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A novel CyclinE/CyclinA-CDK Inhibitor targets p27^{Kip1} degradation, cell cycle progression and cell survival: Implications in cancer therapy

Lu Dai [a], Yuqing Liu [b], Junyang Liu [a], Xiaoming Wen [a], ZhengShuang Xu [a,b], Zhuo Wang [b], Hong Sun [c], Shoubin Tang [a], Anita R. Maguire [d], Junmin Quan [a,*], Hui Zhang [a,c,*], Tao Ye [a,b,*]

[a] Laboratory of Chemical Genomics, School of Chemical Biology and Biotechnology, Shenzhen Graduate School of Peking University, Shenzhen 518055, China
[b] Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong, China
[c] Department of Chemistry, University of Nevada, Las Vegas, NV 89154, USA
[d] Department of Chemistry and School of Pharmacy, Analytical and Biological Chemistry Research Facility, University College Cork, Cork, Ireland

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ABSTRACT

p27^{Kip1} (p27) binds and inhibits the cyclin E- or cyclin A-associated cyclin-dependent kinases (CDKs)2 and other CDKs, and negatively regulates G1–G2 cell cycle progression. To develop specific CDK inhibitors, we have modeled the interaction between p27 and cyclin A-CDK2, and designed a novel compound that mimics p27 binding to cyclin A-CDK2. The chemically synthesized inhibitor exhibited high potency and selective inhibition towards cyclin E/cyclin A-CDK2 kinase in vitro but not other kinases. To facilitate permeability of the inhibitor, a cell penetrating peptide (CPP) was conjugated to the inhibitor to examine its effect in several cancer cell lines. The CPP-conjugated inhibitor significantly inhibited the proliferation of cancer cells. The treatment of the inhibitor resulted in the increased accumulation of p27 and p21$^{Cip1/Waf1}$ (p21) and hypo-phosphorylation of retinoblastoma protein (Rb). The degradation of p27, mediated through the phosphorylation of threonine-187 in p27, was also inhibited. Consequently, exposure of cells to the inhibitor caused cell cycle arrest and apoptosis. We conclude that specific cyclinE/cyclin A-CDK2 inhibitors can be developed based on the interaction between p27 and cyclin/CDK to block cell cycle progression to prevent tumor growth and survival.

1. Introduction

Cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases that play key roles in controlling the entry into and passage through various phases of the cell cycle [1–4]. In the cell cycle, distinct cyclin/CDK complexes are activated to regulate cell cycle progression. For example, while cyclin D/CDK4 or CDK6 regulates the progression of G1 phase, cyclin E/CDK2 is required for the G1/S transition. Another cyclin/CDK complex, cyclin A/CDK2, plays a critical role in the control of S phase and DNA replication. It is also essential for G2 progression. Cyclin A- and cyclin B-associated CDK1 (CDC2) regulates the G2/M phases. Altered activities of cyclin/CDKs, caused by over-expression, translocation, gene amplification, or other aberrant activation of cyclin D, cyclin E, or cyclin A, are associated with various malignant human cancers [1,5]. The cell cycle is also negatively regulated by the presence of CDK inhibitors such as p27^{Kip1}(p27) and p21$^{Cip1/Waf1}$(p21). These inhibitory proteins interact with cyclin E or cyclin A/CDK2 or other cyclin/CDK binary complexes to inhibit their kinase activities [6–9]. p21 is transcriptionally controlled by tumor suppressor protein p53 and loss or mutation of p53 in many cancers leads to the down-regulation of p21. Malignant cancers are also associated with the low or absent expression of p27 protein, which is typically associated with poor prognosis [10]. In the cell cycle, the protein level of p27 is primarily regulated by ubiquitin-dependent proteolysis [11,12]. While the p27 protein level is high in early- and mid-G1 to prevent untimed activation of cyclin E/cyclin A-CDK2 to progress into the S phase, the p27 protein is targeted for degradation at the late G1 or in S phase through its phosphorylation at threonine 187 (T187) by kinases such as cyclin E/CDK2 or cyclin A/CDK2 [13–16]. Evidence indicates the phosphorylation of T187 is a result of phosphorylation at tyrosine 88 in p27 by Src family kinases [17]. When p27 is phosphorylated on the conserved threonine residue 187, the F-box protein Skp2 and its associated CKS1 bind to the phosphorylated p27 and promotes p27 ubiquitin-dependent degradation by the SCF^{SKP2} ubiquitin E3 ligase [14,18,19]. Low p27 protein levels caused by excessive

* Corresponding authors. Addresses: Department of Applied Biology & Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong, China. Tel.: +852 34008722; fax: +852 23649932 (T. Ye). Department of Chemistry, University of Nevada, Las Vegas, 4505 S. Maryland Parkway, Las Vegas, NV 89154, USA (H. Zhang). School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, 518055, China; Tel.: +86 0755 2603 5253 (J. Quan).

E-mail addresses: quanjm@szpku.edu.cn (J. Quan), hui.zhang@unlv.edu (H. Zhang), bctaoye@polyu.edu.hk (T. Ye).

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SCF^{SKP2}-mediated proteolysis of p27 are associated with many types of aggressive tumors [10,13]. Inhibiting cyclin E/cyclin A-CDK activity and prevention of p27 proteolysis should provide an excellent strategy to block the proliferation of malignant human cancers.

Because the elevated activities of CDKs are hallmarks of human cancer, CDKs represent an important therapeutic target for various types of human cancers. Considerable effort has been focused on development of small molecule inhibitors of CDK2 or other CDKs for potential anti-cancer purposes [20–24]. Most of these inhibitors have been developed against the ATP binding domain for these kinases. Although more than 50 CDK inhibitors have been reported [21], the chemical structures that act as CDK inhibitors are quite limited, since most of them are derived from relatively nonspecific protein kinase inhibitor scaffolds that inhibit the binding of ATP to CDKs and other kinases, such as staurosporins, flavonoids, indigoids, paulones, and purines. Structural information indicates that the core of the CDK catalytic center, consisting about 300 amino acid residues, shares significant homology with many other kinases [25]. The high degree of similarity between the kinase domain of CDK family members and other kinases in the ATP binding domain makes the selective inhibition of CDKs difficult.

The identification of CDK inhibitors p27 and p21 provide a new strategy to develop chemical inhibitors for CDKs. In this report, we have designed new CDK inhibitors based on the inhibitory binding of p27 to cyclin A/CDK2. Our data indicate that the inhibitor we have synthesized can selectively inhibit cyclinE- or cyclin A/CDK kinase activities both in vitro and in vivo. Our analysis indicates that the inhibitor can cause the cell cycle arrest and apoptosis of cancer cells.

2. Materials and methods

2.1. Cell lines

The cancer cell lines HeLa, RKO, MCF-7 and PC3 were obtained from American Type Culture Collection (ATCC, Rockville, MD). All cell lines were cultured in DMEM medium containing L-glutamine supplemented with 10% fetal bovine serum (FBS). The immortalized human hepatocyte cell line MIHA was cultured as described before [26].

2.2. Expression and purification of GST-CyclinA/CDK2, GST-CyclinE/CDK2 kinase complexes

Human cDNAs for cyclin A, cyclin E and Cdk2 were cloned into the baculovirus expression vector pVL1392 (Pharmingen, San Diego, CA, USA) which is fused in frame at the carboxy terminal end of glutathione-S-transferase (GST). Active recombinant GST-CyclinA, GST-CyclinE and Cdk2 were produced in Sf9 cells that had been infected with recombinant baculoviruses encoding cDNAs for cyclin (A or E) and Cdk2 using BD Baculogold Transfection Kit (BD Biosciences). Whole-cell lysates were prepared by sonication of the cells in hypotonic buffer (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM EDTA, 1 mM EGTA), and freshly added 1 μM DTT, protease inhibitors cocktail (0.1 mM PMSF, 1 mM DTT, 0.2 mM Na3VO4, 5 μg/ml aprotinin, 30 μg/ml leupeptin, 5 μg/ml pepstatin A and phosphatase inhibitors (25 mM β-glycerophosphate and 1 mM NaF), and centrifuged at 15,000 r.p.m. (18,600 g) for 15 min, collected the supernatant. To assemble GST-CyclinA/CDK2 and GST-CyclinE/CDK2 kinase complex, the supernatants containing GST-CyclinE or GST-CyclinA and CDK2 were mixed with 2 mM ATP at 37 °C for 30 min, followed by pulldown using GST Sepharoase beads (Roche) for the assembled GST-CyclinE/CDK2 or GST-CyclinA/CDK2 complexes. The beads were then washed with NP-40 buffer (0.5% NP-40, 20 mM Tris, pH7.4, 150 mM NaCl), kinase buffer (10 mM HEPES pH 7.4, 10 mM $MgCl_2$, 0.005% Tween-20, 2.5 mM EGTA, 1 mM DTT), and the assembled protein was released from the beads by eluting with 20 mM glutathione.

2.3. In vitro kinase assays

The kinase reactions, containing 2 μl GST-CyclinE/CDK2 (0.5 μg/μl) or 2 μl GST-CyclinE/CDK2 (0.5 μg/μl), 1 μg of histone H1, 10 μCi of [γ-32P]-ATP, 2 μl of 10X kinase buffer and various concentrations of inhibitor **1** or inhibitor **1a** to a final reaction volume of 20 μl, were incubated at 30 °C for 30 min. Each sample was then mixed with 20 μl of 2X SDS sample buffer to stop the reaction, heated for 10 min at 95 °C, and subjected to analysis by SDS–PAGE. The gels were dried and visualized by autoradiography. Similar reactions were used for purified GST-PLK1 and Aurora A kinases except casein or histone H3 was used as substrates for PLK1 or Aurora A kinase, respectively.

2.4. Effects of inhibitor **1** or inhibitor **1a** on cell proliferation

Cells were seeded into 96-well plates and incubated overnight. Inhibitor **1** or inhibitor **1a** was added in serial dilutions in the medium containing 1% FBS and the plates were incubated for another 48 h. Cell proliferation was performed using MTS assay by adding 20 μl of CellTiter96 Aqueous solution (Promega Corp., Madison, WI) into each well containing 100 μl culture medium, and cells were incubated for 2 h at 37 °C according to the manufacturer's instructions. The absorbance at 490 nm was measured using a microplate plate reader (Model 680 Microplate Reader, Bio-Rad Laboratories Ltd, UK).

2.5. Detection of cell cycle by flow cytometry analysis

HeLa and MCF-7 cells were seeded in 6-well plates and treated with inhibitor **1a** at different concentrations for 24 h. Cells were washed, fixed with ice-cold 70% ethanol and then incubated in 400 μl PBS, 50 μl RNase (1 mg/ml) (Sigma, St. Louis, MO), and 10 μl propidium iodide (PI) (2 mg/ml) (Sigma, St. Louis, MO) for 30 min at 37 °C, followed by flow cytometry analysis using FACS calibur (Becton Dickinson) as described before [26]. The percentage of cells in the G_0–G_1 and G_2–M phases was assessed by ModFit LT software (Verity Software House, Topsham, ME).

2.6. Detection of cell apoptosis by flow cytometry analysis

HeLa and MCF-7 cell lines were seeded in 24-well plates and treated with inhibitor **1a** at different concentrations for 24 h. Cells were harvested and resuspended in binding buffer (BD PharMingen, San Diego, CA) at a concentration of 1×10^6 cells/ml. Five μl of Annexin V-FITC (BD PharMingen) and 10 μl of PI (BD PharMingen) were added to 100 μl of resuspended cells. Cells were gently mixed and incubated for 15 min at room temperature in the dark and analyzed within 1 h by FACS calibur (Becton Dickinson) as described before [26].

2.7. Western blot analysis for p27, p21, phospho-p27, Rb, PARP, bcl-2, bcl-x_L and p53 expression

Cells were treated with inhibitor **1a** or inhibitor **1** at various concentrations for 24 h, washed twice with PBS and lysed in lysis buffer (Cell Signaling, Beverly, MA) for 20 min at 4 °C. The lysates were centrifuged at 14,000 g, 4 °C for 10 min, and equal amounts of solubilized proteins were separated by SDS–PAGE, and then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with TBST (20 mM Tris, pH 7.6, 135 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk and then immunoblotted with the following antibodies: p27 (1:2000) (Transduction Laboratory, Lexington, KY), p21 (1:1000), bcl-2 (1:1000), bcl-x_L (1:1000) (Cell Signaling, Beverly, MA), Rb (1:500), phosphor-p27 (T187) (1:1000), p53 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4 °C, followed by detection using HRP-conjugated secondary antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive protein bands were visualized by the ECL system (Amersham Biosciences, Piscataway, NJ) and quantified with Image J software (National Institutes of Health, USA).

2.8. Analysis of p27 levels in the cytoplasm and nuclear fractions

HeLa cells were seeded in tissue culture plates overnight and then treated with different concentrations of inhibitor **1a** for 24 h. Cells were then fractionated into cytoplasm and nuclear fractions using a nuclear extraction kit (Novagen, Darmstadt, Germany). According to the manufacturer's instruction, cells were harvested in NuBuster Reagent 1, and the supernatant (cytoplasm fraction) was collected after centrifugation. The pellet was then resuspended in NuBuster Reagent 2 containing Protease Inhibitor Cocktail and DTT, and nuclear extracts were recovered by centrifugation. p27 expression was evaluated by Western blot as describe before.

2.9. p27 Localization detected by immunofluorescence staining

HeLa cells were plated in chamber slides (IWAKI, Tokyo, Japan) at 1×10^4/side overnight, and then were treated with inhibitor **1a**. After 24 h, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% triton X-100 for 15 min, blocked with 1% BSA for 1 h, and then incubated with p27 antibody (Becton Dickinson, San Jose, CA, USA) at 4 °C overnight, followed by incubating with anti-mouse FITC (Becton Dickinson) for 1 h at room temperature. Cells were visualized by fluorescence microscopy (Nikon Eclipse Ti–U, Tokyo, Japan) and photographed.