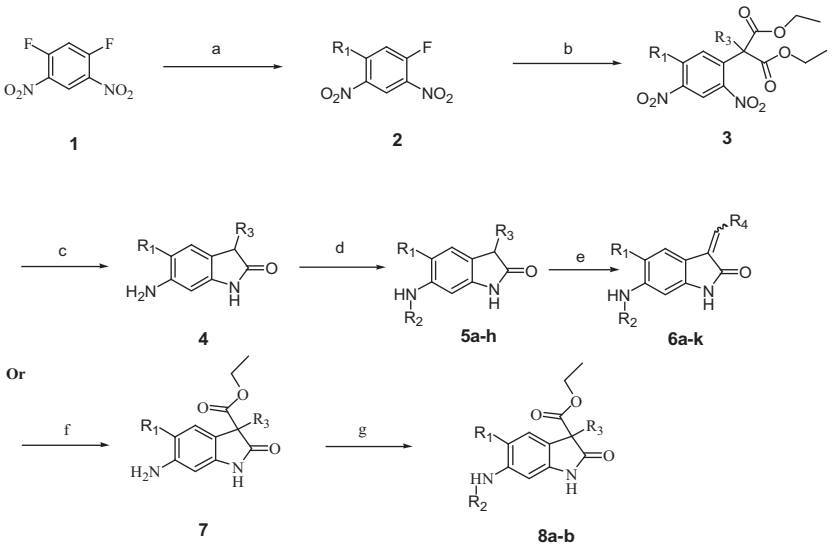
Entry	R^1	R^2	R^3/R^4	% inhibition*
 8				
8a				NA
8b				NA
 6				
6a				NA
6b				31
6c				NA
6d				NA
6e				65
6f				NA
6g				NA
6h				83
6i				97
6j				47
6k				NA

* Percentage inhibition was tested under the concentration of 10 μM, and the values were the average of duplicate measurements.
\# NA: percentage inhibition under the tested concentration was less than 30%.

25 μM ATP, 300 ng/well of Aurora B and 25 μM SB2 in a 50 μL final volume. Notably, we found that less than 2% DMSO in volume could confer a negligible influence on activity of Aurora B. Under the optimal assay condition, the *Z*′-factor was further analyzed with running total of 48 positive wells in the presence of Aurora B and 48 negative wells in absence of Aurora B. Calculations based on these data provided a *Z*′ score of 0.77, indicating that the developed luminescence-based assay was an excellent assay (Fig. 2D).

Three thousand small molecular compounds in our stock compound solution were screened by the developed assay (complete experimental details see in the Supplementary data). Our compound collection were assembled through a scaffold-directed program aiming at developing benzofused heterocyclic privileged structures with drug-like properties.[18–29] Thirteen compounds were greater than 60% inhibition of Aurora B activity under the concentration of 10 μM in the primary screening. Two of them were at 3.3 μM (**13**–**39**) and 8.2 μM (**13**–**15**) with 50% inhibition of Aurora B (Fig. 3).

The two active compounds identified in the preliminary screening belong to indolin-2-one derivatives which have multiple biological activities.[30–32] Table 2 indicated the new designed derivatives of **13**–**39** and **13**–**15** because we were looking for more potent inhibitors against Aurora B. Phenoxyl, dipropylamino, diethylamino, pyrrolidin, piperazin, morpholino were introduced



Scheme 1. Synthesis of compounds **5a**–**h**, **6a**–**k**, **8a**–**b**. Reagents and conditions: R^1–R^4 was defined in Table 2 (a) RR′NH, DIPEA or ROH, K_2CO_3; (b) $RCH(COOC_2H_5)_2$, NaF, THF; (c) Sn, HCl (aq), reflux; (d) $(RCO)_2O$, or RNCO, or RNCS, or RSO_2Cl; (e) When R^3 = H, R_4CHO, pyridine; (f) Pd/C, $HCOONH_4$, rt; (g) $(RCO)_2O$, or RNCO, or RNCS, or RSO_2Cl.

in R^1 position, respectively. Different substituted amides, sulfonamides, ureas and thioureas formed in R^2. Meanwhile, R^3 included benzyl, allyl, pyrrol, furan, thiophen, pyridine groups, etc. Above introduced groups are all common functional groups or pharmacophore in drugs. The preparation of target compounds in Table 2 followed the previously published protocols[23] and was described in Scheme 1. Selective displacement of one of fluorine atoms of DFDNB by secondary amines produced compound **2**. Substitution of another fluorine atom with the anion of diethyl malonate gave compound **3**. **3** was converted to indolin-2-one (compound **4**) with tin powder and hydrochloric acid under reflux condition in a high yield, which is the essential intermediate in synthetic route. Subsequent reaction with anhydride, sulfonyl chloride, isocyanate, or isothiocyanate of **4** produced compound **5a**–**h.** Further condensation of intermediate **5** (when R^3 = H) with an organic base provided compound **6a**–**k**. In addition, compound **3** (when $R^3 \neq H$) could be reduced to compound **7** under Pd-C/$HCOONH_4$, which was subsequently converted to compound **8a**–**b** by anhydride, sulfonyl chloride, isocyanate, or isothiocyanate (R^2).

The luminescent assay described above was used to screen these 21 compounds at initial concentration of 10 μM. Eventually, 2 new active compounds (**6h** and **6i**) were identified. Their IC_{50} values were 2.5 μM and 1.8 μM, respectively (Fig. 3).

Due to limited data, it is difficult to deduce a full scape of SAR from the results. However, it did reveal some SAR trends. The substitution at 3′ position is very important for inhibition effect on Aurora B. When R1 and R2 were respectively substituted with morpholino and fluorophenyl-acetamide, hydrogen or butyl with R3 resulted in significant loss of potency (**5f**, **5g**). When R1 and R2 were respectively substituted with diethylamino and acetamide, it showed little effect on Aurora B when R3 was substituted with thiophen, pyridine and propane (**6f**, **6g**, **6k**). When R1 and R2 were respectively substituted with pyrrolidin, trifluomethylphenyl acetamide, thiophen with R3 led to reduced activity (**6j**). Therefore, double bond and aromatic ring or pyrrol ring at 3′ position are necessary for inhibition activity.

Molecular modeling was further performed by docking compounds **6h** into the ATP binding site of Aurora B.[33] Figure 4 showed that **6h** can properly occupy ATP binding pocket via docking of **6h** to human Aurora B kinase by using LibDock module of Discovery Studio 2.5 software (Libdock score 95.9467). The backbone

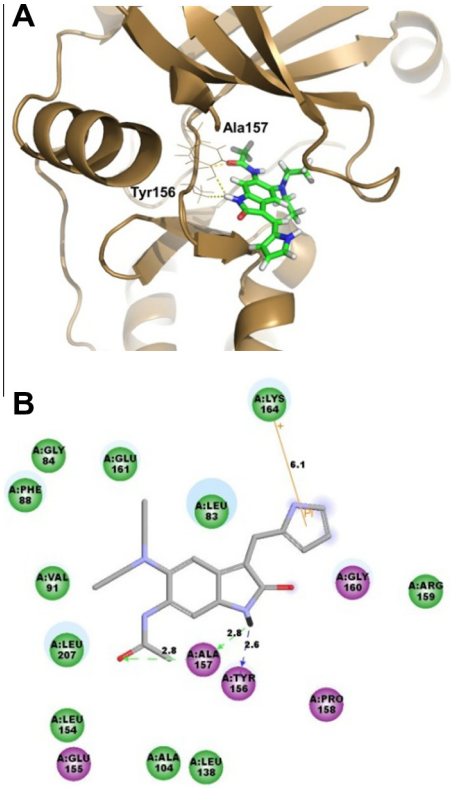


Figure 4. The interactions between **6h** and Aurora B. (A) Docking of the **6h** into the active site of Aurora B. (B) The 2D plot of the interactions between **6h** and Aurora B.

carbonyl group of the residue Ala157 or Tyr156 in Aurora B was able to form a hydrogen bond with amino group of indol in **6h**. The residue Ala157 or Tyr156 was the hydrogen bond receptor or donor in the hinge region of the kinase. Furthermore, the backbone amino group of the residue Ala157 was also able to give an additional hydrogen bond with carbonyl group of **6h**. Therefore, good binding pattern shown in the docking study proved that indolin-2-ones have great potential to be new human Aurora B inhibitors.

In this study, a luminescent kinase assay using Kinase-Glo reagent was developed for high-throughput identification of novel Aurora B inhibitors. In the first round of screening, a total of 3000 compounds were screened and 2 active 3,5,6-substituted indolin-2-ones were identified. Furthermore, twenty-one 3,5,6-substituted indolin-2-one derivatives were designed and synthesized that gave 2 new active compounds with improved inhibitory potency of 2.5 μM (**6h**) and 1.8 μM (**6i**), respectively. Docking study mimicked the binding pattern of 3,5,6-substituted indolin-2-one with Aurora B indicating that two hydrogen bonds occurred between the residue Ala157 or Tyr156 in Aurora B and amino group of indol in **6h** with great potential to be new human Aurora B inhibitors.

Acknowledgments

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Supplementary data

Supplementary data (all target compounds were characterized by ^{1}H NMR and mass spectra analyses. Supplementary information on general peptide substrate synthesis, mass spectrometric data of peptide substrates and luminescent assay protocols) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.05.043.

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