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Specific inhibition of cyclin-dependent kinases and cell proliferation by harmine☆

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Abstract

As key regulators of the cell proliferation cycle, cyclin-dependent kinases (CDKs) are attractive targets for the development of anti-tumor drugs. In the present study, harmine was identified from a collection of herbal compounds to be a specific inhibitor of Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25 with IC_{50} values at low micromoles. It displayed little effect on other serine/threonine and tyrosine kinases tested. The CDK inhibition by harmine is competitive with ATP-Mg^{2+}, suggesting that it binds to the ATP-Mg^{2+}-binding pocket of CDKs. In cytotoxicity assays, harmine exhibited a strong inhibitory effect on the growth and proliferation of carcinoma cells whereas it had no significant effect on quiescent fibroblasts. Further, harmine was found to block DNA replication in the carcinoma cells. Taken together, harmine is a selective inhibitor of CDKs and cell proliferation.

Keywords: Cyclin-dependent kinase; Harmine; β-Carboline alkaloid; Competitive inhibitor; Cell cycle; Cell proliferation

Cyclin-dependent kinases (CDKs) are a family of protein serine/threonine kinases that play a central role in the molecular machinery that runs the cell cycle [1]. The activities of CDKs are tightly regulated at each stage of the cell cycle by sophisticated mechanisms including the binding of regulatory proteins and the phosphorylation/dephosphorylation of CDKs, assuring the completion of respective phases before progressing to the next stage [2]. An array of evidence showed the aberrant regulation of CDKs and their regulators in human cancers [3,4]. In addition to the control of cell division, CDKs are involved in cell differentiation and apoptosis. A particular CDK member, Cdk5, was found to play important roles in a variety of cellular events occurring in neurons of the central nervous system, ranging from differentiation, migration to degeneration, and apoptosis [5,6]. Although Cdk5 is a ubiquitous protein, its enzymatic activity is primarily restricted to the central nervous system where it forms complexes with its neuron-specific activator protein, p35 or p39 [7,8]. In Alzheimer's brains, p35 is proteolytically converted to p25, resulting in the deregulation of Cdk5 [9]. Cdk5/p25 is neurotoxic and induces aberrant hyperphosphorylation of tau in vitro and in animal models [9–11].

Over a long history of traditional Chinese medicine, a number of medicinal herbs are known to have antitumor effects and used in cancer therapy. However, little is known about the active ingredients of these herbs and how they act on tumor cells. From inhibitor screens of Cdk2 and Cdk5, we identified harmine from a collection of compounds isolated from medicinal herbs. Detailed characterization showed that harmine specifically inhibited Cdk1, Cdk2, and Cdk5 in an ATP-competitive manner. Moreover, harmine exhibited a strong inhibition of tumor cell growth and cellular DNA replication,

☆ *Abbreviations:* CDK, cyclin-dependent kinase; ATP, adenosine 5′-triphosphate; Erk, extracellular signal-regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

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whereas it displayed little growth effect on stationary fibroblasts, indicating that harmine is a specific inhibitor of cell proliferation.

Materials and methods

Protein kinase assay. The kinase activities of CDKs were measured using a protocol described previously [12]. Assay mixtures contained 30 mM Mops, pH 7.4, 10 mM $MgCl_2$, 15 μM [γ-^{32}P]ATP (~500 dpm/pmol), and 100 μM of the substrate peptide HS(9–18) (Pro-Lys-Thr-Pro-Lys-Lys-Ala-Lys-Lys-Leu), harmine (Sigma) at concentrations as specified, and CDK enzymes. Reactions were performed at 30 °C for 10 min. Phosphate incorporation into the substrate peptide was measured by scintillation counting. Cdk5/p25 was a complex reconstituted and purified from recombinant Cdk5 and p25 [12]. Cdk2/cyclin A was prepared by reconstitution of recombinant Cdk2 and cyclin A and the complex was activated by the CDK-activating kinase [13]. Cdk1/cyclin B was from New England BioLabs.

The kinase activities of PKA, PKC, and Erk were measured using the protocol above with variations of the substrate peptide. PKA and its substrate Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were from Sigma. PKC and its substrate peptide 4–14 a.a. of myelin basic protein (Glu-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) were purchased from Calbiochem. The PKC enzyme is a tryptic preparation of the catalytic subunit of PKC and does not require Ca^{2+}, phospholipids, and diacylglycerol for its activity. Erk2 was from New England BioLabs and its substrate peptide 94–102 a.a. of myelin basic protein (Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg) was from Upstate Cell Signaling Solutions.

The tyrosine kinases Lck, Lyn, and Fyn were purified from bovine tissues [14,15]. Their kinase activities were assayed in 50 mM Tris–HCl, pH 7.0, 50 mM $MgCl_2$, 7 mg/ml *p*-nitrophenyl phosphate, 50 μM Na_3VO_4, 15 μM [γ-^{32}P]ATP (~500 dpm/pmol), and 300 μM of the substrate peptide Cdc2(6–20) (Lys-Val-Glu-Lys-Ile-Gly-Glu-Gly-Thr-Tyr-Gly-Val-Val-Tyr-Lys) [14].

Cell culture and cytotoxicity assay. HeLa and MCF-7 cells were seeded at 2000 cells/well and SW480 was seeded at 4000 cells/well on 96-well tissue culture plates. The cells were cultured in the medium RPMI-1640 (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. After an overnight culture at 37 °C, harmine was added into the medium at various concentrations. They were then cultured for 5 days before the MTT assay. Swiss 3T3 fibroblasts were seeded at the density of 5000 cells/well and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mg/ml glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. Upon reaching confluence in about 3 days, the cells were subjected to the harmine treatment as above. All assays were performed in quadruplicates.

To stain viable cells, 20 μl of MTT (5 mg/ml in phosphate-buffered saline, Sigma) was added to each well. The cells were then incubated for 4 h at 37 °C. After the media were aspirated carefully without disturbing formazan crystals, the dye was dissolved in 200 μl dimethyl sulfoxide. Sample readings were taken at 570 nm in a microtiter plate reader. ED_{50} is defined as the compound concentration at which the absorption reading is reduced by 50% with respect to the controls. ED_{50} values were determined by interpolation from dose–response curves.

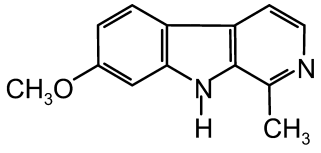
[^{3}H]Thymidine incorporation assay. SW480 cells were seeded into 24-well plates at 50,000 cells/well. The cells reached 80% confluence after 48 h at 37 °C. They were then treated with increasing concentrations of harmine in quadruplets for 24 h. Following the treatment, it was a pulse treatment of [^{3}H]thymidine (5 μCi/ml, Amersham Biosciences) for 2 h in the media. The radioactive media were then removed by aspiration, and the cells were washed twice with the ice-cold serum-free medium. The cells were subjected to two rounds of lysis and precipitation at 0 °C using ice-cold 10% trichloroacetic acid for least 5 min in each round. The lysates were centrifuged to collect precipitates. After the pellets were dissolved in 10% sodium dodecyl sulfate, the amounts of [^{3}H]thymidine were determined by scintillation counting. DNA synthesis rates were expressed relatively as percentages of the activity observed in the control group, which was treated with the harmine solvent but without harmine.

Results

Specific CDK inhibition by harmine

We conducted inhibitor screens to target Cdk2/cyclin A and Cdk5/p25 from compounds isolated from medicinal herbs that are used in cancer therapeutic formulas. From the screens, harmine (Fig. 1) was identified to exhibit strong inhibition towards the targets. The harmine effect was then carefully examined using Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25. It displayed dose-dependent inhibition of the tested CDKs (data not shown). Under the assay condition using 15 μM ATP-Mg^{2+}, IC_{50} values are approximately 17, 33, and 20 μM for Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25, respectively (Table 1).

To test inhibitory specificity, the harmine effect was examined on various protein kinases, including PKA, PKC, Erk, and the Src-related protein tyrosine kinases Lck, Lyn, and Fyn. Kinase activities were measured using respective peptide substrates in the presence of an increasing concentration of harmine. In the assays, poor or negligible effect was detected towards these kinases at low micromoles of harmine and IC_{50} values are at least 10-fold higher than those of Cdk1/cyclin B, Cdk2/cyclin



7-mythoxy-1-methyl-9H-pyridol[3,4-*b*]indole

Fig. 1. Chemical structure of harmine.

Table 1
IC_{50} of harmine

Kinase	Harmine (μM)
Cdk1/cyclin B	17
Cdk2/cyclin A	33
Cdk5/p25	20
PKA	>250
PKC	>250
Erk	>250
Lck	>250
Fyn	>250
Lyn	>250

A, and Cdk5/p25, suggesting that harmine selectively inhibits Cdk1, Cdk2, and Cdk5.

Harmine is an ATP-competitive inhibitor

To probe CDK inhibitory mechanisms, the harmine effect on Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25 was determined at varying ATP-Mg^{2+} concentrations. Results showed that IC_{50} values varied at different ATP-Mg^{2+} concentrations and higher IC_{50}s were observed at higher ATP-Mg^{2+} concentrations (data not shown), indicating a competitive nature between harmine and ATP-Mg^{2+} in the CDK inhibition. Kinetic analysis was then conducted using Cdk5/p25. Assay data demonstrated that harmine is a competitive inhibitor with respect to ATP-Mg^{2+} with a K_i value of 21 μM towards Cdk5/p25 (Fig. 2). Additionally, it is a mixed-type inhibitor with respect to the peptide substrate in the assays (data not shown).

Inhibition of cell proliferation

As Cdk1 and Cdk2 are crucial regulators of the cell cycle, we examined the harmine effect on cell proliferation. Cytotoxicity experiments were conducted on three human carcinoma cell lines, which are HeLa (cervical cancer), MCF-7 (breast cancer), and SW480 (colon cancer). Proliferating cells were treated with harmine at various concentrations. Following the treatment, the amounts of viable cells were determined by the MTT staining. Harmine appeared to inhibit the proliferation of HeLa, MCF-7, and SW480 in a concentration-dependent manner with ED_{50} values of 8, 15, and 22 μM, respectively (Fig. 3). As a comparison, harmine was tested on a confluent culture of Swiss 3T3 fibroblasts, which were in a nondividing and quiescent stage. In contrast to the growth inhibition of the tumor cells, harmine exhibited little effect on the stationary fibroblasts (Fig. 3). Most of the fibroblasts were viable even after the treatment of harmine at a high concentration (100 μM) for 5 days (Fig. 3). Hence, harmine has little cytotoxicity to non-proliferating cells and selectively blocks cell proliferation.

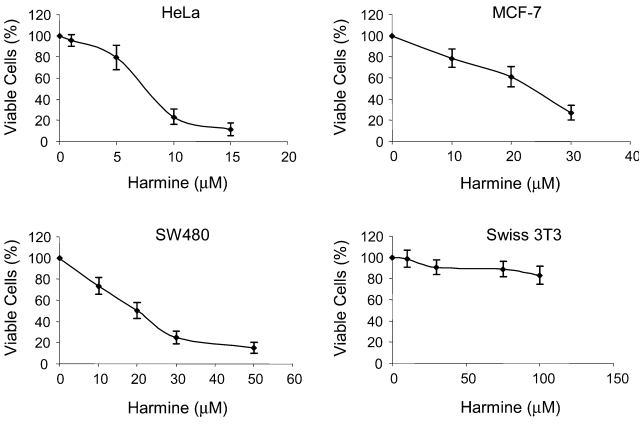


Fig. 3. Anti-proliferation effect of harmine on carcinoma cells. The cytotoxicity of harmine was tested on three tumor cell lines, which are HeLa, MCF-7, and SW480, and quiescent Swiss 3T3 fibroblasts. Proliferating cells of HeLa, MCF-7, and SW480 and confluent Swiss 3T3 fibroblasts were treated with various amounts of harmine as described in the Materials and methods. Viable cells were determined by the MTT staining.

During the cell division cycle, Cdk2 is a key regulator of DNA replication and the S phase progression [16]. Given the result that harmine inhibited the Cdk2 activity, we examined further the anti-tumor cell growth effect of harmine to monitor cellular DNA replication under the harmine treatment. The DNA synthesis of proliferating SW480 was determined by the rate of [^{3}H]thymidine incorporation into DNA. As shown in Fig. 4, harmine strongly inhibited the [^{3}H]thymidine incorporation in the treated cells. Fifty percent inhibition of the incorporation can be achieved at 23 μM of harmine approximately (Fig. 4), displaying a good correlation to its Cdk2 inhibitory activity and its cytotoxicity to SW480. It implicates that the cell proliferation-inhibitory effect of harmine is mainly contributed from its inhibition of CDKs.

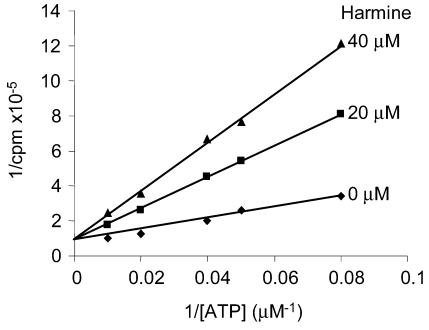


Fig. 2. The CDK inhibition by harmine is competitive with ATP-Mg^{2+}. The kinase activity of Cdk5/p25 was assayed at varying concentrations of ATP-Mg^{2+} in the absence or presence of harmine. One hundred micromolar of the kinase substrate peptide was present in the assays. Double reciprocal plots are from the data of triplicate assays.

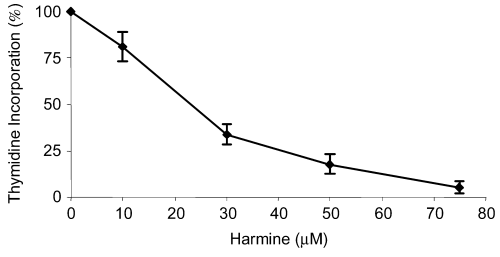


Fig. 4. Effect of harmine on cellular DNA replication. Proliferating SW480 cells were treated with various amounts of harmine. [^{3}H]Thymidine incorporation into DNA was measured as described in Materials and methods.

Discussion

The importance of CDKs in cell proliferation has stimulated a great interest in the development of CDK inhibitors. To date, several classes of CDK inhibitors have been reported, including staurosporine, butyrolactone I, flavopiridol, indirubins, paullones, hymenialdisine, and a series of 2,6,9-substituted purine derivatives such as olomoucine, roscovitine, and purvalanol [17,18]. These compounds exert their inhibitions by competing with ATP-Mg^{2+} for the ATP-binding pocket of CDKs. In the present report, harmine was identified as a novel specific inhibitor of Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25. Like the other CDK inhibitors, it showed a competitive effect with ATP-Mg^{2+} in the inhibition. The inhibition is specific to the CDKs since the activities of other tested protein kinases, which are PKA, PKC, Erk, and the Src-related tyrosine kinases, were not significantly affected by harmine at low micromole concentrations.

Harmine is a β-carboline alkaloid originally isolated from the plant *Peganum harmala* [19]. Powdered seeds of *P. harmala* are used in herbal formulas of Chinese medicine to cure digestive tract tumors. Recently, a number of β-carbolines, including harmine, were shown to have cytotoxicities towards many of the tested human tumor cell lines [20]. However, it is elusive how the β-carbolines exert their anti-tumor growth effects. Although a few cellular enzymes were reported as potential targets of the β-carbolines, such as cytochrome P450, monoamine oxidase A, and DNA topoisomerase I, none of these proteins have a claimed function in the cell cycle control [21–24]. In our assays, we have characterized harmine as a specific CDK inhibitor. Moreover, harmine inhibited DNA replication of the tumor cells and thereby the tumor cell proliferation. Meanwhile, harmine displayed little cytotoxicity to the stationary fibroblasts. Hence, harmine is a specific inhibitor of cell growth and proliferation. The proliferation of eukaryotic cells involves a series of coordinated events, such as DNA replication and mitotic division, which are mediated by CDKs. The present studies provide evidence to indicate that the anti-tumor growth effect of harmine is derived at least partially from its inhibitory activity towards CDKs.

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