The natural product Aristolactam Allla as a new ligand targeting the polo-box domain of polo-like kinase 1 potently inhibits cancer cell proliferation

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Original Article

Introduction

Polo-like kinases (Plks) belong to a family of Ser/Thr protein kinases and play a variety of roles in cell cycle progression[1]. To date, four members of this family have been identified in mammalian cells, termed as Plk1, Plk2 (SNK), Plk3 (PRK/FNK), and Plk4 (SAK)[2–6], of which Plk1 is the best characterized. It has been discovered that Plk1 functions importantly in numerous aspects of mitotic progression, including controlling entry into mitosis through the activation of the cdc2/cyclinB complex[7], centrosome maturation[8], bipolar spindle formation[9], sister chromatid separation[9], anaphase promoting complex activation[10] and affecting cytokinesis by phosphorylating NudC[11], etc. Overexpression of Plk1 has been observed in many human tumors including non-small cell lung cancer, oropharyngeal carcinoma, esophageal carcinoma, gastric carcinoma, melanoma, breast cancer, ovarian cancer, endometrial cancer, colorectal cancer, glioblastoma, papillary carcinoma, pancreatic, prostate, hepatoblastoma, and non-Hodgkin’s lymphoma[12]. In addition, Plk1 expression is also considered to be of prognostic value for patients suffering from varied types of tumors. Furthermore, microinjection of Plk mRNA was proved to be sufficient to drive quiescent cells into mitosis, and constitutive expression of Plk in NIH 3T3 cells could cause oncogenic focus formation[13]. Meanwhile, phosphorothioate antisense oligonucleotides (ASONs) against Plk1 showed potent anti-proliferative effect in cell culture and in mouse xenograft studies[14, 15]. Therefore, all these above-mentioned results have made Plk1 a potent target for the dis-
covery of anti-tumor agents.

Structurally, Plks are homologous and contain two conserved domains, the N-terminal catalytic kinase domain (CD) and the C-terminal polo box domain (PBD) that is composed of so-called polo boxes. The PBD exhibits a critical role in the regulation of Plk1’s kinase activity and the subcellular localization of Plk1[16]. It has been reported that Plk1 interacts, through its PBD, with certain serine/threonine-phosphorylated proteins localized at particular mitotic apparatuses, and binding of the PBD to the primed phosphorylation sites not only serves for targeting the kinase domain to substrates but also simultaneously activates the kinase domain by relieving the inhibitory intramolecular interaction[17]. Therefore, in addition to blocking the ATP-binding or the substrate-binding site, targeting the PBD is also considered as another efficient tactic for the exploration of Plk1 inhibitors.

The first published small molecular Plk1 inhibitor was scytonemin, a natural marine product isolated from cyanobacteria[18, 19], which is a micromolar non-specific ATP competitor. The pharmacophore ON01910 was a non-ATP competitive inhibitor of Plk1, which was probably a substrate-competitive inhibitor of Plk1, as it was a substrate-competitive inhibitor of recombinant casein and CDC25C[20]. The first small molecule inhibitor targeting the PBD was reported recently[21], which could interfere with Plk1 intracellular localization by inhibiting the function of the PBD.

In the current work, by random screening against our in-house natural product library, we discovered that the natural product Aristolactam Allia (Figure 1A), an Aristolactam derivative[22], functions as a new type of ligand targeting the PBD. It could inhibit the proliferation of cancer cells and induce apoptosis and the mitotic arrest at G2/M phase with spindle abnormalities. Different from the published Plk1 inhibitors, this natural product not only targeted both the CD and PBD domains, but also enhanced the CD/PBD interaction. Our findings might help to shed light on the possible mechanism of the Aristolactams inhibition against cancer cell proliferation[23, 24], and Aristolactam Allia might be used as a potential lead compound for further research.

Materials and methods

Plasmid construction

The PBD (residues 326–603 of Plk1) and the catalytic domain of Plk1 (CD, residues 1–370 of Plk1) were amplified by PCR from pUC18-Plk1 (synthesized by Shanghai Sangon Biologi
cal Engineering Technology & Services Co, Ltd, Shanghai, China), and then subcloned into the vector pGEX4T-1 and pFastBacHTb, respectively. For the yeast two-hybrid assay, the DNA fragment encoding the PBD was digested with EcoRI and XhoI (NEB) from the pGEX4T-1-PBD plasmid and the DNA fragment encoding the PBD was cloned into the pGADT7 vector. Similarly, the catalytic domain of Plk1 was cloned into the pGBK7 vector from pFastBacHTA-CD. For overexpression, the PBD and CD were amplified by PCR from pUC18-Plk1 and then subcloned into the vector pCDNA3.1a, respectively.

Protein preparation

By using pGEX4T-1-PBD as the expression plasmid, the recombinant protein GST-tagged PBD was expressed in E. coli BL21 (DE3) cells and purified by glutathione-affinity chromatography. The GST tag was cleaved on column with thrombin (Pharmacia) and the native PBD was obtained by gel filtration. The His-tagged catalytic domain of Plk1 was expressed in TN insect cells (TN-5B1-4, Trichoplusia ni) using standard baculovirus expression protocols and purified with Ni-NTA affinity chromatography.

In vitro enzymatic assays of the full-length Plk1 and its catalytic domain

The enzymatic assays of Plk1 and its catalytic domain were performed using the Cyclex Plk1 assay kit/inhibitor screening kit (Cyclex Co, Japan). Kinase inhibition experiments were carried out according to the protocol provided by the manufacturer. Plates were precoated with the substrate termed recombinant Protein-X, which contains a threonine residue that can be phosphorylated by Plk1. The detector antibody specifically detects the phosphorylated threonine on Protein-X. During the assay, Plk1 or its catalytic domain was dissolved in 10 µL kinase buffer and mixed with 10 µL of compound solution at different concentrations (prepared by diluting 1 µL of DMSO mother liquor into 9 µL kinase buffer). The mixture was finally added to 80 µL of kinase buffer containing 50 µmol/L ATP. After pre-incubation at 4 °C for 80 min, the plates were incubated at 30 °C for 30 min, and the wells were washed five times with 1× wash buffer provided by the manufacturer. Subsequently, 100 µL of anti-phospho-threonine polyclonal antibody (PPT-07) was added to each well and incubated at room temperature for 30 min. The wells were washed five times as described above and incubated with 100 µL of HRP-conjugated anti-rabbit IgG. Following a 30-min incubation at room temperature, the wells were washed five times and incubated with 100 µL of a chromogenic substrate reagent for 5 min. The reaction was terminated with 100 µL stop solution, and the amount of phosphorylated substrate was determined by measuring the absorbance at dual wavelengths of 450/540 nm.

Immunoprecipitation

For Plk1 immunoprecipitation, HeLa cells (4×10^6 cells/well) were seeded on 10-mm dish and incubated overnight. After incubation with nocodazole (5 µg/mL) for 12 h, the cells were lysed with 500 µL cold lysis buffer containing a protease inhibitor cocktail. The cell lysate was treated with 500 µL of lysis buffer containing 10 µL of a prepared protein A/G bead slurry 1 h at 4 °C and then centrifuged at 13 000 r/min for 15 min at 4 °C. The supernatant was carefully collected without disturbing the pellet and transferred to a clean tube, followed by incubation with 10 µL of Plk1 antibody (1:50, 35–306 aa, Abcam) overnight. After incubated with 20 µL pre-cleared protein A/G bead slurry at 4 °C for 3 h on a rotator, the mixture was spun at 13 000 r/min for 2 min at 4 °C. The supernatant was
carefully removed and the beads were washed twice with 50 µL kinase buffer. After the final wash, the agarose beads were resuspended in 50 µL kinase buffer and mixed gently. The catalytic domain of Plk1 (residues 1-370 of Plk1) that was transiently transfected into 293T cells for 48 h was immunoprecipitated with an anti-myc antibody (1:400, Invitrogen).

**Surface plasmon resonance (SPR) technology-based binding assay**

The binding of Aristolactam AIIIa to the PBD or CD was analyzed by SPR technology-based Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). All experiments were carried out using HBS-EP (10 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 3.4 mmol/L EDTA and 0.005% surfactant P20) as running buffer with a constant flow rate of 30 µL/min at 25 °C. The PBD protein, which was diluted in 10 mmol/L sodium acetate buffer (pH 4.2) to a final concentration of 2 µmol/L, was covalently immobilized on the hydrophilic carbamoylmethylated dextran matrix of the CM5 sensor chip (Biacore) using standard primary amine coupling procedure. The CD protein, which was diluted in 10 mmol/L sodium acetate buffer (pH 4.13) to a final concentration of 2 µmol/L, was immobilized on the chip using the same procedure. Aristolactam AIIIa was dissolved in the running buffer at different concentrations ranging from 1 to 10 µmol/L. All data were analyzed by BIAevaluation software, and the sensorgrams were processed by automatic correction for nonspecific bulk refractive index effects. The kinetic analyses of the Aristolactam AIIIa/PBD binding were performed based on the steady state affinity fit model according to the procedures described in the software manual, and the Aristolactam AIIIa/CD binding equilibrium dissociation constant was calculated based on the 1:1 Langmuir binding fit model.

To investigate the effect of Aristolactam AIIIa on the PBD/CD interaction, the purified CD protein was immobilized on a CM5 sensor chip. After the PBD (14 µmol/L) was incubated with different concentrations of Aristolactam AIIIa (0, 24, 34.3, 49, 70, and 100 µmol/L) for 1 h, the samples were applied to the sensor chip at a flow rate of 10 µL/min for 60 s, followed by washing with HBS-EP 180 s. The binding ability of PBD towards the CD was reflected by RUs values recorded directly by the Biacore 3000 instrument.
Yeast two-hybrid assay

Competent cells of the yeast strain AH109 were obtained from Clontech (Plano Alto, CA), and transformations were performed according to the manufacturer’s protocol. First, 500 ng of plasmid DNA was added to 50 µL of competent cells and mixed with 36 µL of 1 mol/L lithium acetate, 5 µL of boiled 10 mg/mL ss-carrier DNA and 240 µL of 50% poly(ethylene glycol) (MW3350) at 30 ºC for 30 min followed by heat-shock at 42 ºC for 30 min and subsequently spread on a drop-out-agar plate that lacked leucine and tryptophan. The plates were incubated at 30 ºC for 48 h to allow for yeast growth. PCR was used to confirm the transformation of the target plasmids. A positive clone was inoculated into SD medium lacking leucine and tryptophan (SD-LT) overnight, the culture was then diluted to OD600 of 0.003 with SD medium lacking leucine, tryptophan, and histidine (SD-LTH) which was supplemented with 2 mmol/L of 3-amino-10,20,40-triazole (SD-LTH+3-AT) and 100 µmol/L of Aristolactam Allla. The medium was shaken at 250 r/min at 30 ºC for 48 h before 200 µL of the culture was added to the well of a 96-well microplate. The absorbance at 600 nm of the culture in the 96-well microplate was then measured by a Benchmark Plus™ microplate spectrophotometer (BIO-RAD). This is an alternative method based on the growth curve analysis for yeast culture that is amenable to a microtiter plate format. It is reproducible and of equal or greater sensitivity compared with the β-galactosidase assay[26]. All the yeast media were prepared according to the standard Protocols Handbook (PT3024-1, Clontech).

Results

Aristolactam Allla inhibits Plk1 activity

To evaluate the inhibition of Aristolactam Allla against Plk1, the Polo-like kinase1 Assay/Inhibitor Screening Kit (Cyclex Co, Japan) was used. During the assay, 1.25 munits of recombinant Plk1 (Cyclex Co, Japan) was mixed with different concentrations of either Aristolactam Allla or DMSO (as a control), and the kinase activity was measured by using Protein-X as substrate. As indicated in Figure 1B, the natural product Aristolactam Allla exhibited a dose-dependent inhibition against Plk1-mediated phosphorylation of Protein-X with an IC₅₀ value of 47.5 µmol/L.

In order to further confirm the inhibition of Aristolactam Allla against Plk1, the kinase activity of endogenous Plk1 immunoprecipitated from Nocodazole-arrested HeLa cells was measured after incubation with Aristolactam Allla (10 µmol/L and 100 µmol/L) or DMSO (as a control). As shown in Figure 1C, Aristolactam Allla also inhibited the endogenous Plk1 activity in a dose-dependent manner.

To investigate the potential selectivity of Aristolactam Allla against Plks, the inhibition of Aristolactam Allla against

Figure 1. Aristolactam Allla could inhibit the Plk1 catalytic activity. (A) Structure of Aristolactam Allla. (B) Inhibition of Aristolactam Allla against Plk1 catalytic activity. Plk1 (1.25×10⁻² units) was pre-incubated with Aristolactam Allla at different concentrations, and the inhibited Plk1 activity was presented as percentage of the maximal inhibition. ▲, Aristolactam Allla (0.1, 1, 5, 10, 50, 100, and 1000 µmol/L). All data were averaged from three independent experiments. Half-maximal inhibitor concentrations (IC₅₀ values) were obtained by sigmoidal fit of inhibitory curves using Origin 7.0. (C) Inhibition of Aristolactam Allla against the endogenous Plk1 enzyme immunoprecipitated from mitotic cells (Nocodazole-arrested HeLa cells). Plk1 was pre-incubated with Aristolactam Allla (10 and 100 µmol/L) or DMSO for 1 h. (D) Inhibition of Aristolactam Allla against Plk3 catalytic activity. Plk3 was pre-incubated with Aristolactam Allla (1, 10, and 100 µmol/L) or DMSO for 1 h.
Plk3 was measured with a Polo-like kinase3 Assay/Inhibitor Screening Kit (Cyclex Co, Japan). As shown in Figure 1D, Aristolactam Allla exhibited a dose-dependent inhibition against Plk3, indicating that Aristolactam Allla had no selectivity for Plks.

Aristolactam Allla inhibits the proliferation of human HeLa, A549, HGC, and HCT-8/V cells

As has been reported[24], some Aristolactam derivatives could exhibit cytotoxicity against KB, P388, A549, HT29, HL60, HeLa, and L1210 cells, but no detailed inhibition mechanism was investigated. Although Aristolactam Allla has been reported to inhibit platelet aggregation induced by collagen and AA, its anti-tumor activity has not yet been elucidated[27]. Here, the anti-proliferation effect of Aristolactam Allla on HeLa, A549, and HGC cancer cell lines was examined. As shown in Table 1 and Figure 2A, Aristolactam Allla inhibited the proliferation of these cancer cells in a dose-dependent manner with IC50 values ranging from 7 to 30 µmol/L. Moreover, to further explore the potential inhibition of Aristolactam Allla against the relevant clinical drug-resistant cancer cell, the Navelbine (NVB)-resistant HCT-8/V cell line was assayed. As indicated in Figure 2B and Table 2, Aristolactam Allla exhibited dose-dependent inhibitory effects on HCT-8/V with an IC50 of 3.55 µmol/L, even while 10 µmol/L of NVB had no effect on HCT-8/V proliferation.

Table 1. Anti-proliferation activity of Aristolactam Allla against different cancer cell lines.

| Cancer cell line | HeLa | A549 | HGC |
|------------------|------|------|-----|
| IC50 (µmol/L)    | 7.98 | 15   | 31  |

Table 2. Susceptible property of clinical drug-resistant cell line HCT-8/V to Aristolactam Allla.

| IC50 (µmol/L) | Aristolactam Allla | NVB |
|---------------|---------------------|-----|
|               | 3.55                | NI  |

NI: no inhibition at 10 µmol/L.

Aristolactam Allla induces cell cycle arrest in the G2/M phase with spindle abnormalities in HeLa cells

Plk1 was shown to be essential to mitotic progression and cytokinesis, and functional down-regulation of Plk1 (either by microinjecting Plk1-specific antibodies or overexpressing dominant-negative Plk1) might induce G2/M arrest[8,29]. With these facts in mind, we thereby investigated the potential effect of Aristolactam Allla on the cell cycle of tumor cells. As shown in Figure 2C, incubation of HeLa cells with 10 µmol/L of Aristolactam Allla for 24 h increased the cell population staying in G2/M phase by 100% compared with the control cells, which thus suggested that Aristolactam Allla was able to induce cell cycle arrest at the G2/M phase. To further confirm this result, the cell cycle distribution pattern of HeLa cells treated for 48 h with Aristolactam Allla or DMSO was also investigated. As shown in Figure S1, similar to the case in 24 h incubation, a 48 h incubation with Aristolactam Allla could also induce cell cycle arrest in G2/M phase.

It has been known that Plk1 is involved in bipolar spindle formation which requires proper spindle assembly. Cells injected with anti-Plk1 antibodies display striking defects in their ability to assemble bipolar spindles, as manifested by the lack of focused spindle poles and unstable attachment of the chromosomes to the spindles[8,29]. To test whether Aristolactam Allla could affect spindle assembly, we used confocal laser microscopy to image the spindle apparatus and chromosomes of Aristolactam Allla-treated cells. HeLa cells were incubated with either DMSO (as a control) or 10 µmol/L of Aristolactam Allla for 12 h, fixed with 4% paraformaldehyde, and then stained with FITC conjugated anti-α-tubulin antibody (to visualize tubulin spindles) and propidium iodide.
to visualize chromosomal DNA). The images showed that the most DMSO-treated cells at mitosis phase exhibited no abnormality while 70% of the Aristolactam Allia-treated cells displayed multipolar spindles and misaligned chromosomes (Figure 2D and 2E). Such results were in agreement with the previous results for Plk1-depleted cells[29], and suggested that Aristolactam Allia could induce aberrant spindle assembly in cells through its inhibition against Plk1.

**Aristolactam Allia induces apoptosis of cancer cells**

Considering that the activation of apoptotic pathways could be detected by examining PARP (Poly [ADP-ribose] polymerase-1) cleavage[30], a marker for caspase activation, we thereby examined the potential effects of Aristolactam Allia on the apoptosis of HeLa cells by determining the induction of the cleaved PARP p85 fragment. Western blot analysis showed PARP cleavage in Aristolactam Allia-treated HeLa cells (Figure 3A), indicating the apoptosis induction in these cells. This result is consistent with the reported effects of Plk1 siRNA[31] and other Plk1 inhibitors on cancer cells[20, 32].

To further confirm the above results, Aristolactam Allia (10 µmol/L) or DMSO-treated HeLa cells were labeled with
Annexin V and PI to determine the extent of cellular apoptosis. As shown in Figure 3B and 3C, Aristolactam AIIIa induced an elevated percentage of apoptotic cells including early phase apoptosis (Annexin V-positive) and late phase apoptosis (Annexin V- and PI-positive) compared with DMSO-treated cells.

Moreover, this phenomenon was also confirmed by the analysis of the cell cycle distribution pattern of HeLa cells with 24 h incubation of Aristolactam AIIIa. As shown in Figure 2C, the population of sub-G0/G1 peaks were 3.67% (DMSO) and 8.85% (Aristolactam AIIIa 10 µmol/L), respectively, indicating Aristolactam AIIIa could induce apoptosis of cancer cells.

Aristolactam AIIIa binds to the catalytic domain (CD) of Plk1 and inhibits its activity
In order to further scrutinize the possible inhibition mechanism of Aristolactam AIIIa against Plk1, the binding and inhibition features of Aristolactam AIIIa against the Plk1 PBD and CD domains were characterized.
exhibits a high binding affinity against CD with a $K_D$ of 1.44 µmol/L.

To test the enzymatic inhibition of Aristolactam AIIIa against the CD, we conducted a similar kinase inhibition assay against the purified recombinant CD (residues 1–370 of Plk1). During the assay, after incubation of the CD with DMSO (as a control) or Aristolactam AIIIa (100 µmol/L and 10 µmol/L), the kinase activity was measured as described above. As indicated in Figure 4B, Aristolactam AIIIa exhibited a dose-dependent inhibition against the CD. To further confirm this inhibition, the kinase activity of the CD immunoprecipitated from the CD transient transfected 293T cells was also measured as incubated with Aristolactam AIIIa (100 µmol/L) or DMSO (as a control). The results listed in Figure 4C have thereby validated the Aristolactam AIIIa inhibition against the CD.

Taken together, the above results have suggested that Aristolactam AIIIa could directly bind the catalytic domain of Plk1 and inhibit its activity.

**Aristolactam AIIIa targets the PBD and increases the PBD/CD interaction**

The auto-inhibitory mechanism of Plk1 by its intrinsic Polo box domain (PBD) has made the PBD as another potential target besides the catalytic domain. To investigate whether Aristolactam AIIIa could interfere with PBD function through its binding to the PBD, the binding affinity of Aristolactam AIIIa against PBD was studied by using the SPR technology-based Biacore 3000 instrument. In the assay, immobilization of the PBD on the Biacore biosensor chip resulted in a resonance signal of 7540 resonance units (RUs). The results in Figure 5A indicated the dose-dependent biosensor RUs for Aristolactam AIIIa, suggesting that this natural product could bind to the PBD in vitro. The steady state affinity fit model was used to determine the equilibrium dissociation constant $K_D$, and the

| Target | $K_D$ (µmol/L)$^*$ | $\chi^2$ |
|--------|-----------------|---------|
| CD     | 1.44            | 0.353   |
| PBD    | 2.99            | 3.28    |

$^*$ $K_D$, equilibrium dissociation constant; $\chi^2$, statistical value in BIAevaluation; CD, catalytic kinase domain; PBD, polo box domain.

**Table 3. SPR technology based kinetic analysis for PBD or CD/Aristolactam AIIIa interaction.**

![Figure 5](image-url)
accuracy of the obtained results were evaluated by χ². The fitted kinetic parameters listed in Table 3 thus demonstrated a strong binding affinity of Aristolactam AIIIa towards the PBD with a $K_D$ value at 2.99 µmol/L.

In addition, cell-based assay was also carried out to further examine the potential targeting of Aristolactam AIIIa against the PBD in the PBD over-expressing HeLa cells. After transient transfection with pCDNA3.1a-PBD, HeLa cells were incubated with Aristolactam AIIIa at 10 µmol/L for 48 h. As shown in Figure 5B, Aristolactam AIIIa could exhibit inhibitory effects by 50% on the proliferation of the PBD over-expression in HeLa cells in the SRB assay. Compared with the 71% inhibition by Aristolactam AIIIa against the proliferation of the HeLa cells transfected with empty vector (Figure 5B), the result supports the finding that Aristolactam AIIIa targets the PBD.

Since the PBD plays a critical role in Plk subcellular localization[23] and Aristolactam AIIIa has been suggested to target the PBD as mentioned above, the localization of endogenous Plk1 in Aristolactam AIIIa or DMSO-treated HeLa cells was thereby examined by using an antibody directed against the N terminus of Plk1 (35–206 aa). As indicated in Figure 5C, in Aristolactam AIIIa-treated cells at mitotic phase, endogenous Plk1 was detected on spindle poles and concentrated close to the midbody in the postmitotic bridges connecting the dividing cells, which is consistent with the founded in DMSO-treated cells.

As has been reported, the PBD is believed to regulate Plk1 activity through its intramolecular interaction with the catalytic domain (CD)[16, 17]. Considering this fact, we have thus inspected the potential influence of Aristolactam AIIIa on the PBD/CD interaction by yeast two-hybrid system and SPR technology. In the yeast two-hybrid assay, the yeast cells transformed with both PBD and CD were incubated with either 100 µmol/L Aristolactam AIIIa or DMSO (as a control) for 48 h. The result in Figure 6A implied that Aristolactam AIIIa increased the PBD binding to the catalytic domain of Plk1 by ~43%. Additionally, such Aristolactam AIIIa-stimulated PBD/CD interaction was also confirmed in vitro by SPR technology-based assay (Figure 6B).

Summarily, all the above results indicated that Aristolactam AIIIa targets both the PBD and CD. It interferes with the PBD function through the inhibition of Plk kinase activity by increasing the PBD/CD interaction without affecting Plk1 localization to the proper subcellular structure.

**Discussion**

Plk1 plays essential roles in numerous aspects of mitotic progression. A number of studies have demonstrated that Plk1 is over-expressed in a broad spectrum of human tumors[12], and Plk1 has been determined as an attractive target for cancer therapy. To date, three kinds of small molecular inhibitors of Plk1 have been discovered: ATP competitive inhibitor, substrate competitive inhibitor and the PBD inhibitor.

In general, the ATP-binding domain is a regular target for protein kinase inhibitor discovery. However, specificity of such target-based inhibitor is difficult to obtain due to the high degree of structural conservation among ATP-binding pockets, and only a few ATP-like inhibitors of Plk1 have so far been published. The first small molecule Plk1 inhibitor was scytonemin, a non-specific ATP competitor, which exhibited comparable potencies against other Ser/Thr and Thr/Tyr kinases including MYT1, CHK1, CDK1, and PKC[18, 19].

Exploration of Plk1 non-ATP competitor is another choice for a Plk1 specific inhibitor in anti-tumor drug discovery. For example, the pharmacophore ON01910 was reported to be a substrate-competitive inhibitor of recombinant casein and CDC25C[20]. Another strategy in the development of Plk1 specific inhibitor is to target the PBD, which has a critical role in the regulation of the kinase activity and the subcellular localization of Plk1[16]. Recently, the first small molecule targeting the PBD was reported, which could interfere with Plk1 intracellular localization by inhibiting the function of the PBD[21].

Aristolactams are phenanthrene lactam alkaloids that are structurally and biogenetically related to aporphines, which have been reported to show inhibitory activity against cancer cell proliferation; however, the relevant detailed data still remain unclear[22, 23]. As a member of Aristolactams, Aristolactam AIIIa demonstrated significant inhibition of platelet
aggregation induced by collagen and AA\textsuperscript{[27]}, while its anti-tumor activity has not yet been elucidated. By random screening against the in-house natural product library, we discovered that Aristolactam Allla functions as a new type of ligand targeting the PBD, as investigated by the SPR and enzymatic inhibition assays. It could inhibit the proliferation of HeLa, A549, HGC, and HCT-8/V cells, influence cell cycle progression and spindle assembly in HeLa cells and induce the apoptosis in HeLa cells.

Enzymatic characterization indicated that Aristolactam Allla could exhibit inhibition activity against the CD, which might contribute to its inhibition against the full length Plk1 \textit{in vitro}, similar to previously published inhibitors\textsuperscript{[20, 32]}

To further investigate the potential inhibition mechanism of Aristolactam Allla against Plk1, the kinetic feature of Aristolactam Allla binding to the PBD was assayed. SPR results demonstrated a strong binding affinity of Aristolactam Allla to the PBD (\(K_D=2.99 \text{µmol/L}\)). Since the PBD has a critical role in the regulation Plk’s kinase activity by interacting with the catalytic domain of Plk1\textsuperscript{[16, 17]}, the result that Aristolactam Allla could enhance the PBD interaction with the CD as indicated by SPR and yeast two-hybrid-based assays has shown that this natural product further strengthened the self-regulatory effect of the PBD on Plk1.

It is noticed that the small molecule inhibitors reported by Reindl \textit{et al}\textsuperscript{[21]} inhibited the function of the Plk1 PBD and interfered with the intracellular localization of Plk1, while Aristolactam Allla did not affect the localization of Plk1. Such discrepancy might possibly come from the different PBD binding sites for the compounds. The reported inhibitors\textsuperscript{[21]} interfered with the interaction between PBD and its intracellular anchoring sites of the substrates, resulting in mis-localization of Plk1. However, Aristolactam Allla enhanced the CD/PBD interaction, thereby inhibiting Plk1 activity without affecting Plk1 localization. All these findings thus revealed that the regulation of PBD in Plk1’s kinase activity and the subcellular localization of Plk1 are closely related to the PBD binding sites.

In summary, we have identified the Aristolactam derivative, Aristolactam Allla as a new type of ligand targeting the PBD by random screening our in-house natural product library. Cell-based assays indicated that this natural product could inhibit the proliferation of HeLa, A549, HGC, and HCT-8/V cells, induce mitotic arrest at the G\textsubscript{2}/M phase with spindle abnormalities and promote apoptosis. Different from the published Plk1 inhibitors, Aristolactam Allla could bind to the interaction interface of CD/PBD due to targets both the catalytic domain and the polo-box domain of Plk1 kinase, and enhances the CD/PBD interaction. Our current work is expected to shed light on the potential antitumor mechanism of Aristolactam Allla, and this determined Plk1 inhibitor may also be used as a lead compound for further research.

\textbf{Abbreviations}

Plk1: Polo-like kinase 1; CD: catalytic domain of Plk1; PBD: Polo box domain; HeLa: cell line taken from Henrietta Lacks alias Helen Lane; A549: human lung cancer A549 cell line; HGC: human gastric cancer cell line; HCT-8/V: human colon adenocarcinoma cell line resistant to vincristine; SPR: Surface Plasmon Resonance; Plks: Polo-like kinases; Plk2: Polo-like kinase 2; SNK: serum inducible kinase; Plk3: Polo-like kinase 3; PRK: proliferation-related kinase; FNK: fibroblast growth factor-inducible kinase; Plk4: Polo-like kinase 4; SAK: Snk/Plk-okin kinase; cd2c: cell division cycle 2 (cell cycle-dependent protein kinase); NudC: nuclear distribution gene C; NIH 3T3: mouse embryonic fibroblast cell line; ASONs: phosphorothioate antisense oligonucleotides; CDC25C: cell division cycle 25 homolog C; DMSO: dimethyl sulfoxide; HRP: horseradish peroxidase; 293T cell line: a derivative of human embryonic kidney cells line that stably express the large T-antigen of SV40; SRB: sulphorhodamine B; NBV: navelbine; PBS: phosphate-buffered saline; FITC: fluorescein isothiocyanate; PI: propidium iodide; PARP: poly [ADP-ribose] polymerase-1; ss-carrier DNA: salmon sperm-carrier DNA; SD medium: synthetic defined medium; 3-AT: 3-amino-10, 20, 40-triazole; IC\textsubscript{50}: concentration giving 50% of maximal inhibition; KB: human carcinoma cell (strain KB); P388: murine lymphoma cell (strain P388); HT29: a colon adenocarcinoma cell line ( HT-29); HL60: human promyelocytic leukemia 60 cell line; L1210 cells: L1210 mouse leukemia cells; AA: arachidonic acid; K\textsubscript{D}: dissociation constant; RU5: resorcinol units; MYT1: membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase; CHK1: checkpoint kinase 1; CDK1: cyclin dependent kinase 1; PKC: protein kinase C.

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\textbf{Author contribution}

This study was designed by Xu WANG, Li LI, Jing CHEN, and Li-hong HU. The surface plasmon resonance (SPR) technology-based assay, enzymatic inhibition assay and cell-based assays which were used to screen and further identify Plk1 inhibitor were performed by Xu WANG, Li LI, Hong DING, and Jing CHEN. The experiments about investigating the potential inhibition mechanism of active compound against Plk1 were performed by Hong DING, Yu ZHANG, and Jing CHEN. Xu SHEN, Hua-liang JIANG, and Li-hong HU supervised the project. Li LI, Jing CHEN, Tian-cen HU, and Xu SHEN contributed to the manuscript writing. All authors read and approved the final manuscript.

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