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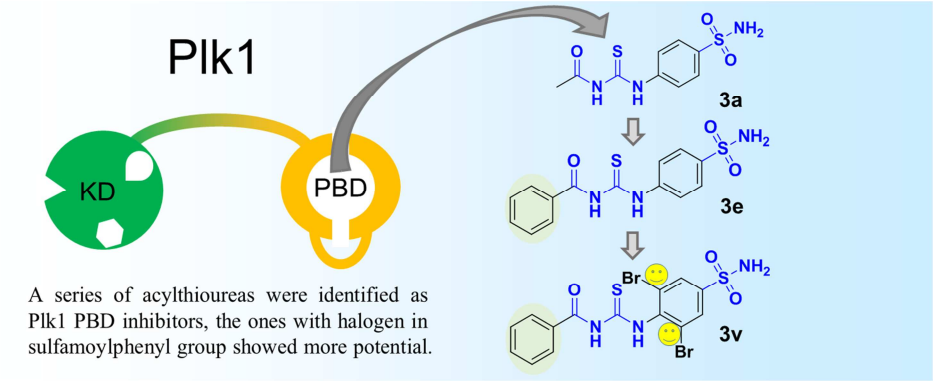

A series of acylthioureas were identified as Plk1 PBD inhibitors, the ones with halogen in sulfamoylphenyl group showed more potential.

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Identification of acylthiourea derivatives as potent Plk1 PBD inhibitors

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ABSTRACT: Thiourea derivatives have drawn much attention for their latent capacities of biological activities. In this study, we designed acylthiourea compounds as polo-like kinase 1 (Plk1) polo-box domain (PBD) inhibitors. A series of acylthiourea derivatives without pan assay interference structure (PAINS) were synthesized. Four compounds with halogen substituents exhibited binding affinities to Plk1 PBD in low micromole range. The most potent compound (**3v**) showed

selectivity over other subtypes of Plk PBDs and inhibited the kinase activity of full-length Plk1.

Keywords: Polo-like kinase 1; Polo-box domain; Small molecular inhibitor; Halogenosulfamoylphenyl acylthiourea; Binding affinity; *In vitro* assay

1. Introduction

Polo-like kinases (Plks) belong to a conserved serine/threonine protein kinases family. Five Plks (Plk1-5) were found in *Homo sapiens*[1]. The structures and functions of Plk4-5 are distinct from Plk1-3[2]. Though with similar structure, Plk1-3 have different roles in the cell cycle. Plk1 participates in mitosis and cytokinesis. Plk2 functions as a tumor suppressor and participates in centriole duplication, G1/S transition[3, 4]. Plk3 is a tumor suppressor and plays an important role in stress response, cell cycle regulation and Golgi disassembly[3, 5]. Among five Plks, Plk1 is the most studied one. Its overexpression was found in a variety of cancer cells[6-9] and correlated with poor prognosis[10]. Plk1 is considered as a promising anti-cancer target and its selective inhibition over other Plks is necessary[11].

Plk1 consists of a highly conserved kinase domain (KD) and a polo-box domain (PBD). KD is the catalytic domain and similar to other protein kinases, while PBD is a unique phosphopeptide-binding module involved in Plk target binding. It also blocks KD to regulate its kinase activity[12]. PBD inhibitors affect the function of Plk1 through blocking the interaction between PBD and its partner[13-17]. According to references[3, 18-23], inhibitors targeting PBD can be divided into two categories: phosphopeptides and small molecules. Phosphopeptide inhibitors showed high affinity and specificity. Their K_d values were ranged from 2.0 nM to 2.5 μM, while

the affinities against Plk2 or Plk3 PBD were two orders of magnitude less[19]. Phosphopeptide mimics were also developed[20]. The phosphate group plays an important role in binding. But phosphate ester, which is with negative charges and prone to be hydrolyzed by intracellular phosphatases, leads to poor cell permeability and proteolytic stability[20]. Reported small molecular PBD inhibitors have different structures (Fig. 1) and may avoid the conundrum of phosphopeptide inhibitors. Thymoquinone and Poloxin were identified through high throughput screening with quinones scaffold[21]; while (-)-epigallocatechin (EGC), containing polyphenol skeleton, was got by combined virtual and experimental screenings[22]. In addition to blocking PBD binding to its partner, PBD inhibitors with terphenyl moiety were reported to inhibit the kinase activity of full-length Plk1 with an IC_{50} value of 151 μM[23]. We also found several small molecule Plk1 PBD inhibitors with diverse scaffolds and some of them could induce mitotic arrest of HeLa cells at low micromolar concentrations[24].

Put **Fig. 1.** Here

Thiourea derivatives have diverse biological properties[25-32], like anti-inflammatory[25], anticancer[26], antimalarial[27], antituberculosis and antithyroid. And some of them are applied in clinical practice: thioacetazone is used in the treatment of tuberculosis[28]; and propylthiouracil is applied to treat hyperthyroidism by decreasing of thyroid hormone[29]. Acylthiourea derivatives have been studied by several research groups. Saeed *et al.* synthesized hybrid molecules containing sulfanilamide and acylthiourea templates as urease inhibitors[30]. Mahdavi *et al.* reported 3-aroyl-1-(4-sulfamoylphenyl)thiourea derivatives as 15-lipoxygenase inhibitors[31]. Singh *et al.* reported N-(phenylcarbamothioyl)benzamide as histone deacetylase 8 activator[32]. Until now, no Plk1 inhibitors with thiourea sub-structure

have been reported. Considering the druggability of thiourea, acylthiourea derivatives may be valuable for developing PBD inhibitors.

In the present study, we tried to design acylthiourea derivatives as potent PBD inhibitors. N-((4-sulfamoylphenyl)carbamothioyl)acetamide (**3a**), derived from screening an in-house chemical library with modest binding affinity to Plk1 PBD *in vitro*, was chosen as the starting molecule. Based on **3a**, a series of acylthiourea derivatives were synthesized. Their binding affinities against Plk1 PBD were tested. Competitive experiments were carried to verify whether the compounds bind to the phosphopeptide binding site. Ligand binding assays were applied versus different Plk subtypes to assure the selectivity. And kinase assays were performed to identify the inhibition against Plk1 kinase activity.

2. Results and discussion

2.1. Chemistry

As pan assay interference structure (PAINS) may interfere in screening technologies and cause false positives[33], non PAINS ones were designed for more effective research. To explore the structure activity relationships, the general formula $R_1CONHCSNHR_2$ was used. Acylthiourea fragment was kept, R_1 was substituted with aliphatic, alicyclic or aromatic hydrocarbons, R_2 was substituted with sulfamoylphenyl or its bioisosteric groups. 24 compounds (**3a-x**) were designed and synthesized. The synthetic route is illustrated in Scheme 1.

Carbonyl isothiocyanate derivatives **2** were got by nucleophilic substitution reaction between commercially available acyl chloride **1** and potassium thiocyanate. Then the title compounds **3** were prepared by addition-elimination reaction. Through

effective combinations of eleven carbonyl isothiocyanate (**2**) and ten commercially aromatic amines, title compounds (**3a-t)** were obtained. **3u-x** contained halogen in the phenyl ring of R_2. Their intermediate aromatic amines ($\mathbf{I_{u-x}}$) were got by different methods (Scheme 2). For halogenated disubstituted intermediates, $\mathbf{I_u}$ and $\mathbf{I_v}$ were got by reacting sulfanilamide with halogen acid in hydrogen peroxide. Iodinated disubstituted sulfanilamide, 4-amino-3,5-diiodobenzenesulfonamide, was also synthesized by reacting sulfanilamide with iodine monochloride in acetic acid[34]. Unfortunately, the corresponding product was not obtained due to steric hindrance effects of iodine atoms. Halogenated monosubstituted sulfanilamides, 4-amino-3-bromobenzenesulfonamide ($\mathbf{I_w}$) was got by reacting sulfanilamide with N-bromosuccinimide; 4-amino-3-iodobenzenesulfonamide ($\mathbf{I_x}$) was prepared by substitution reaction between sulfanilamide and iodine[35].

The yields of **3a-x** range from 10% to 90%. R_1 with aromatic rings usually led to good yields, such as **3e-k**. ^{1}H NMR spectra of **3a-x** showed two single signals both at 11-13 ppm corresponding to the iminos of thiourea, and ^{13}C NMR revealed two signals more than 157 ppm, presented two carbon atoms of acylthiourea fragment, formatting characteristics of this kind of compounds.

Among the title compounds, fourteen of them (**3b-c**, **3k-s**, **3v-x**) have not been reported.

Put **Scheme 1** here

Put **Scheme 2** here

2.2. Biological evaluation

2.2.1. Binding against Plk1

We used microscale thermophoresis (MST) to measure the binding affinities between Plk1 PBD and small molecules. MST is a method developed recently to measure protein-ligand binding by detecting the mobility of molecules in temperature gradients[36]. Phosphopeptide PLHSpT was used as a positive reference according to previous studies[20, 37, 38]. Poloxin was also chosen as another reference.

The modest binding affinity of **3a** to Plk1 PBD is 73.7±6.3 μM (Table 1), indicating the potential of acylthiourea as Plk1 PBD inhibitors. Then synthesized compounds (**3b-x)** have been tested for their interactions with PBD. Ten compounds (**3b-k**) have difference at R_1. Changing R_1 from methyl in **3a** to ethyl (**3b**) or phenyl (**3e**) group did not alter the binding affinities significantly, while the other R_1 groups like cyclopropyl (**3c**) or benzyl (**3d**) deteriorated binding (data not shown). We then studied the influence of R_2 on binding. Moving the sulfonamide group from the para-position to the meta-position (**3l**) destroyed binding and substituents on the sulfonamide (**3m-q**) also deteriorated binding (data not shown). Starting from **3e**, we further tested halogen substituents in the sulfamoylphenyl group at R_2 (**3u-x**). Binding affinities of all the four compounds were significantly improved with one order of magnitude. Mono-substitutions (**3w-x**) improved binding significantly and di-substitutions (**3u-v**) were much stronger. The dissociation constants of the four compounds are all at single-digit micromole, stronger than PLHSpT (K_d = 14.0±1.3 μM) and Poloxin (K_d = 10.5 ± 0.9 μM) under our assay condition. The dibromosubstituted compound, **3v**, showed the strongest binding affinity with a K_d of 2.3±0.1 μM.

Put **Table 1.** here

We further tested the inhibition of **3v** to Plk1 PBD by fluorescence polarization assay. **3v** (IC_{50} = 39.8 ± 3.5 μM) blocked the binding of fluorescein-labeled phosphopeptide to Plk1 PBD (Fig. S2) and its inhibition is comparable to Poloxin (IC_{50} = 29.4 ± 5.8 μM) under our assay condition.

To verify the binding site of the compounds, the binding affinity of **3v** was measured in presence of PLHSpT (with a concentration of 240 μM) by MST. The thermophoresis signal dropped greatly (Fig. S3), suggesting that **3v** competes with the phosphopeptide for the same binding site in PBD.

*2.2.2. Selectivity of **3v** against other Plk family members*

Selective inhibiting Plk1 hopes to get anticancer drugs. We test the selectivity of **3v**: ligand binding assays were applied against Plk2 and Plk3 PBDs. Primary amines (Lys or Arg) of different Plk subtypes were labeled to form stable dye-protein-conjugates for fluorescence detection with MST. No significant signal changes and concentration dependence were observed, indicating that **3v** is highly selective and does not bind with Plk2 (Fig. S4A) or Plk3 PBD (Fig. S4B).

2.2.3. Inhibition of full-length Plk1

Whether **3v** can inhibit the kinase activity of full-length Plk1 or not was tested, with *in vitro* kinase assay by detecting the formation of ADP. The IC_{50} value of **3v** is 95±21 μM (Fig. 2), which is better than the most effective PBD inhibitor reported by Mita *et al.* (IC_{50} = 151 μM)[23]. This suggests that **3v** not only binds to PBD affecting its interaction with phosphopeptide, but also inhibits the kinase activity of full-length Plk1. Molecular mechanism of these dual activities needs to be further explored.

Put **Fig. 2.** Here

2.3. Molecular docking study

In the complex structure of Plk1 PBD with PLHSpT (PDB code: 3HIK)[39], the minimal phosphopeptide occupies two pockets of PBD: its SpT motif acts as an anchor to occupy the phosphate-binding pocket (His538/Lys540), and the Pro residue occupies the pyrrolidine-binding pocket (Trp414/Arg516/Phe535)[20, 39].

Compound **3a** occupies the same binding sites, both phosphate-binding pocket and pyrrolidine-binding pocket, as PLHSpT does. In the binding mode of **3a**, acylthiourea fragment plays an important role. The imino groups of thiourea form hydrogen bonds to the main-chain oxygen atom of Leu491. And the sulfur atom of thiourea forms a hydrogen bond to the side-chain of the positively charged residue Lys540. For the sulfanilamide moiety, its amino group of R_2 forms a hydrogen bond to the main-chain oxygen atom of Trp414 (Fig. 3A). There is still unoccupied space between phosphate-binding pocket and pyrrolidine-binding pocket (Fig. 3B). Elongation of compound to occupy this space may improve its binding to PBD.

The binding poses of our synthesized compounds are similar in modelling. Compounds **3e** and **3v** are analyzed as examples (Fig. 3C). Their hydrogen-bonding interactions are similar: both compounds contact to Trp414, Leu491 and Lys540; and their phenyl ring of sulfonamide moiety at R_2 both forms a T-shape π-π stacking with Trp414.

However, in experiment, the binding affinities of our compounds are different: the ones with halogen in R_2 (**3u-x**) are higher than the others (such as **3a** and **3e**). For the unique properties of **3v**, one bromine atom might form a halogen bond with the side-chain indole of Trp414, meanwhile the halogen atom occupies the unoccupied space between phosphate-binding pocket and pyrrolidine-binding pocket. The other

bromine atom might form another halogen bond with the main-chain amide hydrogen atom of Leu491 (Fig. 3D). It is concluded that halogen atoms have crucial effects on binding abilities. Chemical properties of halogen atoms have been reported to render halogen bonding which is useful in drug design[40]. Sometimes halogen bonding plays an important role in molecular recognition with comparable energetic contribution as hydrogen bonding[39].

Put **Fig. 3.** Here

3. Conclusion

In this study, we explored the possibility of using acylthiourea derivatives as potent Plk1 PBD inhibitors. The starting compound, N-((4-sulfamoylphenyl)carbamothioyl)acetamide (**3a**) showed moderate binding strength to Plk1 PBD. Based on **3a**, we designed a series of derivatives and tested their binding affinities to Plk1 PBD. The important optimizations were the addition of functional group in the phenyl ring of R_2 to occupy the vacant space between phosphate-binding pocket and pyrrolidine-binding pocket, and introducing halogen atoms to form the halogen bonds. Both lead to the binding affinities increased. The best compound **3v** binds to Plk1 PBD with 30-fold higher affinity than **3a** and 5-fold higher affinity than PLHSpT. **3v** is proved with selectivity over Plk2 and Plk3 PBDs and also inhibited full-length Plk1 kinase activity. Being a dual functional compound, **3v** can be further optimized for its activities and used as a molecular probe to study the regulatory mechanism of Plk1.

4. Experimental section

4.1. Synthesis

The reagents and solvents were commercially available and purified according to conventional methods. Melting points were determined on an X-4 microscopic melting point apparatus and uncorrected. ^{1}H spectra were recorded at 400 MHz and ^{13}C spectra were recorded at 100 MHz on a Bruker Ascend spectrometer. The chemical shift values (δ) are reported in parts per million (ppm) relative to tetramethylsilane as internal standard in DMSO-d6. ^{1}H spectra in methanol-d4 were recorded furthermore to assign active hydrogens of some compounds. High resolution mass spectra (HRMS) were obtained on a Bruker Apex IV FTMS mass spectrometer using electrospray ionization (ESI). Elemental analyses were performed on an Elementar Vario EL CUBE instrument. Analyses of representative compounds indicated within ± 0.4 % of the theoretical values (C, H, N).

4.1.1. General procedure for the synthesis of compounds $\boldsymbol{I_{u\text{-}x}}$

4.1.1.1. 4-amino-3,5-dichlorobenzenesulfonamide ($\boldsymbol{I_u}$*).*

Sulfanilamide (0.43 g, 0.0025 mol) was stirred in 15 mL of 6 N halogen acid (0.08 mol) at room temperature, and 2 ml of 30% hydrogen peroxide (0.02 mol) was slowly added. After 5 h, the product was filtered and recrystallized from ethanol. Pink solid. Yield: 70%. ^{1}H NMR (DMSO-d6, 400MHz): δ 7.61 (s, 2H, ArH), 7.23 (s, 2H, SO_2NH_2), 6.29 (s, 2H, NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 144.2, 131.3, 125.7, 117.0.

4.1.1.2. 4-amino-3,5-dibromobenzenesulfonamide ($\boldsymbol{I_v}$*).*

Sulfanilamide (0.43 g, 0.0025 mol) was stirred in 15 mL of 6 N halogen acid (0.08 mol) at room temperature, and 2 ml of 30% hydrogen peroxide (0.02 mol) was slowly added. After 5 h, the product was filtered and recrystallized from ethanol. White solid.

Yield: 62%. ^{1}H NMR (DMSO-d6, 400MHz): δ 7.79 (s, 2H, ArH), 7.26 (s, 2H, SO_2NH_2), 6.11 (s, 2H, NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 145.8, 132.7, 129.5, 106.1.

*4.1.1.3. 4-amino-3-bromobenzenesulfonamide (**I_w**).*

4-amino-benzenesulfonamide (1 g, 5.81 mmol) was stirred in DMF (5 mL) at 0 ^{o}C, and 1.03 g NBS (5.81 mmol) was added. Stirred for 1 h, the mixture was quenched by water. The product was filtered and recrystallized from ethanol. White solid. Yield: 74%. ^{1}H NMR (DMSO-d6, 400MHz): δ 7.76 (m, 1H, ArH), 7.74 (dd, J = 8.5, 2.1 Hz, 1H, ArH), 7.07 (s, 2H, SO_2NH_2), 6.82 (d, J = 8.5 Hz, 1H, ArH), 6.04 (s, 2H, NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 148.8, 131.7, 130.2, 126.3, 114.0, 105.5.

*4.1.1.4. 4-amino-3-iodobenzenesulfonamide (**I_x**).*

4-amino-benzenesulfonamide (1 g, 5.81 mmol) was dissolved in glacial acetic acid (5 mL), and 0.65 g iodine (2.56 mmol) was added. After being stirred at 85 ^{o}C for 1 h, sodium bisulfite (0.16 g, 1.54 mmol) was added to destroy the excess iodine. 10 mL water was added. The product was filtered and recrystallized from ethanol. Silver solid. Yield: 54%. ^{1}H NMR (DMSO-d6, 400 MHz): δ 7.94 (d, J = 2.1 Hz, 1H, ArH), 7.48 (dd, J = 8.5, 2.1 Hz, 1H, ArH), 7.05 (s, 2H, SO_2NH_2), 6.77 (d, J = 8.5 Hz, 1H, ArH), 5.93 (s, 2H, NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 151.5, 136.5, 132.2, 127.0, 112.8, 80.7.

*4.1.2. General procedure for the synthesis of compounds **3a-x***

A solution of acyl chloride (5 mmol) and potassium thiocyanate (5.5 mmol) in acetone (20 mL) was reacted under reflux for 1 h. Aromatic amine (5 mmol) was added and the mixture was stirred under reflux for 2 h. The reaction mixture was

cooled to room temperature. The precipitate was filtered, washed with water and recrystallized from ethanol.

*4.1.2.1. N-((4-sulfamoylphenyl)carbamothioyl)acetamide (**3a**).* White solid. Yield: 60%. m.p. 238-240 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.63 (s, 1H, NH), 11.61 (s, 1H, NH), 7.84 (m, 4H, ArH), 7.40 (s, 2H, SO_2NH_2), 2.17 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.1, 172.9, 141.0, 140.6, 126.2, 124.3, 23.8. HRMS (ESI): calcd for $C_9H_{11}N_3O_3S_2$, [$(M+H)^+$], 274.0315, found 274.0314.

*4.1.2.2. N-((4-sulfamoylphenyl)carbamothioyl)propionamide (**3b**).* White solid. Yield: 67%. m.p. 206-208 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.66 (s, 1H, NH), 11.56 (s, 1H, NH), 7.84 (m, 4H, ArH), 7.38 (s, 2H, SO_2NH_2), 2.48 (m, 2H, CH_2), 1.06 (t, J = 7.5 Hz, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.1, 176.3, 141.0, 140.7, 126.2, 124.3, 29.2, 8.5. HRMS (ESI): calcd for $C_{10}H_{13}N_3O_3S_2$, [$(M+H)^+$], 288.0471, found 288.0474.

*4.1.2.3. N-((4-sulfamoylphenyl)carbamothioyl)cyclopropanecarboxamide (**3c**).* White solid. Yield: 45%. m.p. 229-231 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.66 (s, 1H, NH), 11.91 (s, 1H, NH), 7.84 (m, 4H, ArH), 7.37 (s, 2H, SO_2NH_2), 2.12 (td, J = 7.9, 4.0 Hz, 1H, CH), 0.97 (m, 4H, CH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 178.7, 175.9, 141.2, 140.7, 126.2, 124.1, 14.2, 9.4. HRMS (ESI): calcd for $C_{11}H_{13}N_3O_3S_2$, [$(M+H)^+$], 300.0471, found 300.0468.

*4.1.2.4. 2-phenyl-N-((4-sulfamoylphenyl)carbamothioyl)acetamide (**3d**).* Pale yellow solid. Yield: 71%. m.p. 222-224 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.53 (s, 1H, NH), 11.82 (s, 1H, NH), 7.82 (s, 4H, ArH), 7.33 (m, 7H, ArH and SO_2NH_2), 3.82 (s, 2H, CH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.1, 173.2, 141.3, 140.6, 134.1,

129.5, 128.4, 127.0, 126.2, 124.3, 42.4. HRMS (ESI): calcd for $C_{15}H_{15}N_3O_3S_2$, [$(M+H)^+$], 350.0628, found 350.0629.

*4.1.2.5. N-((4-sulfamoylphenyl)carbamothioyl)benzamide (**3e**).* Pale yellow solid. Yield: 56%. m.p. 218-220 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.71 (s, 1H, NH), 11.70 (s, 1H, NH), 7.99 (m, 2H, ArH), 7.92 (m, 2H, ArH), 7.85 (m, 2H, ArH), 7.67 (m, 1H, ArH), 7.55 (m, 2H, ArH), 7.40 (s, 2H, SO_2NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.4, 168.2, 141.3, 140.9, 133.2, 132.1, 128.7, 128.5, 126.3, 124.3. HRMS (ESI): calcd for $C_{14}H_{13}N_3O_3S_2$, [$(M+H)^+$], 336.0471, found 336.0473.

*4.1.2.6. 4-methyl-N-((4-sulfamoylphenyl)carbamothioyl)benzamide (**3f**).* White solid. Yield: 54%. m.p. 227-229 °C. ^{1}H NMR (400MHz, DMSO): 12.76 (s, 1H, NH), 11.59 (s, 1H, NH), 7.89 (m, 6H, ArH), 7.37 (m, 4H, ArH and SO_2NH_2), 2.40 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.4, 168.1, 144.0, 141.1, 140.9, 129.2, 128.9, 128.6, 126.3, 124.4, 21.1. HRMS (ESI): calcd for $C_{15}H_{15}N_3O_3S_2$, [$(M+H)^+$], 350.0628, found 350.0626.

*4.1.2.7. 3-methyl-N-((4-sulfamoylphenyl)carbamothioyl)benzamide (**3g**).* Pale yellow solid. Yield: 71%. m.p. 215-217 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.73 (s, 1H, NH), 11.61 (s, 1H, NH), 7.88 (m, 5H, ArH), 7.78 (d, J = 7.7 Hz, 1H, ArH), 7.47 (m, 2H, ArH), 7.39 (s, 2H, SO_2NH_2), 2.40 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.4, 168.4, 141.1, 140.9, 138.1, 133.9, 131.8, 128.9, 128.5, 126.3, 125.7, 124.5, 20.7. HRMS (ESI): calcd for $C_{15}H_{15}N_3O_3S_2$, [$(M+H)^+$], 350.0628, found 350.0627.

*4.1.2.8. 4-nitro-N-((4-sulfamoylphenyl)carbamothioyl)benzamide (**3h**).* Pale yellow solid. Yield: 48%. m.p. 208-210 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.48 (s, 1H, NH), 12.05 (s, 1H, NH), 8.36 (d, J = 8.0 Hz, 2H, ArH), 8.18 (d, J = 8.2 Hz, 2H, ArH), 7.87 (m, 4H, ArH), 7.40 (s, 2H, SO_2NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.1,

166.6, 149.8, 141.4, 140.8, 138.0, 130.3, 126.3, 124.4, 123.4. HRMS (ESI): calcd for $C_{14}H_{12}N_4O_5S_2$, [(M+H)$^+$], 381.0322, found 381.0325.

*4.1.2.9. 3-nitro-N-((4-sulfamoylphenyl)carbamothioyl)benzamide (**3i**).* Pale yellow solid. Yield: 50%. m.p. 214-216 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.52 (s, 1H, NH), 12.12 (s, 1H, NH), 8.79 (s, 1H, ArH), 8.50 (d, J = 8.3 Hz, 1H, ArH), 8.38 (d, J = 7.3 Hz, 1H, ArH), 7.87 (m, 5H, ArH), 7.40 (s, 2H, SO_2NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.2, 166.2, 147.4, 141.4, 140.8, 135.1, 133.8, 130.2, 127.4, 126.3, 124.4, 123.7. HRMS (ESI): calcd for $C_{14}H_{12}N_4O_5S_2$, [(M+H)$^+$], 381.0322, found 381.0326.

*4.1.2.10. 4-[3-(Furan-2-carbonyl)-thioureido]-benzenesulfonamide (**3j**).* White solid. Yield: 56%. m.p. 208-210 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.46 (s, 1H, NH), 11.44 (s, 1H, NH), 8.09 (s, 1H, ArH), 7.87 (m, 5H, ArH), 7.39 (s, 2H, SO_2NH_2), 6.78 (dd, J = 3.4, 1.4 Hz, 1H, ArH). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.0, 157.5, 148.6, 144.6, 141.3, 140.9, 126.2, 124.4, 118.8, 112.7. HRMS (ESI): calcd for $C_{12}H_{11}N_3O_4S_2$, [(M+H)$^+$], 326.0264, found 326.0261.

*4.1.2.11. N-((4-sulfamoylphenyl)carbamothioyl)-1-naphthamide (**3k**).* White solid. Yield: 86%. m.p. 219-221 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.75 (s, 1H, NH), 12.10 (s, 1H, NH), 8.20 (dd, J = 43.9, 8.2 Hz, 2H, ArH), 7.94 (m, 6H, ArH), 7.64 (m, 3H, ArH), 7.42 (s, 2H, SO_2NH_2). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.3, 169.9, 141.3, 140.9, 133.0, 131.6, 131.3, 129.5, 128.5, 127.4, 127.2, 126.5, 126.3, 124.8, 124.4, 122.4. HRMS (ESI): calcd for $C_{18}H_{15}N_3O_3S_2$, [(M+H)$^+$], 386.0628, found 386.0630.

*4.1.2.12. N-((3-sulfamoylphenyl)carbamothioyl)acetamide (**3l**).* Pink solid. Yield: 33%. m.p. 207-209 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.58 (s, 1H, NH), 11.60

(s, 1H, NH), 8.11 (t, *J* = 2.0 Hz, 1H, ArH), 7.83 (m, 1H, ArH), 7.69 (m, 1H, ArH), 7.59 (t, *J* = 7.9 Hz, 1H, ArH), 7.44 (s, 2H, SO_2NH_2), 2.17 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): *δ* 179.2, 172.8, 144.5, 138.2, 129.3, 127.6, 123.3, 121.4, 23.8. HRMS (ESI): calcd for $C_9H_{11}N_3O_3S_2$, $[(M+H)^+]$, 274.0315, found 274.0320.

*4.1.2.13. N-((4-(N-carbamimidoylsulfamoyl)phenyl)carbamothioyl)acetamide (**3m**).* White solid. Yield: 45%. m.p. 239-241 °C. ^1H NMR (DMSO-d6, 400MHz): *δ* 12.61 (s, 1H, NH), 11.58 (s, 1H, NH), 7.78 (m, 4H, ArH), 6.73 (br, 4H, guanidylH), 2.16 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): *δ* 178.9, 172.8, 158.1, 141.8, 140.1, 126.1, 123.9, 23.8. HRMS (ESI): calcd for $C_{10}H_{13}N_5O_3S_2$, $[(M+H)^+]$, 316.0533, found 316.0532.

*4.1.2.14. N-((4-(N-acetylsulfamoyl)phenyl)carbamothioyl)acetamide (**3n**).* White solid. Yield: 20%. m.p. 219-221 °C. ^1H NMR (DMSO-d6, 400MHz): *δ* 12.73 (s, 1H, NH), 12.12 (s, 1H, NH), 11.64 (s, 1H, NH), 7.92 (m, 4H, ArH), 2.17 (s, 3H, CH_3), 1.94 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): *δ* 179.0, 172.9, 169.2, 142.2, 135.9, 128.3, 124.0, 23.8, 23.1. HRMS (ESI): calcd for $C_{11}H_{13}N_3O_4S_2$, $[(M+H)^+]$, 316.0420, found 316.0424.

*4.1.2.15. N-((4-(N-(thiazol-2-yl)sulfamoyl)phenyl)carbamothioyl)acetamide (**3o**).* Pale yellow solid. Yield: 35%. m.p. 230-232 °C. ^1H NMR (DMSO-d6, 400MHz): *δ* 12.79 (s, 1H, NH), 12.64 (s, 1H, NH), 11.59 (s, 1H, NH), 7.82 (m, 4H, ArH), 7.27 (d, *J* = 4.6 Hz, 1H, ArH), 6.85 (d, *J* = 4.6 Hz, 1H, ArH), 2.16 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): *δ* 179.0, 172.9, 168.9, 140.9, 139.2, 126.4, 124.4, 124.3, 108.4, 23.8. HRMS (ESI): calcd for $C_{12}H_{12}N_4O_3S_3$, $[(M+H)^+]$, 357.0144, found 357.0140.

*4.1.2.16. N-((4-(N-(pyrimidin-2-yl)sulfamoyl)phenyl)carbamothioyl)acetamide (**3p**).* White solid. Yield: 60%. m.p. 247-249 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.69 (s, 1H, NH), 11.88 (s, 1H, NH), 11.61 (s, 1H, NH), 8.52 (d, J = 4.9 Hz, 2H, ArH), 7.95 (m, 4H, ArH), 7.07 (t, J = 4.9 Hz, 1H, ArH), 2.16 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 178.9, 172.9, 158.3, 156.6, 141.6, 137.2, 128.2, 123.8, 115.7, 23.7. HRMS (ESI): calcd for $C_{13}H_{13}N_5O_3S_2$, [$(M+H)^+$], 352.0533, found 352.0528.

4.1.2.17.

*N-((4-(N-(3,4-dimethylisoxazol-5-yl)sulfamoyl)phenyl)carbamothioyl)acetamide (**3q**).* White solid. Yield: 54%. m.p. 211-213 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.71 (s, 1H, NH), 11.64 (s, 1H, NH), 11.09 (s, 1H, NH), 7.94 (m, 2H, ArH), 7.77 (m, 2H, ArH), 2.17 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 1.64 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 179.0, 172.9, 161.7, 155.2, 142.0, 136.7, 127.3, 124.4, 105.4, 23.8, 10.2, 5.7. HRMS (ESI): calcd for $C_{14}H_{16}N_4O_4S_2$, [$(M+H)^+$], 369.0686, found 369.0686.

*4.1.2.18. N-((4-hydroxyphenyl)carbamothioyl)acetamide (**3r**).* Brown solid. Yield: 20%. m.p. 222-224 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.26 (s, 1H, NH), 11.37 (s, 1H, NH), 9.55 (s, 1H, OH), 7.34 (m, 2H, ArH), 6.76 (m, 2H, ArH), 2.14 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 178.7, 172.7, 155.7, 129.1, 125.8, 115.1, 23.8. HRMS (ESI): calcd for $C_9H_{10}N_2O_2S$, [$(M+H)^+$], 211.0536, found 211.0539.

*4.1.2.19. 4-(3-acetylthioureido)benzamide (**3s**).* Pale yellow solid. Yield: 10%. m.p. 240-242 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.64 (s, 1H, NH), 11.56 (s, 1H, NH), 7.99 (s, 1H, CONH), 7.89 (m, 2H, ArH), 7.75 (m, 2H, ArH), 7.39 (s, 1H, CONH), 2.17 (s, 3H, CH_3). ^{1}H NMR (methanol-d4, 400MHz): δ 7.88 (d, J = 3.6 Hz, 4H, ArH), 2.17 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 178.7, 172.9, 167.5, 140.3,

131.5, 128.0, 123.4, 23.8. HRMS (ESI): calcd for $C_{10}H_{11}N_3O_2S$, [$(M+H)^+$], 238.0645, found 238.0645.

*4.1.2.20. N-((4-acetamidophenyl)carbamothioyl)benzamide (**3t**).* White solid. Yield: 90%. m.p. 211-213 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.53 (s, 1H, NH), 11.53 (s, 1H, NH), 10.04 (s, 1H, NH), 7.98 (d, J = 7.0 Hz, 2H, ArH), 7.57 (m, 7H, ArH), 2.06 (s, 3H, CH_3). ^{13}C NMR (DMSO-d6, 100MHz): δ 178.9, 168.3, 168.2, 137.5, 133.1, 132.8, 132.2, 128.6, 128.4, 124.8, 119.0, 24.0. HRMS (ESI): calcd for $C_{16}H_{15}N_3O_2S$, [$(M+H)^+$], 314.0958, found 314.0959.

*4.1.2.21. N-((2,6-dichloro-4-sulfamoylphenyl)carbamothioyl)benzamide (**3u**).* White solid. Yield: 52%. m.p. 222-224 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.10 (s, 1H, NH), 11.98 (s, 1H, NH), 8.03 (d, J = 7.4 Hz, 2H, ArH), 7.92 (s, 2H, ArH), 7.70 (m, 3H, ArH and SO_2NH_2), 7.56 (t, J = 7.7 Hz, 2H, ArH). ^{1}H NMR (methanol-d4, 400MHz): δ 7.99 (m, 4H, ArH), 7.68 (t, J = 7.4 Hz, 1H, ArH), 7.56 (t, J = 7.7 Hz, 2H, ArH). ^{13}C NMR (DMSO-d6, 100MHz): δ 180.9, 168.0, 144.6, 137.8, 134.6, 133.4, 131.8, 128.8, 128.5, 125.4. HRMS (ESI): calcd for $C_{14}H_{11}Cl_2N_3O_3S_2$, [$(M+H)^+$], 403.9692, found 403.9693.

*4.1.2.22. N-((2,6-dibromo-4-sulfamoylphenyl)carbamothioyl)benzamide (**3v**).* White solid. Yield: 35%. m.p. 232-234 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.16 (s, 1H, NH), 11.98 (s, 1H, NH), 8.05 (m, 4H, ArH), 7.70 (m, 3H, ArH and SO_2NH_2), 7.56 (t, J = 7.8 Hz, 2H, ArH). ^{13}C NMR (DMSO-d6, 100MHz): δ 180.5, 168.0, 145.0, 140.4, 133.4, 131.7, 128.9, 128.8, 128.5, 124.8. HRMS (ESI): calcd for $C_{14}H_{11}Br_2N_3O_3S_2$, [$(M+H)^+$], 491.8681, found 491.8673.

*4.1.2.23. N-((2-bromo-4-sulfamoylphenyl)carbamothioyl)benzamide (**3w**).* White solid. Yield: 74%. m.p. 226-227 °C. ^{1}H NMR (DMSO-d6, 400MHz): δ 12.70 (s, 1H,

NH), 11.96 (s, 1H, NH), 8.12 (m, 2H, ArH), 8.01 (d, *J* = 7.6 Hz, 2H, ArH), 7.86 (dd, *J* = 8.4, 2.1 Hz, 1H, ArH), 7.69 (t, *J* = 7.8 Hz, 1H, ArH), 7.56 (m, 4H, ArH and SO_2NH_2). ^{1}H NMR (methanol-d4, 400MHz): δ 8.46 (d, *J* = 8.6 Hz, 1H, ArH), 8.20 (d, *J* = 2.0 Hz, 1H, ArH), 7.99 (m, 2H, ArH), 7.90 (dd, *J* = 8.6, 2.0 Hz, 1H, ArH), 7.68 (t, *J* = 7.5 Hz, 1H, ArH), 7.57 (t, *J* = 7.7 Hz, 2H, ArH). ^{13}C NMR (DMSO-d6, 100MHz): *δ* 180.4, 168.5, 143.3, 140.0, 133.3, 131.8, 129.8, 129.0, 128.8, 128.5, 125.1, 119.4. HRMS (ESI): calcd for $C_{14}H_{12}BrN_3O_3S_2$, [$(M+H)^+$], 413.9576, found 413.9575.

*4.1.2.24. N-((2-iodo-4-sulfamoylphenyl)carbamothioyl)benzamide (**3x**).* White solid. Yield: 72%. m.p. 230-232 °C. ^{1}H NMR (DMSO-d6, 400MHz): *δ* 12.52 (s, 1H, NH), 11.92 (s, 1H, NH), 8.31 (s, 1H, ArH), 8.02 (d, *J* = 7.5 Hz, 2H, ArH), 7.87 (s, 2H, ArH), 7.69 (t, *J* = 7.4 Hz, 1H, ArH), 7.59 (m, 4H, ArH and SO_2NH_2). ^{13}C NMR (DMSO-d6, 100MHz): *δ* 180.6, 168.4, 143.5, 143.4, 135.8, 133.4, 131.8, 129.0, 128.8, 128.5, 125.7, 97.7. HRMS (ESI): calcd for $C_{14}H_{12}IN_3O_3S_2$, [$(M+H)^+$], 461.9438, found 461.9433.

4.2. Biology

4.2.1. Expression and purification of PBDs

Protein expression and purification were performed similar as previously described[24]. *Escherichia coli* strain Rosetta (DE3) carried plasmids were grown until the OD_{600} reached 0.6, and introduced with IPTG (final concentration 0.4 mM). The cells were grown overnight at 20 °C.

Cells were resuspended and then lysed in buffer contained 20 mM HEPES, pH 7.4, 300 mM NaCl, 0.1 mM PMSF and 1 mM DDT. The cell lysates were clarified. The supernatants of Plk1 PBD were filtered and then applied to nickel column (GE

Healthcare) and further purified by Sephacryl S200 gel-filtration column (GE Healthcare) that had been equilibrated with buffer contained 20 mM HEPES, pH 7.4, 300 mM NaCl. The supernatants of Plk2 PBD, Plk3 PBD were filtered, applied to GSTrap column (GE Healthcare) and further purified by Sephacryl S200 gel-filtration column (GE Healthcare) respectively. The final purities of PBDs were more than 90%.

4.2.2. Expression and purification of full-length Plk1

Protein expression and purification were performed similar as previously described[24]. *Escherichia coli* strain Rosetta (DE3) carried plasmids were grown until the OD_{600} reached 0.6, and introduced with IPTG (final concentration 0.6 mM). The cells were grown overnight at 25 °C for 8 h.

Cells were resuspended and then lysed in buffer contained 40 mM MOPS, pH 7.4, 200 mM NaCl, 0.1 mM PMSF and 1 mM DDT. The cell lysates were clarified. The supernatants were filtered and then applied to nickel column (GE Healthcare) and further purified by Sephacryl S200 gel-filtration column (GE Healthcare) that had been equilibrated with buffer contained 40 mM MOPS, pH 7.4, 200 mM NaCl. The final purity of full-length Plk1 was more than 90%.

4.2.3. Microscale thermophoresis based ligand binding assay

The peptide of PLHSpT (purity judged by HPLC > 99%) was from GL Biochem (Shanghai) Ltd. Poloxin (purity > 98%) was purchased from MedChem Express.

According to the manufacturer's protocol the PBD of Plks was labeled with the red fluorescent dye NT-647. Measurements were carried out in the solution contained 20 mM HEPES, pH 7.4, 300 mM NaCl, 0.05% tween-20, 5% DMSO, by using 20%

LED power and 20% MST power. The concentration of PBD was adjusted by the fluorescence and was about 0.2 μM. DMSO was used as negative control.

Nanotemper Analysis software V1.5.41 was applied to analyze data.

4.2.4. Fluorescence polarization assay

The fluorescein-labeled peptide of 5-FAM-DPPLHSpTAI-OH (purity judged by HPLC > 96%) was from GL Biochem (Shanghai) Ltd.

The FP assay procedure was based on the reference with slight modifications[37]. Fluorescein-labeled peptide (final concentration: 20 nM) was incubated with Plk1 PBD (final concentration: 300 nM). Final concentrations of the buffer were as follows: 10 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.1% Nonidet P-40 and 5% DMSO. Fluorescence polarization was analyzed 1 h after mixing all components in the 96-well format at 25 °C. Assays were run as triplicates. Results were monitored with micro-plate reader Synergy4 (BIOTEK).

4.2.5. Kinase assay

Kinase assay was performed as previously described[41]. Amplite^{TM} Universal Fluorimetric Kinase Assay Kit from AAT Bioquest Inc. was applied to measure kinase activity according to the manufacturer's protocols. The kit monitors kinase activity based on ADP formation. 2 μL peptide of ALMDASFADQ was mixed with 2 μL Plk1, then 16 μL kinase buffer containing ATP and compound was added. The final concentration of buffer components was 0.34 μM Plk1, 83 μM ALMDASFADQ, 200 μM ATP and 5% DMSO. The mixture was incubated at 30 °C for 40 min. For detection of kinase activity of Plk1, 20 μL of ADP Sensor Buffer and 10 μL of ADP Sensor were added and incubated at 30 ^{o}C for 40 min. Finally, fluorescence was read

with excitation (Ex)/emission (Em) = 540 nm/590 nm. Assays were run as triplicates. IC_{50} values were fitted by Hill equation with OriginPro 8.

4.3. Molecular modeling

The crystal structure of Plk1 PBD-PLHSpT complex (PDB code: 3HIK[39]) was used for the docking study. The peptide was removed from the structure first. Cavity V1.1[42, 43] was used to analysis the binding sites and pharmacophores of Plk1 PBD. The structures of the compounds were prepared using Ligprep with the OPLS_2005 force field in Schrödinger software package. Docking calculations were performed using Glide[44, 45] XP mode with default settings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found.

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CAPTION FOR FIGURES

Fig. 1. Structures of known small molecule PBD inhibitors.

Fig. 2. Dose-response curve of **3v** against full-length Plk1.

Fig.3. The predicted binding models. Figures are prepared by Pymol. Hydrogen bonds are shown with green dashed lines. A, Binding mode of **3a**. B, Binding modes of PLHSpT and **3a**. Phosphopeptide is colored by magenta and **3a** is colored by orange. C, Binding modes of **3e** and **3v**. **3e** is colored by green and **3v** is colored by yellow. D, Binding mode of **3v**. Halogen bonds are shown with magenta dashed lines.

CAPTION FOR SCHEMES

Scheme 1. Synthesis of compounds **3a-x**. (i), KSCN, acetone; (ii), the amine derivative of R_2, acetone.

Scheme 2. Synthesis of intermediates $\mathbf{I_{u\text{-}x}}$. (i), haloid acid, hydrogen peroxide; (ii), NBS, DMF; (iii), iodine, glacial acetic acid.

CAPTION FOR TABLES

Table 1. Dissociation constants measured by MST for synthesized compounds with significant binding signals. Detailed results are shown in Fig. S1.

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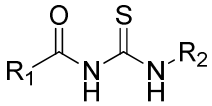
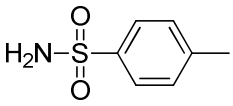
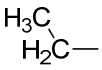
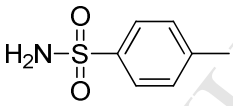
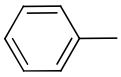
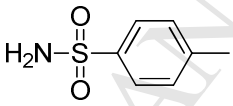
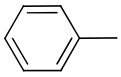
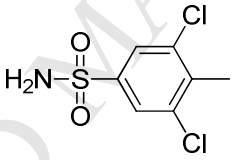
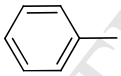
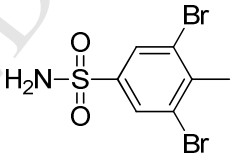
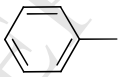
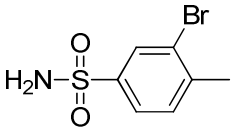
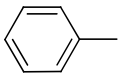
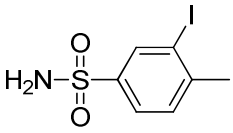
Table 1.



Compds	R_1	R_2	K_d (μM)
PLHSpT	-	-	14.0±1.3
Poloxin	-	-	10.5±0.9
3a			73.7±6.3
3b			64.3±3.8
3e			68.8±3.7
3u			5.1±0.3
3v			2.3±0.1
3w			6.9±0.6
3x			5.6±0.3

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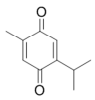
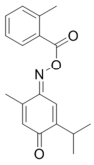
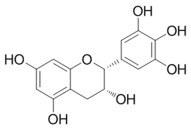


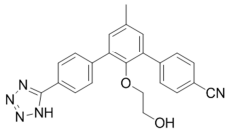
Thymoquinone



Poloxin



(-)-epigallocatechin (EGC)



2'-(2-hydroxyethoxy)-5'-methyl-4''-(1H-tetrazol-5-yl)-[1,1':3',1''-terphenyl]-4-carbonitrile

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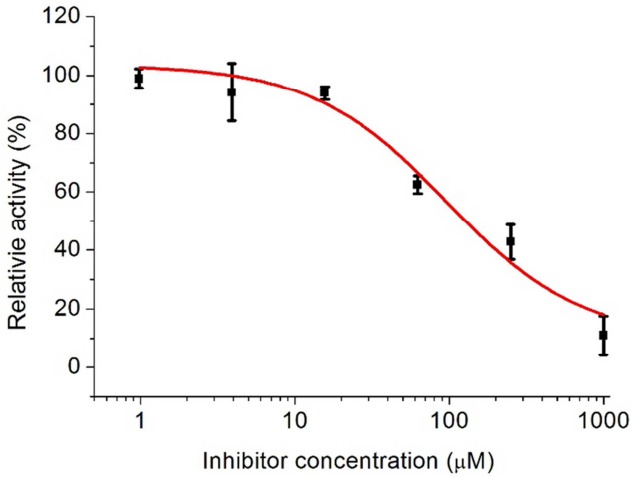

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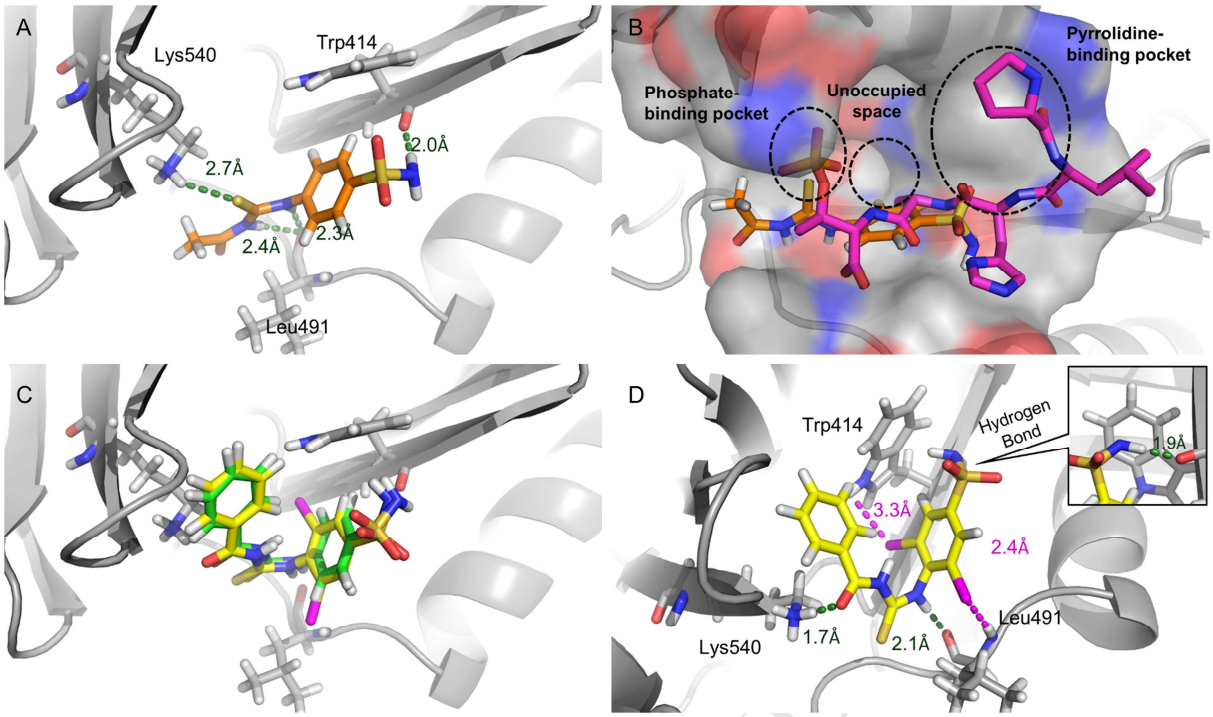

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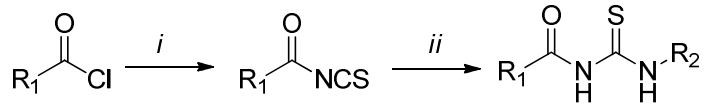
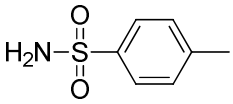
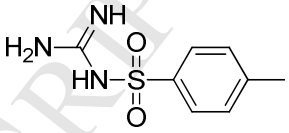
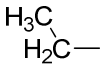
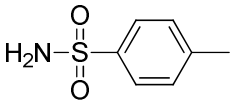
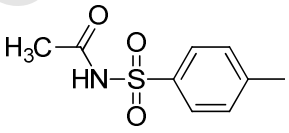
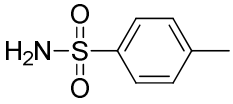
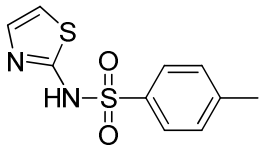
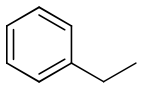
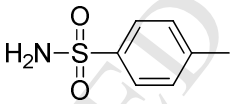
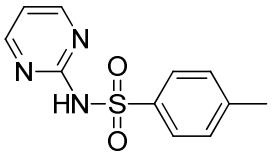
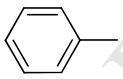
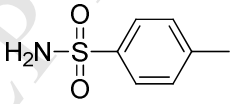
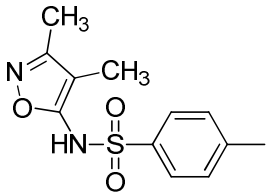
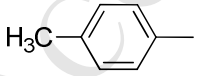
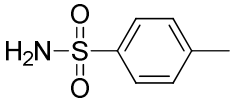
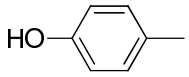
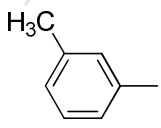
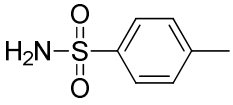
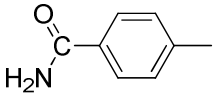
Scheme 1.



1 **2** **3a-x**

Compds	R_1	R_2	Compds	R_1	R_2
3a			**3m**		
3b			**3n**		
3c			**3o**		
3d			**3p**		
3e			**3q**		
3f			**3r**		
3g			**3s**		

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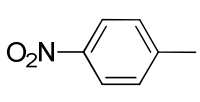
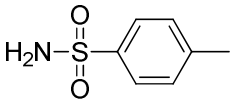
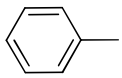
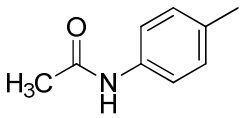
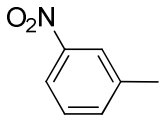
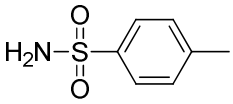
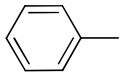
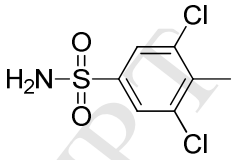
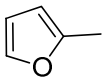
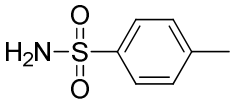
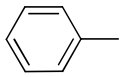
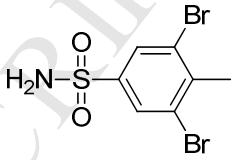
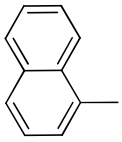
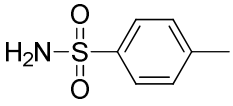
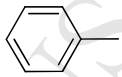
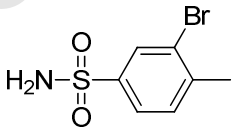
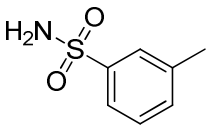
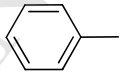
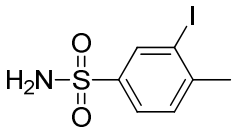
3h			**3t**		
3i			**3u**		
3j			**3v**		
3k			**3w**		
3l			**3x**		

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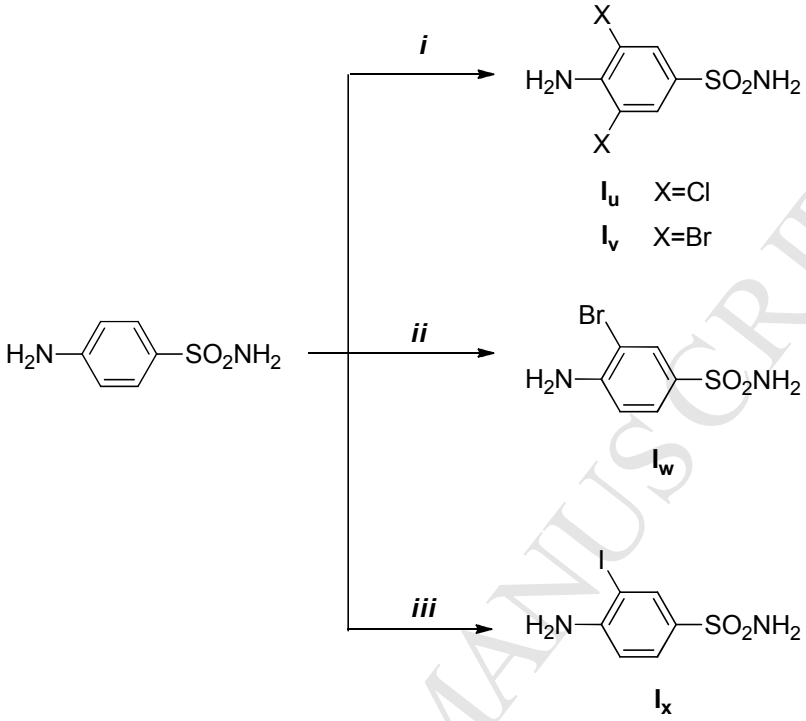
Scheme 2.



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A series of acylthioureas were designed and synthesized as Plk1 inhibitors.

Compounds with halogen in sulfamoylphenyl group showed better binding affinities.

The best compound **3v** binds to Plk1 PBD with a K_d of 2.3±0.1 μM.

Compound **3v** also inhibits the kinase activity of full-length Plk1.

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