Synthesis and investigation of new Hesperadin analogues antitumor effects on HeLa cells

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Abstract Hesperadin is one of the indolinones that was designed against the ATP-binding site of Aurora kinase. This molecule inhibits Aurora B kinase by phosphorylation of histone H3. In this study, new derivatives of Hesperadin containing an amide group in their structures were synthesized through sequential Ugi/palladium-catalyzed approach and in vitro antitumor activity of new compounds were evaluated by cell proliferation assay. The results show that compounds 6f, 6i, 6l, and 6o were dose-dependently inhibited in different concentrations, and IC₅₀ values were between 35 and 43 nM. It seems that lipophilic substitution on the indolinone core with the ability to form additional hydrogen bond might lead to increased stability of structure and activity of new Hesperadin analogues.

Keyword Aurora kinase · Hesperadin · Antitumor effects · Analogues

Introduction

The Aurora kinases are a family of three highly homologous serine threonine protein kinases that play a critical role in

regulating many of the processes that are key roles to mitosis [19]. Aurora A is mainly involved in centrosome function, mitotic entry, and spindle assembly, whereas Aurora B participates in chromatin modification, microtubule-kinetochore attachment, spindle check point and cytokinesis. Aurora C is also a chromosomal passenger protein [4, 5, 7, 8].

Aurora kinases are known to be oncogenic and over-expressed in various forms of tumor cells. These kinases were recently identified as a potential target in anticancer therapy, and various Aurora A and Aurora B kinase inhibitors are being developed [14].

Aurora B is the catalytic component of the chromosomal passenger complex, which is composed of three additional noncatalytic subunits that direct its activity: survivin, INCEP, and borealin [1, 23]. When Aurora B is inhibited, cells fail to biorient their chromosomes and resulting in induction of polyploidy in cells as a consequence of cytokinesis failure [13]. On the other hand, high-level expression of Aurora B in model systems has been linked to chromosome instability [12, 18].

The first Aurora inhibitors to be described were small-molecule inhibitors of Aurora kinase, including Hesperadin, VX-680, and ZM447439 [6, 10, 11].

The small-molecule Hesperadin, a 3-(anilinoaryl)methylene-2-oxindole that acts as an Aurora B kinase inhibitor, was designed against the ATP-binding site of Aurora kinase. Like VX-680 and ZM447439, Hesperadin also inhibits phosphorylation of histone H3 on serine 10 [3, 7]. This compound induces polyploidy in HeLa cells [11] and stops cell growth of prostate and breast cancer cells [15].

In this study, we investigated the antitumor effect of new Hesperadin analogues containing an amide group in their structures on cancer cell line (Table 1). These potentially bioactive 3-(anilinoaryl)methylene-2-oxindoles have been

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Table 1 Characterization of Hesperadin new analogues

Compound	Formula	Structure	IC50 (nm) ^a	Molecular Docking Results	
				Binding Energy (Kcal/mole)	Inhibition Constant (Ki) (nM)
Hesperadin	C ₂₉ H ₃₂ N ₄ O ₃ S		NC	-10.8	12.07
6c	C ₃₆ H ₃₅ N ₃ O ₃		NC	-9.66	82.76
6d	C ₃₆ H ₃₅ N ₃ O ₂		56.73	-9.82	63.12
6e	C ₃₆ H ₃₂ F ₃ N ₃ O ₂		96.47	-9.99	47.42
6f	C ₃₅ H ₃₂ BrN ₃ O ₂		36.38	-10.88	10.53
6i	C ₃₆ H ₃₇ N ₃ O ₂		42.91	-10.42	22.87
6j	C ₃₆ H ₃₅ N ₃ O ₃		NC	-9.5	108.26
6k	C ₃₄ H ₃₁ N ₅ O ₄		NC	-9.23	171
6l	C ₃₄ H ₃₂ FN ₃ O ₂		35.05	10.13	37.34
6m	C ₃₄ H ₃₉ N ₃ O ₂		69.70	-9.69	79.18
6o	C ₃₄ H ₃₃ N ₃ O ₂		38.10	-10.33	26.92

NC not calculated

^a Inhibition of proliferation of HeLa cells measured by cell counts after 48 h of incubation

prepared by a highly efficient palladium-catalyzed method. Ugi/Heck carbocyclization/Buchwald reaction sequences were used for the stereoselective synthesis of Hesperadin analogues with *Z*-configuration.

Material and methods

Synthesis

Some 3-(anilinoaryl)methylene)-2-oxindoles as Hesperadin analogue derivatives were prepared through designing the sequential Ugi/Heck/Buchwald reaction sequencing by palladium-catalyzed approach [2].

In this approach, simple starting materials were selected to generate *N*-substituted-2-alkynamides **I**, which could be synthesized via four-component reactions of benzaldehydes **1**, 2-iodoaniline **2**, phenyl propiolic acid **3**, and isocyanides **4** to form desired *N*-substituted-2-alkynamides **I**. The reaction of intermediate **I** with aniline derivatives in the presence of Pd(OAc)₂ (5 mol %), Cs₂CO₃ and *rac*-BINAP leads to form products (**6a-o**) (Fig. 1).

General procedure for the synthesis of *N*-substituted-2-alkynamides **Ia-o**

2-Iodoaniline (219 mg, 1 mmol), aldehyde (1 mmol), and MeOH (5 mL) were stirred for 30 min. Then, phenyl propiolic acid (146 mg, 1 mmol) and, after 15 min, isocyanide (1 mmol) were added, and the mixture was stirred for 24 h. The progress of reaction was monitored by TLC (eluent hexane/ethyl acetate 5:1). The mixture was washed with saturated NaHCO₃ (30 mL) and was extracted with ethyl acetate (3 × 20 mL). Organic phase was dried with Na₂SO₄. The solvent was removed under reduced pressure.

General procedure for the synthesis of 3-(anilinoaryl)methylene)-2-oxindoles **6a-o**

The Ugi adduct was added to a flask which contained toluene (50 mL), Pd(OAc)₂ (11 mg, 0.05 equiv), cesium carbonate (652 mg, 2 mmol), *rac*-BINAP (62 mg, 0.1 equiv), and aniline derivatives (2 mmol). The mixture was heated under reflux condition for 7 h. After cooling to room temperature, the reaction mixture was washed with brine (2 × 30 mL) and organic phase was collected. The combined organic layers were dried with sodium sulfate, concentrated to dryness in vacuo, and purified by column chromatography on silica gel (hexane/ethyl acetate 10:3) to give **6a-o** with 59–96 % (Fig. 1).

Cell culture

Human cervical cancer cell line (HeLa) was obtained from Pasteur Institute of Iran. Cells were cultured in RPMI 1640 medium supplemented with 10 % (V/V) heat-inactivated fetal bovine serum (Gibco, USA) and 100 IU/mL penicillin, 100 µg/mL streptomycin (Invitrogen). Cells were incubated at 37 °C in an atmosphere containing 5 % CO₂.

Cell proliferation assay

To determine the antiproliferation activity of the new analogues dose-dependent proliferation, experiments were performed. In brief, the following steps were carried out: 1,500 cells were plated in multiple 96-well dishes (Greiner Bio-one, Germany). After 24 h, the cells were treated with 12.5, 25, 50, and 100 nM concentrations of each analogue and control cells were plated in medium containing 1 % DMSO. This treatment was repeated during 2 days. After 48 h, cells were trypsinized and after Trypan-blue staining, the viable cells were counted with Neubauer-type hemocytometer and the data was expressed as the percentage of control. All proliferation assays were performed at least three times. It is necessary to be mentioned the polyploidy in cells case false negative result

Fig. 1 **a** MeOH, rt, 24 h, **b** 5 mol% Pd(OAc)₂, 10 mol% *rac*-BINAP, 2 eq. Cs₂CO₃, aniline derivatives, Toluene, reflux [2]

in proliferation assay by XTT kit. Because of the increase in cell diameter, the DNA content could not be measured by flow cytometry.

Cell staining

Cytomorphological changes in treated HeLa cells were observed with an Olympus phase-contrast microscope. Cell aneuploidy was confirmed by using nuclear staining with a vital dye such as propidium iodide (PI) according to the manufacturer's instructions. Briefly, HeLa adherent cells were washed with the culture medium, and then, PI was added (1 $\mu\text{g/mL}$). After rinsing with culture medium to remove excess dyes, the cells were observed by fluorescent microscope (Olympus).

Statistical analysis

Statistical analysis was done by ANOVA test using SPSS software for Windows. The IC₅₀ was calculated from dose–response curves and also using ED50V10 software for Microsoft Excel Add Ins [22].

Molecular docking simulations

Docking study of new Hesperadin analogues with Aurora kinase B (PDB_ID: 2BFY) was performed in order to find specific binding model using AutoDock 4.0 [16, 17]. The binding affinity of a given analogues is determined via AutoDock; the program gives affinity in terms of binding energy (kcal/mol) and inhibition constant (K_i). All ligand structures were prepared and optimized with HyperChem program. The grid parameter file of receptor was generated using AutoDock 4.0. The grid size is set such that the box containing the entire receptor binding site and accommodate ligand to move freely. The number of grid points in x -, y -, and z -axes were $126 \times 126 \times 126$ Å. The spacing of distance between grid

points was 0.153 Å. The center of the ligand in the X-ray crystal structure was used as the center of the grid box.

Lamarckian genetic algorithm (LGA) [9] was chosen as the docking search parameter for receptor-rigid and ligand-flexible docking calculations. The number of search attempts (ga_run parameter) was set on 100. Other docking parameters were set to the software's default values. After complete execution of AutoDock, 100 conformations of ligand in complex with the receptor were obtained, which were finally ranked on the basis of binding energy and inhibition constant (K_i). The resulting conformations were visualized in the AutoDock Tools 1.5.6.

Results

Synthesis

Potentially bioactive 3-(anilinoaryl)methylene)-2-oxindoles as Hesperadin analogues were synthesized stereoselectively via a two-step procedure: (a) an Ugi-4CR and (b) reaction of the Ugi adduct with aniline in the presence of a palladium catalyst via *domino* Heck/Buchwald reaction. In all cases, a single isomer with the *Z*-configuration was obtained in good to high yields [2], which is related to intramolecular hydrogen bonding.

The structures of the products 6a-o were characterized using as a single *Z*-stereo isomer in all cases. The *Z*-configuration of the product was indicated by the signal at δ 5.98–6.08 ppm for the H-4 oxindole proton. This unusual chemical shift is related to anisotropy of phenyl ring. Also, the -NH protons are deshielded and the chemical shifts were observed at 11.86–12.13 ppm. This result was not surprising due to the intramolecular hydrogen bond that can exist between the amino group and the carbonyl group in *Z*-stereo isomer.

Fig. 2 Effect of different concentrations of new analogues on HeLa cell line. Each column represents the cell number percent in comparison with control. The data are representative of three independent experiments. Analysis of variance: $P < 0.005$

Fig. 3 Treatment of HeLa cell line with the new analogues, **a** HeLa cells (control), **b** HeLa cells after treatment with 50 nM compound 6l and staining with PI, **c** and **d** HeLa cells after treatment with 50 nM compounds 6f and 6o, respectively, and staining with hematoxylin (Arrows show the aneuploid form of cells)

Effect of Hesperadin analogues on cell proliferation

Cell proliferation was inhibited with different concentrations of the new analogues (12.5, 25, 50, 100 nM). Figure 2 shows a comparison of the cell frequency of

the cancer cell line after treatment with the new analogues and Hesperadin.

HeLa cells, treated with DMSO (as a control), showed a normal cell cycle profile. In contrast, after 48 h of new analogues treatments, these compounds induced polyploidy

Fig. 4 Interaction of Hesperadin and new analogues with Aurora kinase B. **a** 1 Hesperadin, 2 6f, and 3 6l compounds in Aurora B active site. (The residues involve in binding site are presented in stick form, and red and

blue circles indicate groups involved in hydrogen bonds). **b** LIGPLOT view of the interaction of 1 Hesperadin, 2 6f, and 3 6l compounds, with Aurora kinase B

in the cells (Fig. 3). The majority of the cells had double DNA contents (4N). Among the analogues synthesized, compounds 6l, 6f, 6o, and 6i inhibited cell proliferation with IC₅₀ values 35, 36.3, 38.1, and 42.9 nM, respectively (Table 1).

Molecular docking

The docking simulation technique was performed by using AutoDock 4.2 to show molecular interactions of Aurora kinase B with new Hesperadin analogues. The docked structure of new analogues with Aurora kinase B is shown in Fig. 4.

The lowest energy docked conformation of the most populated cluster (the best cluster) was selected and taken into account. The binding energy and inhibition constant (K_i) of each analogues are illustrated in Table 1. The results showed a better interaction of 6f with Aurora kinase B in terms of inhibition constant and binding energy, although the other analogues showed a good interaction in the following order: 6f > 6i > 6o > 6e > 6d > 6l. LIGPLOT view of the interaction of Hesperadin and new analogues with Aurora kinase B indicated that they occupied a similar position in the active site (Fig. 4).

Discussion

The ATP-binding pocket of protein kinases represents an ideal target for pharmacological therapy, and differences in the nature of residues lining the ATP-binding cavity explain the selectivity of many kinase inhibitors [13, 20]. Hesperadin as an inhibitor of ATP-binding site of Aurora kinase inhibits the activity of Aurora kinase B with IC₅₀ values of 250 nM in vitro [14]. In contrast, HeLa cells treated with 50 nM of Hesperadin stopped proliferation followed by polyploidization [5, 11]. Our cell proliferation assay result showed that compounds 6f, 6i, 6l, and 6o were dose-dependently inhibited in different concentrations, and IC₅₀ values were between 35 and 43 nM which are lower than that of Hesperadin.

Crystal structure studies of Aurora B:INCENP:Hesperadin complex clarify mechanism of Hesperadin binding to the Aurora B active site. Hesperadin binds to a conformation of Aurora B in which the activation loop is fully stretched. The indolinone moiety of Hesperadin sits in the catalytic cleft. At one end of the indolinone ring, the central phenyl ring is in Van der Waals contact with the side chains of Leu99, Val107, and Glu177 and points toward the entry site to the catalytic cleft. The following phenylamine is squeezed between Gly176 and the side chain of Leu99 and precedes a piperidine group exposed to solvent. At the opposite end of the indolinone, the sulfonamide moiety points into the active site, with the sulfur and oxygen atoms roughly occupying the position of the α -phosphate of ATP [21]. Our docking data showed that new analogues have located in Aurora B active site like Hesperadin.

Also, 6f and 6l compounds occupied a similar position of Hesperadin and binds to almost the same amino acids in active site. In other hand, compound 6f showed the binding energy and inhibition constant better than Hesperadin.

According to our results, it suggested that lipophilic substitution on the indolinone core with the ability to form additional hydrogen bond lead to increased stability of structure and its activity.

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