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Design, Synthesis, and Evaluation of Non-ATP-Competitive Small-Molecule Polo-Like Kinase 1 (Plk1) Inhibitors

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A series of small-molecule Plk1 inhibitors targeting the substrate-binding pocket were designed through rational drug design for the first time. The designed compounds were synthesized and their activities were evaluated *in vitro*. Some of the targeted compounds showed potent Plk1 inhibitory activities and anti-proliferative characters. Particularly, **5i** showed Plk1 inhibitory activity with an IC_{50} value of 0.68 μM. Compound **5i** also showed cell growth inhibitory activity on HeLa cells with an IC_{50} value of 0.51 μM, which is about four times more potent compared to thymoquinone. The mechanism of action suggested that **5i** was an ATP-independent and substrate-dependent Plk1 inhibitor. Compound **5i** demonstrated excellent Plk1 inhibitory selectivity against Plk2, Plk3, and five serine/threonine and tyrosine kinases. Our discovery and structure–activity relationship study may provide useful lead compounds for further optimization of non-ATP-competitive Plk1 inhibitors.

Keywords: Non-ATP-competitive / Polo-like kinase 1 / Rational drug design / Small-molecule / Structure–activity relationship

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Introduction

The serine/threonine kinase Polo-like kinase 1 (Plk1) is a member of the Polo-like kinases family (Plk1–5 in humans; collectively termed Plks) that act as a regulator in multiple stages of mitotic progression. Plk1 is considered an attractive anti-cancer drug target due to its ability to promote tumorigenesis [1]. Until now, two distinct drug targets have been identified in Plk1: an N-terminal catalytic domain and a C-terminal Polo-box domain (PBD). For a long period, discovery of Plk1 inhibitors has been focused on ATP-competitive compounds targeting the N-terminal catalytic domain [1]. However, as the catalytic domain of Plk1 is closely related to several members of the superfamily of protein kinases, these efforts suffered from a lack of target specificity. Like other Plks, Plk1 contains a characteristic substrate-binding PBD, which has been proved to be essential for bringing the catalytic activity of Plk1 to its binding targets at specific subcellular locations [2–4]. Thus, the PBD is ideally suited to the exploration of the feasibility of inhibiting a serine/threonine kinase by interfering with its intracellular localization, rather than targeting the conserved ATP-binding site [5].

Several series of peptides were designed and synthesized targeting Plk1 PBD with high affinity and specificity [6–12]. Some small-molecule compounds (Fig. 1) were also identified as Plk1 PBD inhibitors [5, 13–16]. To our knowledge, all the reported small-molecule Plk1 PBD inhibitors were discovered by screening. Herein, we report the identification of a series of small-molecule Plk1 PBD inhibitors through rational drug design according to the binding pocket of Plk1 PBD.

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Results

Rational design

Rational design of the target compounds is depicted in Fig. 2. Compound **CJ-032** was obtained from the screening of a series of in-house compounds. The Plk1 IC_{50} value of **CJ-032** was 5.04 μM. Then **CJ-032** was docked in to the Plk1 PBD to do some modification.

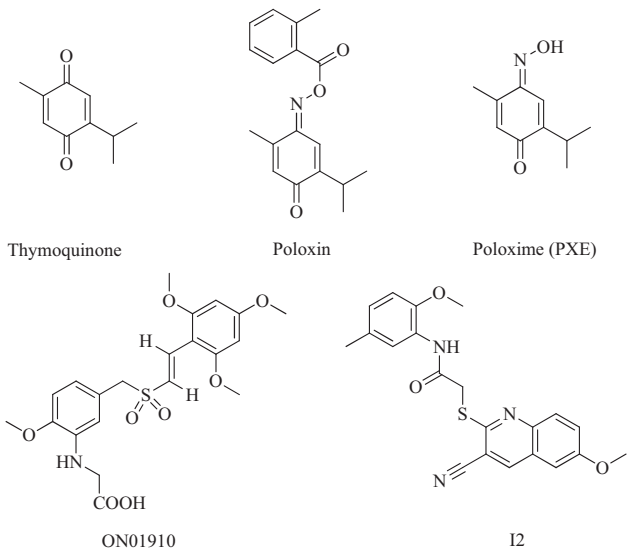


Figure 1. Chemical structures of the reported small-molecule Plk1 PBD inhibitors.

According to the docking result of CJ-032 shown in Fig. 2, the benzimidazole residue of CJ-032 may form hydrophobic interaction with the indole ring of Trp414. The 2-methyl phenyl group in CJ-032 may interact with a hydrophobic pocket formed by Trp410, Asn496, Arg557, and Phe559. Based on these, a substituted or unsubstituted phenyl group was introduced through an amide linker. The modified structure was then validated by docking again. As shown in Fig. 2, the introduced phenyl group linked with an amide merged into the hydrophobic pocket formed by Trp410, Asn496, Arg557, and Phe559. Interestingly, the carbonyl group could form an additional H-bond with the amino group in Lys540 as shown in Fig. 2. The benzimidazole ring could be substituted by an indole group as an isostere. A series of benzimidazoles and indoles were designed as shown in Table 1.

Chemistry

The synthesis of designed compounds **5** and **6** is provided in Scheme 1. Commercially available benzimidazole or indole (**1**) was reacted with 1-bromo-3-chloropropane in acetone at reflux temperature to give **2**. Compound **2** was reacted with 2-nitrophenol in DMF to give nitro **3**. Aniline **4** was obtained after reduction of nitro **3**. Then, the designed compounds **5** or **6** were obtained after acylation or benzylation.

In vitro Plk1 and cancer cell line growth inhibition assays

Designed compounds were synthesized and evaluated by Plk1 inhibitory assay [15] and *in vitro* cell line proliferation inhibition assay. As shown in Table 1, compounds with benzamide substitutions (**5a–m**) displayed similar inhibition

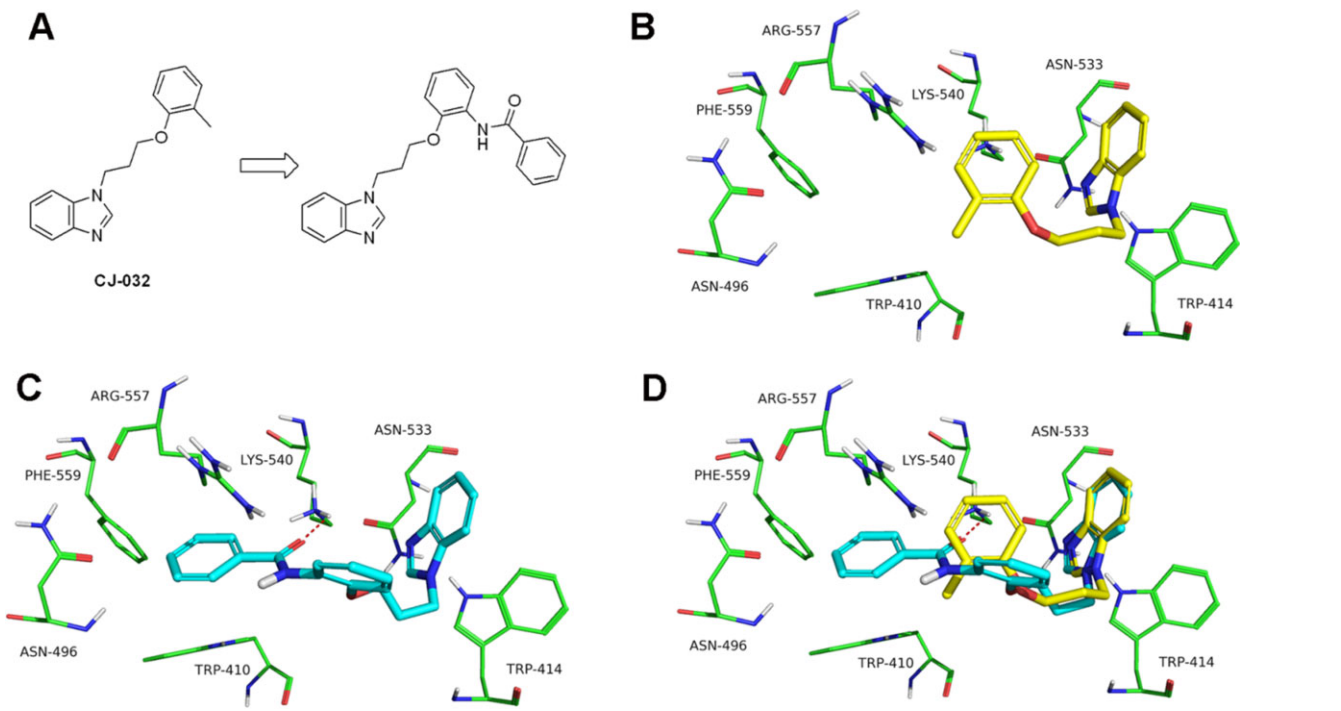
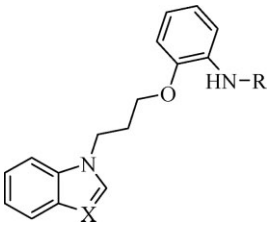


Figure 2. Design of new small-molecule Plk1 inhibitors targeting PBD. (A) Modification of CJ-032. (B) Docking result of CJ-032 (yellow) to Plk1 PBD. (C) Docking result of designed compound (blue) to Plk1 PBD. (D) Docking result of CJ-032 (yellow) merged with designed compound (blue) to Plk1 PBD.

Table 1. ***In vitro*** **Plk1 and cancer cell line growth inhibition of 5 and 6.**



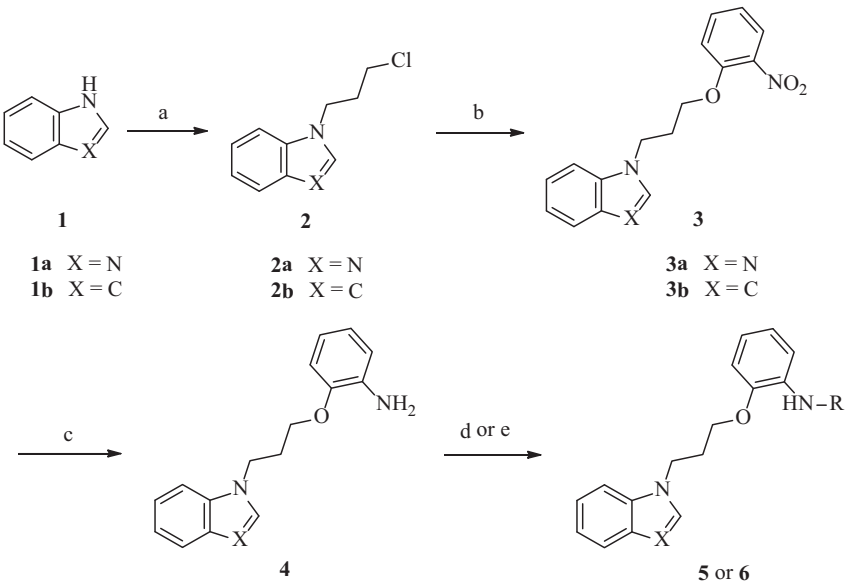
Compound	X	R	Plk1 inhibition, $IC_{50} \pm SD$ (μM)[a)]	Cell growth inhibition, $IC_{50} \pm SD$ (μM)[a)]	
				HeLa	MCF-7
5a	N	Benzoyl-	6.33 ± 1.06	10.03 ± 3.02	>10
5b	N	4-Methoxybenzoyl-	0.93 ± 0.20	2.18 ± 1.41	5.24 ± 0.86
5c	N	4-Methylbenzoyl-	7.09 ± 2.64	5.81 ± 0.62	>10
5d	N	2-Methylbenzoyl-	2.60 ± 0.82	>10,	>10
5e	N	4-Nitrobenzoyl-	>10	–	–
5f	N	2-Nitrobenzoyl-	>10	–	–
5g	N	Acetyl-	6.83 ± 0.52	>10	9.28 ± 1.25
5h	C	Benzoyl-	>10	–	–
5i	C	4-Methoxybenzoyl-	0.68 ± 0.07	0.51 ± 0.18	3.73 ± 1.02
5j	C	2-Methylbenzoyl-	5.59 ± 0.89	2.97 ± 0.74	>10
5k	C	4-Nitrobenzoyl-	>10	–	–
5l	C	2-Nitrobenzoyl-	>10	–	–
5m	C	Acetyl-	4.70 ± 0.48	>10	8.03 ± 2.07
6a	N	Benzyl-	9.48 ± 0.95	>10	>10
6b	N	4-Chlorobenzyl-	3.22 ± 0.22	>10	>10
6c	N	4-Methoxybenzyl-	2.05 ± 0.51	>10	>10
6d	N	4-Methylbenzyl-	4.66 ± 1.01	2.83 ± 1.25	6.56 ± 1.83
6e	N	4-Nitrobenzyl-	>10	–	–
6f	C	Benzyl-	7.02 ± 1.41	5.14 ± 1.00	>10
Thymoquinone	–	–	2.18 ± 0.31	2.06 ± 0.27	6.19 ± 0.97

[a)]Values are the average of three independent experiments.

to Plk1, compared to compounds containing corresponding benzylamine group (**6a**–**f**). Compounds with benzimidazole core also showed similar Plk1 inhibitory activity compared to those with indole core. *N*-Acetyl compounds (**5g** and **5m**) showed similar Plk1inhibitory activity as CJ-032. Compounds **5a** and **6a**, which is *N*-benzoyl and *N*-benzyl substituted, showed similar Plk1 inhibition to CJ-032. The substitution on the phenyl ring had big effect to both the inhibition to plk1 and anti-proliferative activities. Compounds with electron withdrawing groups (**5e**, **5f**, **5k**, **5l**, and **6e**) on the phenyl ring displayed decreased inhibition to Plk1. Electron donating groups gave an improvement for the Plk1 inhibition. For example, compounds **5b** and **5i** showed Plk1 inhibitory activity with an IC_{50} value of 0.93 and 0.68 μM, respectively, about five and eight times more potent than CJ-032. Compounds **5b** and **5i** were two to three times more potent than thymoquinone, a reported natural product as Plk1 PBD inhibitor. This result suggests that the electronic density on the phenyl ring is critical for Plk1 inhibitory activity. Compounds **5b** and **5i** also showed potent *in vitro* anti-proliferative activities. For example, **5i** showed inhibitory activity to HeLa cell line with an IC_{50} value of 0.51 μM, which is about four times more potent compared to thymoquinone.

Plk1 inhibition characters of 5i

The effects of increasing concentrations of ATP or the substrate Cdc25C on the inhibitory activity of the compound were examined using the method reported by Gumireddy et al. [15]. Ten nanograms of recombinant Plk1 was mixed with different concentrations of **5i**, followed by the addition of a reaction mixture containing γ^{32}P-ATP and varying concentrations of ATP (20, 40, 80, and 160 μM). The values from individual samples were analyzed and plotted as a function of inhibitor concentration using Prism 4 Graphpad software. The results are displayed as Fig. 3. These analyses showed that the IC_{50} values for the inhibitor were similar in the presence of increasing concentrations of ATP, suggesting that **5i** is not an ATP-competitive inhibitor (Fig. 3A). Then, the



1a X = N
1b X = C

2a X = N
2b X = C

3a X = N
3b X = C

4a X = N
4b X = C

5a X = N, R = benzoyl-
5b X = N, R = 4-methoxybenzoyl-
5c X = N, R = 4-methylbenzoyl-
5d X = N, R = 2-methylbenzoyl-
5e X = N, R = 4-nitrobenzoyl-
5f X = N, R = 2-nitrobenzoyl-
5g X = N, R = acetyl-
5h X = C, R = benzoyl-
5i X = C, R = 4-methoxybenzoyl-
5j X = C, R = 2-methylbenzoyl-
5k X = C, R = 4-nitrobenzoyl-
5l X = C, R = 2-nitrobenzoyl-
5m X = C, R = acetyl-

6a X = N, R = benzyl-
6b X = N, R = 4-chlorobenzyl-
6c X = N, R = 4-methoxybenzyl-
6d X = N, R = 4-methylbenzyl-
6e X = N, R = 4-nitrobenzyl-
6f X = C, R = benzyl-

Scheme 1. Synthetic route of compounds **5** and **6**. Reagents and conditions: (a) 1-Bromo-3-chloropropane, K_2CO_3, acetone, reflux, 12 h; (b) 2-nitrophenol, KOH, DMF, 80°C, 12 h, 17.7–31.8% yield for two steps; (c) 10% Pb/C, MeOH, H_2, r.t., 4 h, 87.6–91.6%; (d) acyl chloride, CH_2Cl_2, TEA, r.t., 2 h, 42.0–94.5%; and (e) benzyl bromide, K_2CO_3, acetone, reflux, 12 h, 36.7–62.3%.

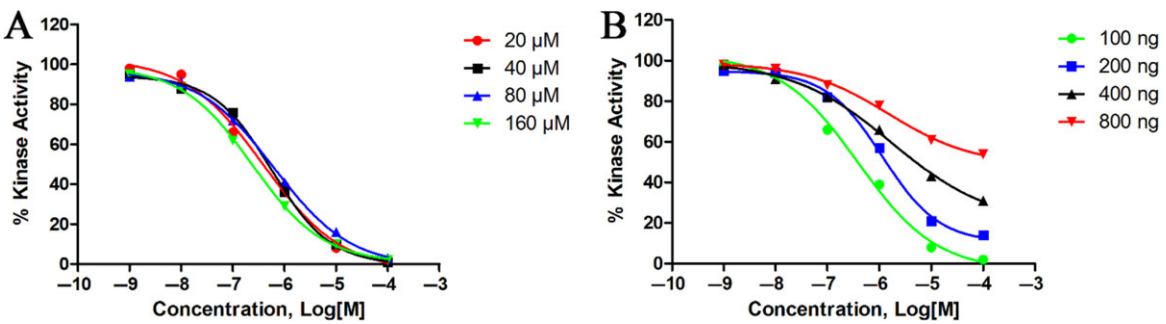


Figure 3. Character of Plk1 inhibition by **5i**. (A) IC_{50} curves for Plk1 with different concentrations of **5i** in the presence of varying concentrations of ATP. (B) IC_{50} curves for Plk1 with different concentrations of **5i** in the presence of varying concentrations of Cdc25C substrate.

Table 2. *In vitro* kinase inhibitory profile of 5i.

Protein kinase	$IC_{50} \pm SD(\mu M)$[a)]	
	5i	Thymoquinone
Plk1	0.68 ± 0.07	2.18 ± 0.31
Plk2	>50	14.72 ± 4.07
Plk3	28.05 ± 5.31	>50
Aurora-A	>50	–
CDK1	21.46 ± 4.78	–
CDK2	>50	–
EGFR	>50	–
Src	>50	–

[a)]Values are the average of three independent experiments.

effects of increasing concentrations of substrate Cdc25C (100, 200, 400, and 800 ng) on the inhibitory activity of the compound in the presence of a constant amount of ATP (100 μM) were examined. It showed that increasing the concentration of the substrate resulted in increased IC_{50} values of the inhibitor (Fig. 3B; IC_{50} curves shift right). Thus, inhibitor **5i** demonstrated the substrate-dependent and ATP-independent nature of inhibition. This result may also confirm indirectly that inhibitor **5i** binds to the binding pocket of Cdc25C rather than the ATP-binding pocket.

We further carried out a specificity test for **5i** against two closely related kinases Plk2 and Plk3 using the protocol described in Section 4. Plk2 and Plk3 were available as recombinant proteins purchased from commercial sources (Abnova, Inc.). As shown in Table 2, thymoquinone showed excellent Plk1 inhibitory selectivity against Plk3 ($IC_{50} > 50$ μM) and moderate inhibitory selectivity against Plk2 ($IC_{50} = 14.72 \pm 4.07$ μM, about sevenfold higher than Plk1 IC_{50}). Compound **5i** showed excellent Plk1 inhibitory selectivity against Plk2 ($IC_{50} > 50$ μM) and Plk3 ($IC_{50} = 28.05 \pm 5.31$ μM, about 40-fold higher than Plk1 IC_{50}). The inhibitory effects of **5i** on five serine/threonine and tyrosine kinases (Aurora-A, CDK1, CDK2, EGFR, and Src) were also examined. These studies showed that at 30-fold higher concentrations ($IC_{50} = 21.46 \pm 4.78$ μM), **5i** exhibits inhibitory activity against one of the other kinases tested, the CDK1. To the other kinases, IC_{50} value of **5i** was more than 50 μM (Table 2). Among these different kinases, **5i** was the most active against Plk1. These results proved that the **5i** was a highly selective inhibitor of Plk1.

In the pursuing of potent and selective Plk1 inhibitors, several series of Plk1 inhibitors (ATP-competitive or non-ATP-competitive) have been reported. Some of these inhibitors show excellent characters as promising candidates.

ON01910 was initially reported to inhibit Plk1 ($IC_{50} = 9$ nM) in a substrate-dependent and an ATP-independent manner [15]. ON01910 has been shown to inhibit the cell proliferation in more than 100 cancer cell lines with IC_{50} values ranging from 50 to 250 nM. This candidate was more potent and selective than **5i**, and the potent cancer cell line proliferative inhibition profiles made it a promising compound. However, later studies showed that ON01910 exhibited little activity against Plk1 *in vitro*. Cell-based phenotypes do not correlate with Plk1 inhibition but are consistent with affecting microtubule dynamics [17].

BI 2536 is a dihydropteridinone developed by Boehringer Ingelheim, which exhibits a potent inhibitory activity against Plk1 *in vitro* ($IC_{50} = 0.83$ nM). This compound shows more than 1000-fold selectivity against a large panel of protein kinases, but it affects the activities of other Plk family members, such as Plk2 ($IC_{50} = 4$ nM) and Plk3 ($IC_{50} = 9$ nM) [18]. BI 6727 is another dihydropteridinone developed by Boehringer Ingelheim. It strongly inhibits Plk1, Plk2, and Plk3 at IC_{50} values of 0.87, 5, and 56 nM, respectively [19, 20]. BI 2536 and BI 6727 were both ATP-competitive Plk1 inhibitors. Compared to **5i**, BI 2536 and BI 6727 were more potent but less selective Plk1 inhibitors. BI 2536 and BI 6727 are now in phase II clinical trials [20, 21].

SBE13 is a type II Plk1 inhibitor, which stabilizes an inactive conformation of the ATP-binding site. SBE13 had subnanomolar inhibitory activity against Plk1 ($IC_{50} = 0.2$ nM) and was found to be selective for Plk1 over Plk2 ($IC_{50} > 66$ μM) or Plk3 ($IC_{50} = 875$ nM) [22], which was more potent and selective than **5i**. However, SBE13 showed moderate proliferative inhibition to HeLa cells ($EC_{50} = 18$ μM) [22].

Conclusion

In this study, a series of benzimidazole and indole-containing compounds were designed as new Plk1 inhibitors through rational design according to the substrate-binding pocket of Plk1. Two compounds showed potent Plk1 inhibitory activity together with potent anti-proliferative activity. The SARs revealed that the electronic density on the phenyl ring was critical for the Plk1 inhibition. The mechanism of action of the optimal compound **5i** was also examined, which suggested that **5i** was an ATP-independent and substrate-dependent Plk1 inhibitor. In the specificity test, **5i** showed excellent Plk1 selectivity against Plk2 and 3, it also showed excellent selectivity against a panel of serine/threonine and tyrosine kinases. Our discovery may provide useful lead compounds for further optimization of non-ATP-competitive Plk1 inhibitors.

Experimental

Chemistry

All reagents were purchased from commercial sources and were used without further purification. Melting points (m.p.) were determined on a Mel-Temp II apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker ACF-300 spectrometer (300 MHz). Chemical shifts are expressed in ppm related to tetramethylsilane. Mass spectra were obtained on either a Mariner Mass Spectrum or a MAT-212 mass spectrometer.

*1-(3-(2-Nitrophenoxyl)propyl)-1H-benzo[d]imidazole (**3a**)*
Benzimidazole (3.0 g, 25.4 mmol), 1-bromo-3-chloropropane (6.0 g, 38.2 mmol), K_2CO_3 (4.2 g, 30.4 mmol) and acetone (30 mL) were mixed and heated to reflux for 12 h. Then, the insoluble was filtered off, the solvent was removed, and the residue was used without further purification. The residue was mixed with 2-nitrophenol (5.22 g, 37.5mmol), KOH (2.1 g, 37.5 mmol), and DMF (45 mL) and the mixture was allowed to stir at 80°C for 12 h. Then, the reaction mixture was diluted with water (50 mL) and extracted with ethyl acetate (50 mL × 3). The combined organic layer was washed with brine (100 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (hexane/EtOAc, 2:1 v/v) to give compound **3** as a yellow oil (2.36 g, 31.8% yield for two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.96–6.92 (m, 9H), 4.55 (t, J = 6.42 Hz, 2H), 3.95 (t, J = 5.46 Hz, 2H), 2.38 (m, 2H) EI-MS (*m/z*): 297 [M].

*1-(3-(2-Nitrophenoxy)propyl)-1H-indole (**3b**)*
Compound **3b** was prepared under similar conditions as described for **3a** (yield 17.7% for two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81–6.43 (m, 10H), 4.35 (t, J = 6.42 Hz, 2H), 3.78 (t, J = 5.55 Hz, 2H), 2.21 (m, 2H). EI-MS (*m/z*): 296 [M].

*1-(3-(2-Aminophenoxyl)propyl)-1H-benzo[d]imidazole (**4a**)*
To a solution of **3** (0.85 g, 2.86 mmol) in methanol (15 mL), 10% Pd-C (0.05 g) was added. The mixture was hydrogenated at normal pressure for 4 h. The insoluble was filtered off and the solvent was removed to gain **4** as a brown oil (0.70 g, yield 91.6%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.92–6.70 (m, 9H), 4.45 (t, J = 6.6 Hz, 2H), 4.00 (t, J = 5.64 Hz, 2H), 3.10 (s, 2H), 2.40 (m, 2H). EI-MS (*m/z*): 267 [M].

*1-(3-(2-Aminophenoxyl)propyl)-1H-indole (**4b**)*
Compound **4b** was prepared under similar conditions as described for **4a** (yield 87.6%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.62–6.48 (m, 10H), 4.37 (t, J = 6.48 Hz, 2H), 3.93 (t, J = 5.67 Hz, 2H), 3.77 (s, 2H), 2.31 (m, 2H). EI-MS (*m/z*): 266 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-benzamide (**5a**)*
To a solution of **4a** (0.20 g, 0.75 mmol) in CH_2Cl_2 (5 mL), TEA (0.11 g, 1.13 mmol) was added. Then, the solution of benzoyl chloride (0.13 g, 0.90 mmol) in CH_2Cl_2 (5 mL) was added in drops. After the addition of benzoyl chloride, the mixture was allowed to stir at room temperature for 2 h. Compound **5a** was obtained after silica gel column chromatography (hexane/EtOAc, 1:1 v/v) as a brown solid (0.21 g, yield 76.9%). m.p. 106–108°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.38 (m, 2H), 7.97–6.75 (m, 13H), 4.35 (t, J = 6.9 Hz, 2H), 4.02 (t, J = 5.79 Hz, 2H), 2.36 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.1, 151.1, 134.4, 131.6, 128.4, 127.5, 126.7, 126.1, 125.1, 124.5, 120.3, 116.4, 112.2, 111.9, 64.4, 42.4, 28.6. EI-MS (*m/z*): 371 [M].

The synthetic methods for the targeted compounds **5b**–**m** were similar to the procedure for the synthesis of **5a**.

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-4-methoxybenzamide (**5b**)*
Yellow solid, yield 90.6%, m.p. 88–90°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.46 (s, 1H), 8.25 (m, 1H), 7.91–6.92 (m, 12H), 4.12 (t, J = 6.9 Hz, 2H), 3.85 (s, 3H), 3.71 (t, J = 5.7 Hz, 2H), 2.61 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.6, 161.8, 151.0, 142.2, 134.7, 131.7, 129.5, 127.0, 126.5, 125.8, 125.0, 124.7, 120.3, 116.2, 113.7, 112.1, 112.0, 64.4, 55.4, 42.6, 28.6. EI-MS (*m/z*): 401 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-4-methylbenzamide (**5c**)*
White solid, yield 83.0%, m.p. 174°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.55 (s, 1H), 9.48 (s, 1H), 7.85–7.02 (m, 12H), 4.63 (t, J = 6.57 Hz, 2H), 4.07 (t, J = 5.19 Hz, 2H), 2.50 (m, 2H), 2.38 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.9, 150.9, 141.5, 131.6, 131.2, 131.0, 129.0, 127.5, 126.9, 126.1, 125.9, 125.7, 125.0, 120.4, 115.0, 112.8, 112.2, 64.4, 43.2, 28.4, 20.9. EI-MS (*m/z*): 385 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-2-methylbenzamide (**5d**)*
Pale pink solid, yield 87.0%, m.p. 234–236°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.58 (s, 2H), 7.94–7.01 (m, 12H), 4.70 (t, J = 6 Hz, 2H), 4.05 (s, 2H), 2.41 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.8, 150.7, 145.4, 141.4, 136.9, 135.3, 131.6, 131.0, 130.5, 129.6, 127.3, 126.6, 126.2, 125.9, 125.7, 125.6, 120.3, 114.9, 112.2, 64.4, 43.3, 28.4, 19.5. EI-MS (*m/z*): 385 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-4-nitrobenzamide (**5e**)*
Green solid, yield 92.8%, m.p. 80–82°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.01 (s, 1H), 9.57 (s, 1H), 8.33 (d, J = 8.67 Hz, 2H), 8.21 (d, J = 8.73 Hz, 2H), 7.85–7.05 (m, 8H), 4.12 (t, J = 6.63 Hz, 2H), 4.09 (t, J = 5.43 Hz, 2H), 2.38 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.6, 151.4, 149.0, 145.7, 141.3, 140.0, 130.9, 130.8, 130.6, 129.2, 126.6, 126.2, 126.1, 125.7, 123.4, 120.3, 114.8, 112.9, 112.4, 64.4, 43.4, 28.3. EI-MS (*m/z*): 416 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-2-nitrobenzamide (**5f**)*
Yellow solid, yield 70.4%, m.p. 116–118°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 10.05 (d, J = 12.69 Hz, 1H), 9.56 (d, J = 12.69 Hz, 1H), 8.34 (d, J = 8.7 Hz, 1H), 8.21 (d, J = 8.73 Hz, 1H), 7.94–7.05 (m, 10H), 4.65 (t, J = 6.69 Hz, 2H), 4.08 (m, 2H), 2.39 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.8, 151.4, 147.7, 141.4, 131.9, 130.9, 127.8, 127.6, 127.0, 126.9, 126.1, 125.9, 125.7, 125.0, 120.4, 114.9, 112.8, 112.2, 64.5, 43.3, 28.3. EI-MS (*m/z*): 416 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-acetamide (**5g**)*
Pale pink solid, yield 42.0%, m.p. 104–106°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.03 (s, 1H), 8.23 (s, 1H), 7.81–6.91 (m, 8H), 4.49 (t, J = 6.87 Hz, 2H), 3.94 (t, J = 5.88 Hz, 2H), 2.28 (m, 2H), 2.11 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.3, 149.1, 144.0, 143.4, 133.7, 127.3, 124.5, 122.9, 122.2, 121.4, 120.2, 119.4, 111.9, 110.2, 64.9, 40.9, 28.9, 20.6. EI-MS (*m/z*): 309 [M].

*N-(2-(3-(1H-Indol-1-yl)propoxy)phenyl)benzamide (**5h**)*
Brown solid, yield 51.5%, m.p. 104–106°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.53 (m, 2H), 7.89–6.48 (m, 14H), 4.36 (t, $J = 6.75$ Hz, 2H), 4.05 (t, $J = 5.88$ Hz, 2H), 2.39 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.6, 146.7, 135.3, 134.8, 131.3, 128.4, 128.1, 127.3, 127.2, 126.4, 123.5, 121.2, 121.0, 120.6, 119.7, 119.0, 110.4, 108.5, 101.2, 64.9, 42.4, 29.4. EI-MS (*m/z*): 392 [M].

*N-(2-(3-(1H-Indol-1-yl)propoxy)phenyl)-4-methoxybenzamide (**5i**)*
Brown solid, yield 46.5%, m.p. 122–124°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.51 (m, 1H), 8.47 (s, 1H), 7.85–6.49 (m, 13H), 4.37 (t, $J = 6.63$ Hz, 2H), 4.05 (t, $J = 5.73$ Hz, 2H), 3.87 (s, 3H), 2.39 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 161.9, 146.6, 128.2, 127.5, 127.2, 126.9, 123.2, 121.2, 121.0, 120.6, 119.6, 119.0, 113.6, 110.4, 108.5, 101.2, 65.0, 54.9, 42.5, 29.4. EI-MS (*m/z*): 422 [M].

*N-(2-(3-(1H-Indol-1-yl)propoxy)phenyl)-2-methylbenzamide (**5j**)*
Brown solid, yield 54.2%, m.p. 118–120°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.53 (s, 1H), 8.07 (s, 1H), 7.61–6.43 (m, 13H), 4.3 (t, $J = 6.66$ Hz, 2H), 3.99 (t, $J = 5.88$ Hz, 2H), 2.56 (s, 3H), 2.32 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.2, 146.7, 136.1, 136.0, 135.3, 131.0, 129.9, 128.1, 127.2, 126.2, 125.6, 123.6, 121.2, 120.9, 120.6, 119.8, 119.0, 110.5, 108.5, 101.1, 64.9, 42.4, 29.3, 19.6. EI-MS (*m/z*): 406 [M].

*N-(2-(3-(1H-Indol-1-yl)propoxy)phenyl)-4-nitrobenzamide (**5k**)*
Brown solid, yield 49.3%, m.p. 124–126°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.46 (d, $J = 3.69$ Hz, 1H), 8.35 (m, 4H), 7.95–6.45 (m, 10H), 4.35 (t, $J = 6.54$ Hz, 2H), 4.09 (t, $J = 5.82$ Hz, 2H), 2.40 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 163.6, 151.6, 149.1, 140.2, 135.5, 130.6, 129.0, 128.5, 128.0, 126.7, 126.2, 125.6, 123.6, 120.8, 120.3, 120.2, 118.8, 112.2, 109.4, 100.5, 64.8, 42.1, 29.4. EI-MS (*m/z*): 415 [M].

*N-(2-(3-(1H-Indol-1-yl)propoxy)phenyl)-2-nitrobenzamide (**5l**)*
Brown solid, yield 46.6%, m.p. 106–108°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.48–8.46 (d, $J = 6.72$ Hz, 1H), 8.38 (s, 1H), 8.34–6.44 (m, 13H), 4.35 (t, $J = 6.48$ Hz, 2H), 4.09 (t, $J = 5.94$ Hz, 2H), 2.41 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 162.4, 149.2, 146.7, 140.0, 130.5, 128.1, 127.5, 127.1, 126.5, 124.3, 123.6, 121.2, 121.0, 120.7, 119.9, 119.1, 110.5, 108.3, 101.3, 65.4, 42.7, 29.3. EI-MS (*m/z*): 415 [M].

*N-(2-(3-(1H-Indol-1-yl)propoxy)phenyl)acetamide (**5m**)*
Brown solid, yield 94.5%, m.p. 64–66°C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.03 (s, 1H), 8.23 (s, 1H), 7.81–6.91 (m, 9H), 4.49 (t, $J = 6.87$ Hz, 2H), 3.94 (t, $J = 5.88$ Hz, 2H), 2.28 (m, 2H), 2.11 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.6, 146.3, 135.3, 128.1, 127.3, 127.1, 123.3, 121.2, 120.8, 120.7, 119.9, 119.0, 110.3, 108.6, 101.1, 65.2, 42.8, 29.2, 24.3. EI-MS (*m/z*): 308 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-benzylaniline (**6a**)*
The mixture of **4a** (0.25 g, 0.9 mmol), benzyl bromide (0.19 g, 1.1 mmol), K_2CO_3 (0.16 g, 1.1 mmol), and acetone (10 mL) was mixed and heated to reflux for 12 h. Then, the insoluble was filtered off, the solvent was removed and the residue was purified using silica gel column chromatography (hexane/EtOAc, 1:1 v/v) to obtain **6a** as a brown oil (0.18 g, yield 53.9%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.80–6.63 (m, 13H), 4.40 (m, 4H), 4.00 (t, $J = 5.64$ Hz, 2H), 2.37 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 142.6, 137.7, 128.1, 127.8, 127.6, 126.8, 126.7, 126.4, 122.7, 121.9, 121.4, 119.7, 116.3, 110.3, 110.1, 109.1, 64.1, 47.5, 41.3, 29.0. EI-MS (*m/z*): 357 [M].

The synthetic methods for the targeted compounds **6b–f** were similar to the procedure for the synthesis of **6a**.

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-4-chlorobenzylaniline (**6b**)*
Brown oil, yield 40.8%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.95 (s, 1H), 7.81–6.53 (m, 12H), 4.42 (t, $J = 6.81$ Hz, 2H), 4.34 (s, 2H), 4.01 (t, $J = 5.67$ Hz, 2H), 2.39 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 144.9, 143.3, 142.6, 137.7, 137.3, 133.2, 132.2, 129.0, 128.2, 128.1, 127.9, 122.6, 121.8, 121.3, 119.9, 116.5, 110.2, 110.1, 109.0, 64.2, 46.8, 41.3, 28.9. EI-MS (*m/z*): 391 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-4-methoxybenzylaniline (**6c**)*
Brown oil, yield 57.3%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.81–6.67 (m, 12H), 4.39 (t, $J = 6.81$ Hz, 2H), 3.99 (t, $J = 5.67$ Hz, 2H), 3.80 (s, 2H), 3.79 (s, 3H), 2.37 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 158.1, 142.6, 130.4, 128.9, 128.2, 122.5, 121.7, 121.4, 119.8, 116.2, 113.6, 113.2, 110.1, 109.1, 64.1, 54.7, 47.0, 41.2, 29.0. EI-MS (*m/z*): 387 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-4-methylbenzylaniline (**6d**)*
Yellow oil, yield 36.7%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.88 (s, 1H), 7.82–6.58 (m, 12H), 4.39 (t, $J = 6.8$ Hz, 2H), 4.35 (s, 2H), 3.99 (t, $J = 5.7$ Hz, 2H), 2.42–2.29 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.0, 143.2, 142.6, 137.7, 136.3, 136.0, 133.2, 128.9, 128.2, 128.0, 126.9, 122.6, 121.7, 121.4, 119.9, 116.2, 110.2, 110.1, 109.1, 64.0, 47.3, 41.2, 29.0, 20.6. EI-MS (*m/z*): 371 [M].

*N-(2-(3-(1H-Benzo[d]imidazol-1-yl)propoxy)phenyl)-4-nitrobenzylaniline (**6e**)*
Yellow oil, yield 55.2%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.20 (s, 1H), 8.17 (s, 1H), 8.03 (m, 1H), 7.80–6.38 (m, 10H), 4.47 (t, $J = 6.33$ Hz, 4H), 4.06 (t, $J = 5.7$ Hz, 2H), 2.44 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 147.2, 145.0, 143.3, 142.6, 136.7, 133.2, 128.2, 127.0, 123.3, 123.1, 122.5, 121.7, 121.3, 119.9, 116.8, 110.2, 110.0, 109.0, 64.5, 46.7, 41.6, 28.9. EI-MS (*m/z*): 402 [M].

*N-(2-(3-(1H-Indol-1-yl)propoxy)phenyl)benzylaniline (**6f**)*
Pale yellow oil, yield 62.3%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.62–6.46 (m, 15H), 4.60 (s, 1H), 4.35(m, 4H), 3.95 (t, $J = 5.67$ Hz, 2H), 2.32 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.4, 139.3, 137.9,

135.6, 128.3, 127.6, 127.0, 126.8, 121.3, 121.2, 120.7, 119.0, 116.4, 110.3, 110.1, 108.9, 101.0, 64.6, 47.7, 42.6, 29.5. EI-MS (*m/z*): 402 [M].

Docking study

Glide was selected as the molecular docking tool. The crystallized structure of Plk1-PBD in complex with thymoquinone (PDB ID: 4HCO) was prepared using the Protein PreparationWizard workflow. Ligand was deleted and a receptor grid was generated on the center of the cocrystallized ligand, which was defined as the ligand-binding site search region. The compounds to be docked were confirmed by an enclosing box that was similar in size to the cocrystallized ligand. Furthermore, the compound set was minimized using the LigPrep module. The best conformation of each compound was output on the basis of the Glide score and interactions formed between the compounds and the active site. Finally, the potential compounds were flexibly docked into the binding site using the extra precision (XP) docking mode. All the remaining parameters were kept as default.

Plk1 enzyme inhibition assay

Following the method reported by by Gumireddy et al. [15], an ADP-Glo kinase assay (Promega) was used to determine the Plk1 inhibition of the designed compounds using thymoquinone as a positive control. Ten nanograms of recombinant Plk1 was incubated with different concentrations of test compounds in a 15 μL reaction mixture (50 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 0.01% NP-40 [pH 7.5]) for 30 min at room temperature. Kinase reactions were performed for 20 min at 30°C in a volume of 20 μL (15 μL enzyme + inhibitor, 2 μL 1 mM ATP), 2 μL of γ^{32}PATP (40 μCi), and 1 μL of recombinant Cdc25C (100 ng) substrates. Reactions were terminated by boiling for 2 min in 20 μL of 2× Laemmli buffer. Phosphorylated substrates were separated by 18% SDS–PAGE. The gels were dried and exposed to X-ray film for 3–10 min.

In vitro cancer cell line proliferation inhibition assay

The tested cells were introduced into each well of a 96-well plate, with a density of 2500 cells/well. The cells were then exposed to compounds of different concentrations (0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 μM) (100 μL/well). Controls were performed in which only culture media was added into wells containing cells. After 72 h incubation, 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (20 μL/well) was added and cultured for 4 h, and then the supernatant was discarded and dimethyl sulfoxide (DMSO) was added in (100 μL/well), respectively. The suspension was placed on micro-vibrator for 5 min and the absorbance (*A*) was measured at 570 nm by the Universal Microplate Reader (EL800, Bio-Tek Instruments, Inc.). Triplicate experiments were performed in a parallel manner for each concentration point and the results were reported and presented as mean. Cell inhibitory ratio was calculated by the following formula:

$$\text{Inhibitory ratio}\,(\%) = \left[\frac{(A_{\text{control}} - A_{\text{treated}})}{A_{\text{control}}}\right] \times 100\%$$

The IC_{50} was taken as the concentration that caused 50% inhibition of cell proliferation.

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