Research in Pharmaceutical Sciences, June 2017; 12(3): 196-203
Received: June 2016
Accepted: January 2017

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

In vitro evaluation of dihydropyridine-3-carbonitriles as potential cytotoxic agents through PIM-1 protein kinase inhibition

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Abstract

PIM-1 protein kinase inhibitor belongs to a novel class of serine/threonine kinases. As PIM-1 is overexpressed in cancer cells and possesses oncogenic functions, its inhibition provides a new option in cancer therapy. In this study, *in vitro* inhibitory effects of seven analogues of 1, 2-dihydropyridine-3-carbonitrile derivatives **Ia-c, IIa-d** on the activity of recombinant PIM-1 were evaluated using dimethylthiazol diphenyltetrazolium bromide (MTT) assay. The PIM-1 protein kinase inhibitory potencies and the cytotoxicity effects of tested compounds were respectively as follows: **Ic** > **IIa** > **Ia** > **IIb** > **Ib** > **IId** > **IIc** and **IIb** > **IIa** > **Ia** > **IIc** > **Ic** > **Ib** > **IId,** respectively**.** The compound **Ic** with methylthio imidazole substituent at C-3 position and benzodioxole substituent at C-6 position of 2-imino-1, 2-dihydropyridine-3-carbonitrile structure showed the strongest PIM-1 inhibitory effect (IC_{50} = 111.01 nM), while the compound **IIc** with methythio imidazole substituent at C-3 position and benzodioxole substituent at C-6 position of 2-oxo-1, 2-dihydropyridine-3- carbonitrile structure exhibited the least inhibition activity (IC_{50} = 433.71 nM). The docking results showed that all tested compounds localized appropriately in the middle of binding cavity after docking procedure, demonstrating suitable interactions between ligands and protein. This study demonstrated that the PIM-1 inhibitory potencies of newly synthesized compounds were in submicromolar concentrations (IC_{50} < 150 nM) while they exhibited low cytotoxicity on HT-29 cell line (IC_{50}> 130 µM). Altogether, our data indicated that compounds **Ic**, **IIa**, **Ia** could be considered as new potent non-toxic PIM-1 inhibitors which could be used in combination with routine anti-proliferative drugs.

Keywords: PIM-1; Protein kinase inhibition; Dihydropyridine-3- carbonitrile; Anticancer; Cytotoxicity

INTRODUCTION

The proto-oncogenic protein, PIM-1, is a unique serine/threonine kinase because it has a constant rate of synthesis regardless of physiological demands or the concentration of substrate. The level of PIM-1 enzymatic activity in a cell is dependent on the absolute amount of protein present (1). Previously it was shown that overexpression of PIM-1 leads to tumour formation in mice, while silencing of this protein exhibited no detectable effect (2). It has been reported that PIM-1 contributes to cancer development in different major ways, for example by inhibiting apoptosis and promoting cell proliferation instability (3-6). In hematopoietic malignancies and in numerous solid tumours, including pancreatic, prostate, Burkit lymphoma and colon cancer the overexpression of PIM-1 has been shown to be correlated with the development of malignancies compared with normal tissues that slightly express PIM-1 (6-9). It has been proved that PIM-1 phosphorylates and regulates the activities of several proteins involved in apoptosis, cellular division, signal transduction pathways and transcriptional regulation which are overall linked to cell survival (10).

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Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362.207200

PIM-1 has been demonstrated to be synergistically related to various oncogenic transcription factors like NFATc1, RUNX, c-Myb, SOCS1, RelA/p65 and c-Myc, and consequently stimulates expression of cytokines and survival factors (11-14). Furthermore, PIM-1 kinases inactivate the pro-apoptotic BAD protein, which is a pro-apoptotic protein regulated by phosphorylation and thereby inhibit apoptosis (15). It has been shown that the overexpression of PIM-1 can contribute to malignant transformation (16). In this regard, it was found that in hematopoietic malignancies, prostate and colorectal cancers, PIM-1 promotes tumour onset and progression. Therefore, PIM-1 knockdown or inhibition led to antitumour effects (17,18). These characteristics make PIM-1 an attractive therapeutic target for development of novel anti-proliferative agents.

Over the past decade, great efforts have been made to develop novel PIM-1 kinase inhibitors with different chemical structures, including indolocarbazoles, bisindolyl-maleimides, naphthyridines, pyridazines and isoxazoles to thiazolidine-2,4-diones, thieno-pyrimidinones, pyridones and isoxazolo-quinolines (19-21). In previously published work possible correlation between cytotoxicity and phosphodiesterase and PIM-1 inhibition of 4,6-diaryl-2-imino-1,2-dihydropyridine-3 carbonitriles and their isosteric 4,6-diaryl-2-oxo-1,2-dihydropyridine-3-carbonitriles has been reported (22). In this study in continuation of our previously published work (22), PIM-1 inhibitory effects of seven 4-imidazolyl-6-aryl-2-imino-1,2-dihydropyridine-3- carbonitriles **(Ia-c)** and their relative 2-oxo isoesters **(IIa-d)** were evaluated both *in silico* and *in vitro*. Moreover, the *in vitro* cytotoxicities of these compounds were tested against human colorectal adenocarcinoma cell line (HT-29).

MATERIALS AND METHODS

Chemistry

All tested compounds were synthesized as reported previously (22) and used in the present study to examine their inhibitory effects on the activity of recombinant PIM-1 using MTT assay.

Preparation of 6-(4-bromophenyl)-4-imidazolyl-2-imino-1,2-dihydropyridines-3-carbonitriles and 6-(1,3-benzodioxol-5-yl)-4-imidazolyl-2-imino-1,2-dihydropyridines-3-carbonitriles (Ia–c)

A mixture of *p*-bromoacetophenone or 3,4-methylenedioxy acetophenone (2.5 mmol), 2-akylthio-1-benzyl-imidazole-5-carbaldehyde (2.5 mmol), malononitrile (0.16 g, 2.5 mmol) and ammonium acetate (1.54 g, 20 mmol) in ethanol anhydrous (50 mL) was heated under reflux for 18-24 h. The reaction mixture was cooled and the formed precipitate was filtered, washed with ethanol, then washed successively with water, dried and crystallized from DMF/ethanol 1:2, respectively.

Preparation of 6-(4- bromophenyl)-4-imidazolyl-2-oxo-1,2-dihydropyridin-3-carbonitriles and 6-(1,3-benzodioxol-5-yl)-4-imidazolyl-2-oxo-1,2-dihydropyridin- 3-carbonitriles (IIa–d)

A mixture of *p*-bromoacetophenone or 3,4-methylenedioxy acetophenone (2.5 mmol), appropriate aldehyde (2.5 mmol), ethyl cyanoacetate (0.28 g, 2.5 mmol) and ammonium acetate (1.54 g, 20 mmol) in ethanol (50 mL) was heated under reflux for 10-20 h. The reaction mixture was cooled and the formed precipitate was filtered, washed with ethanol, then washed successively with water, dried and crystallized from DMF/ethanol 1:2, respectively.

In vitro evaluation

Cell culture

HT-29 cell line was obtained from the National Cell Bank of Pasteur Institute of Iran (Iran, Tehran). Cells were cultured in the RPMI medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere (95%) containing 5% CO_2 for three days.

MTT assay

Cells were seeded in a 96-well plate (2×10^4 cells/well) and exposed to various concentrations of synthesized analogues (6.25 μM, 12.5 μM, 25 μM, 50 μM, 75 μM, 100 μM and 200 μM) at 37 °C, 5% of CO_2 for 24 h (23). Then, dimethylthiazol diphenyltetrazolium bromide (MTT) (Sigma, USA) was added to each well at final

concentration of 0.5 mg/mL. Subsequently, microtiter plate was incubated for an additional 4 h at 37 °C and medium was then removed. Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystal formed by viable cells. Absorbance was measured at 545 nm with background of 630 nm using a microplate reader and demonstrated as a percent relative to untreated control cells.

PIM-1 inhibitory assay

PIM-1 activity was calculated using Cyclex PIM-1 kinase assay/inhibitor screening kit (Cat No. CY-1167). In this kit, PIM-1 enzyme, in presence of Mg^{2+} and ATP, phosphorylates the immobilized p21waf1, PIM-1 substrate. The amount of phosphorylated substrate was semi quantified following incubation with anti Thr 145 phospho-p21waf1 polyclonal antibody (PWT-01), horseradish peroxidase conjugated anti-rabbit IgG, and chromogenic substrate tetra-methylbenzidine (TMB). Absorbance was measured at 450 nm using a Synergy H4 Hybrid Multi-Mode Microplate Reader (Biotek, Model: H4MLFPTAD). The absorbance values reflected the relative amount of PIM-1 activities in the presence of different concentrations of newly synthesized compounds.

Docking

Docking studies were employed to investigate mode of interactions between PIM-1 kinase and synthesized compounds. Synthesized compounds were drowned by ChemDraw Ultra 8.0 software and 3D structures were prepared by Hyperchem 7 software using AM1 semi-empirical calculation following molecular mechanic force filed pre-optimization. The crystal coordinate of human PIM-1 kinase co-crystallized with its inhibitor compound protein data bank (PDB ID code: 20BJ) was downloaded from protein data bank database (www.rcsb.org).

Further preparations including water molecules removal and polar hydrogens addition was performed by MOE software. Docking was run in MOE software adjusting all residues in 5 A° around of co-crystallized ligand as binding site and synthesized analogues as ligand. Refinement was set to force field and all other parameters were set as default. Validity of docking procedure was examined using co-crystallized inhibitor as ligand and above-mentioned binding site and protocol.

Statistical analysis

All data shown represent means ± SD from triplicate experiments performed in a parallel fashion. Statistical differences between groups were analyzed by one-way ANOVA analysis followed by Tukey-Kramer test for multiple comparisons using Prism 6 software.

RESULTS

Chemistry

In this study the dihydropyridine carbonitrile derivatives were prepared as reported previously (22). The derivatives **Ia-c, IIa-d** were prepared by substitution of methylthio or ethylthio imidazole substituent at C-3 position and bromophenyl or benzodioxole substituent at C-6 position on 2-oxo-1, 2-dihydropyridine-3- carbonitrile or 2-imino-1,2-dihydropyridine-3- carbonitrile structures. Briefly, *p*-bromoacetophenone or 1-(1, 3-benzodioxol-5-yl) ethanone were reacted with the 2-akylthio-1-benzyl-imidazole-5-carbaldehyde, in the presence of malononitrile or ethyl cyanoacetate and ammonium acetate for synthesis of 4-imidazolyl-6-aryl-2-imino-1,2-dihydropyridine-3-carbonitriles (**Ia–c**) and 4-imidazolyl-6-aryl-2-oxo-1,2-dihydropyridine-3-carbonitriles (**IIa–d**). Chemical structures of synthesized compounds are shown in Fig. 1.

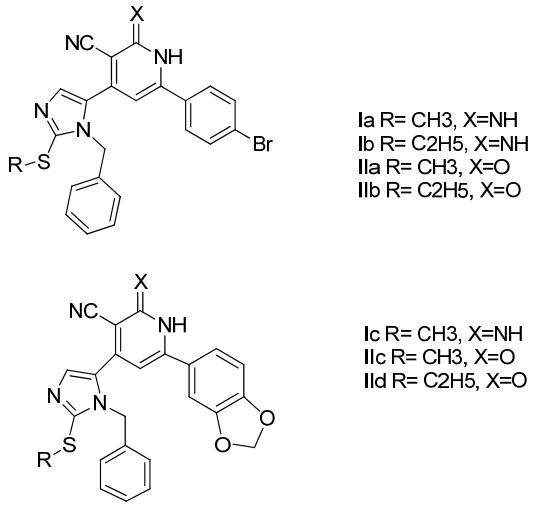

Fig. 1. Chemical structure of synthesized compounds.

Cytotoxic effects of synthesized compounds were evaluated using MTT assays (Fig. 2). The HT-29 cells were incubated with different concentrations of newly synthesized compounds **Ia-c, IIa-d** for 24 h. Our data show that analogue **IIb** was the most toxic compound with IC_{50} = 97 µM on HT-29 cell line among the newly synthesized compounds (**P* < 0.05 *vs* **IIa**, ***P* < 0.001 *vs* **Ia**, and *****P* < 0.0001 *vs* **Ib**, **Ic**, **IIc**, **IId**), while the analogue **IId** exhibited lowest cytotoxicity (IC_{50} = 393 µM).

Our data (Fig. 3) showed that analogues **Ic**, **IIa** and **Ia** were stronger PIM-1 inhibitors than analogues **IIc, IId** and **Ib,** while compound **IIb** showed moderate PIM-1 inhibitory effect. Low PIM-1 inhibitory activity of analogues **IId** and **IIc** compared with other compounds indicates that the benzodioxole group at C-6 position of 2-oxo-1, 2-dihydropyridine-3-carbonitrile structures reversely influenced the PIM-1 inhibitory activities of synthesized compounds.

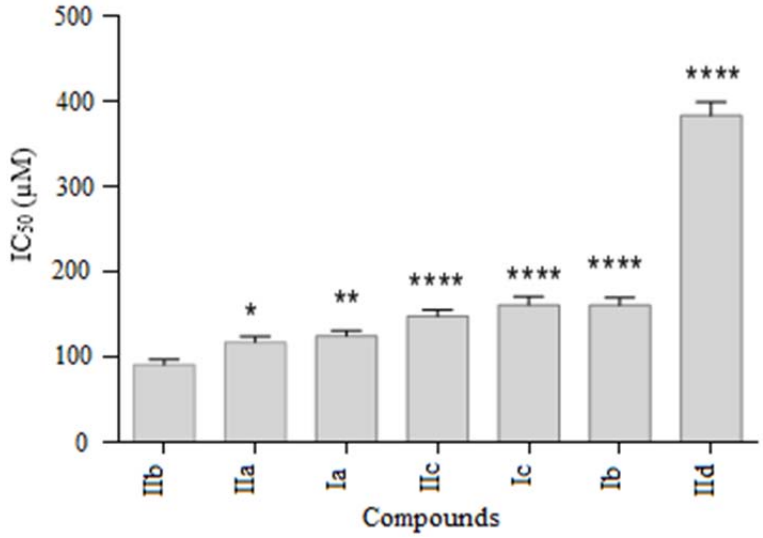

Fig. 2. IC_{50} values of cytotoxicity of synthesized compounds in human colon HT-29 cancer cell line. All compounds were compared with **IIb**. **P* < 0.05, ***P* < 0.001 and *****P* < 0.0001.

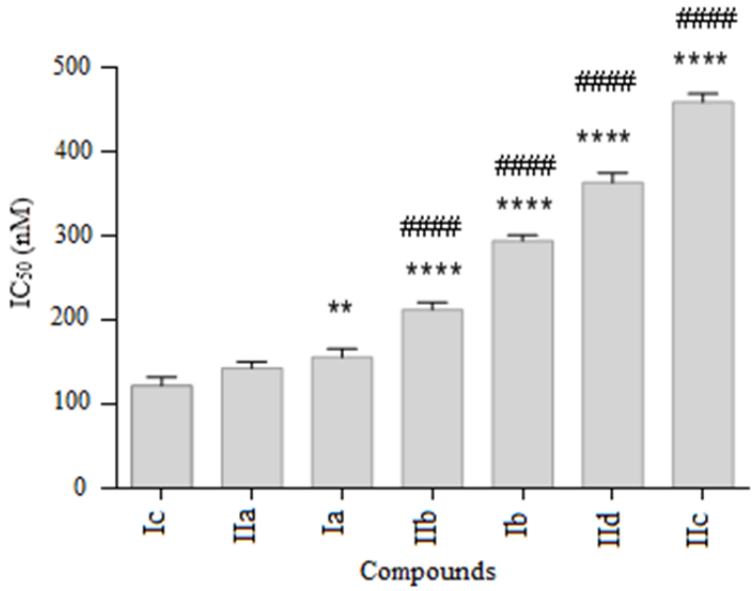

Fig. 3. IC_{50} values of PIM-1 inhibitory potency of synthesized compounds in human colon HT-29 cancer cell line. ***P* < 0.01 *vs* **Ic**, *****P* < 0.0001 *vs* **Ic**, ####*P* < 0.0001 *vs* **IIa**.

Among newly synthesized dihydropyridine carbonitrile derivatives, **Ic**, **IIa** and **Ia** showed efficient PIM-1 kinase inhibitory effects (IC_{50} = 111.01, 115.43 and 132.47 nM, respectively), with very low cytotoxicity (IC_{50} > 130 µM) in HT-29 cell line. Statistical analysis of data showed that compounds Ic and IIa were significantly more potent inhibitors for PIM-1 than other compounds (***P* < 0.01 *vs* **Ia**, *****P* < 0.0001 *vs* **Ib**, **IIb**, **IIc**, **IId** ####*P* < 0.0001 *vs* **Ib**, **IIb**, **IIc**, **IId**).

The root mean square deviation (RMSD) between the best pose of co-crystallized inhibitor docked into the binding site and the one in the crystal structure for PIM-1 kinase was 0.698 A° (Fig. 4). As shown in Fig. 5 seven synthesized compounds localized appropriately in the middle of binding cavity after docking procedure. Benzodioxol group in compound **Ic** also demonstrated another opportunity for hydrogen bond formation via Lys-67 and may justify potent inhibitory effect of this analogue (Fig. 6).

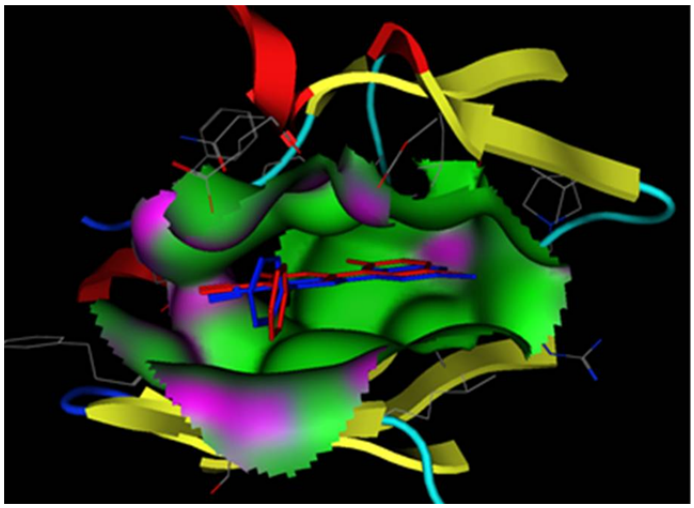

Fig. 4. 3D representation of the best pose of co-crystallized inhibitor (blue color) docked into the binding site and superimposed on co-crystallized inhibitor (red color) in the crystal structure of PIM-1 kinase was also shown (PDB ID: 20BJ).

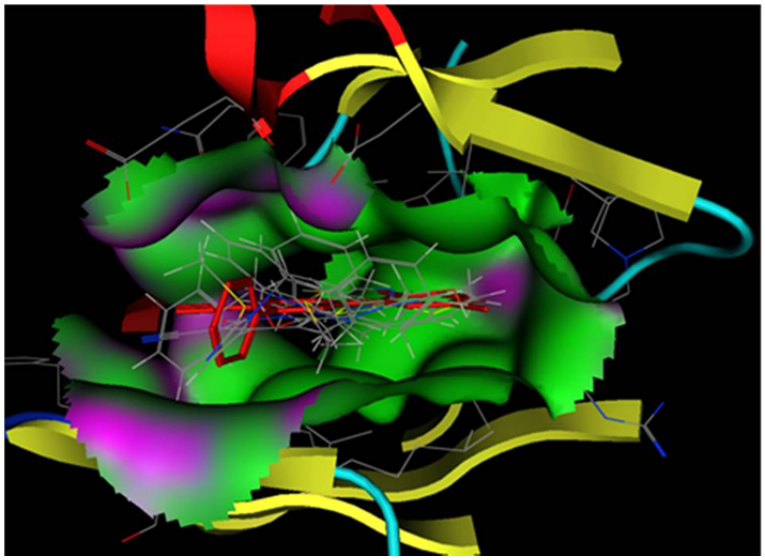

Fig. 5. Seven docked compounds superimposed in binding cavity of PIM-1 kinase. Compounds represent as line and colored by atom. Co-crystallized inhibitor is also shown as stick and red. Green hydrophobic, violet hydrogen bond and blue as middle polar represent electrostatic map of binding cavity.

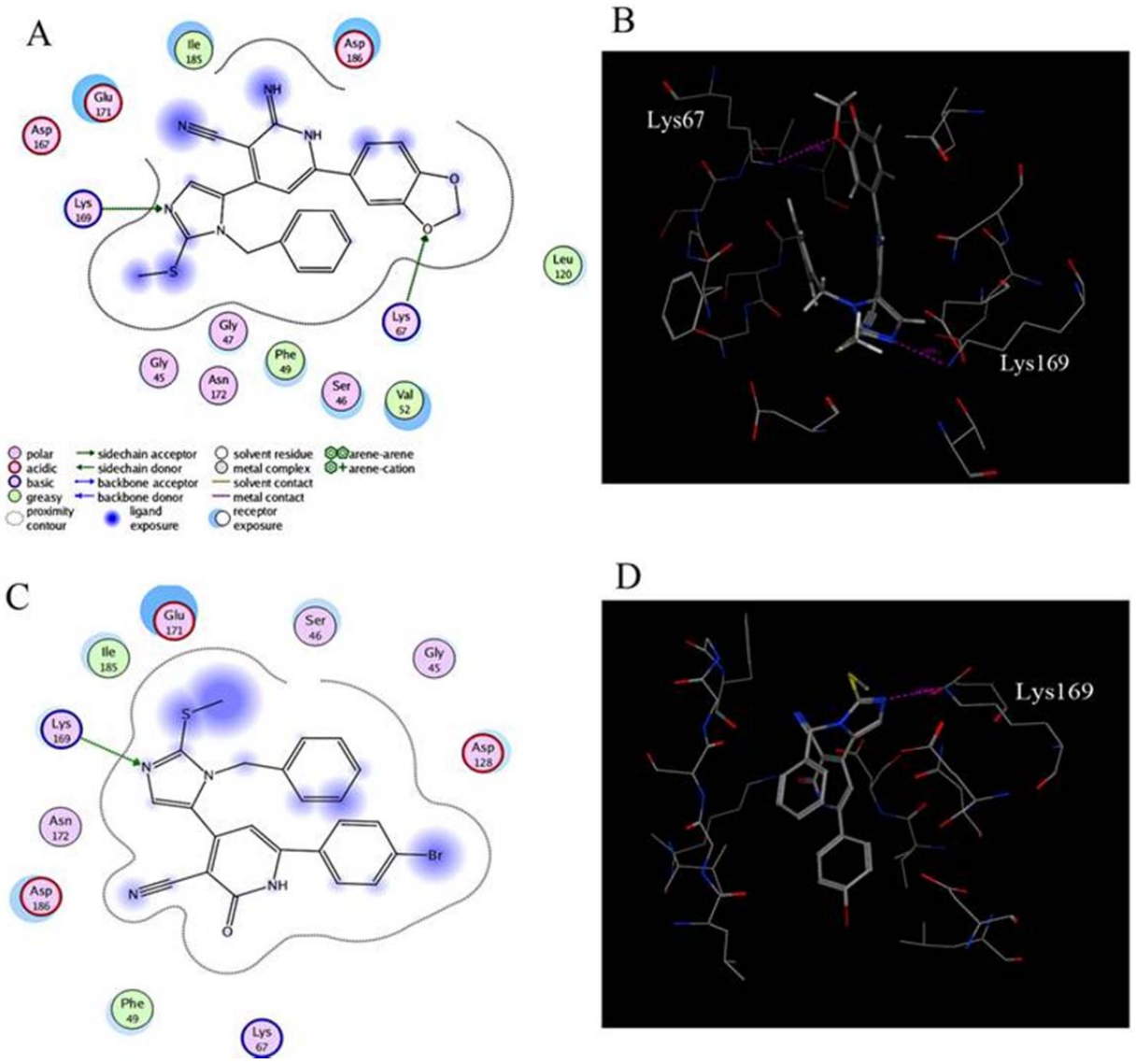

Fig. 6. 2D graphs of interactions between compounds, (A) **Ic**, (C) **IIa**, and PIM-1 kinase (PDB ID: 20bj). Graphs are made using MOE software. In this plots hydrophobic/aromatic residues are colored in green, whereas polar amino acids in magenta. H bonds are shown as green dotted lines. The active site contour is also shown. Part B and D demonstrate 3D position of compound **Ic** and **IIa** (respectively) in active site of enzyme, hydrogen bonds are also shown as violet dotted lines.

DISCUSSION

In our previous study, the cytotoxic effects of the aforementioned synthetized compounds were evaluated on MCF-7 and HeLa cell lines and the findings demonstrated notable cytotoxicity of compounds **Ia, Ib, Ic** and **IIb** against both cell lines (22). In the present study compounds showed less toxicity on HT-29 cell line. It should be considered that different cytotoxic activity of the same compounds on various cell lines may be, in part, due to the different characteristics of cancerous cell lines which makes HT-29 cell line is less sensitive to the tested compounds compared to MCF-7 or HeLa cells.

The significant difference between cytotoxicity of analogue **IId** compared to other analogues (especially **IIb** and **Ic**) demonstrated the effect of benzodioxole aromatic substitution on C-6 position of 2-oxo-1, 2-dihydropyridine carbonitrile structure on reduction of anti-proliferative activity of synthesized compounds. The significant reduction in cytotoxicity of **IId** in comparison with **IIc** suggested that changing the alkyl

chain length of the substituent on position C-2 in imidazole ring from methylthio to ethylthio decreased antiproliferative activity. Our results suggest that the presence of shorter alkyl chain on position 2 in imidazole substituent, i.e. methylthio instead of ethylthio, result in elevation of PIM-1 inhibitory activities of synthesized compounds which is consistent with previous study (22).

Although some studies have shown that PIM-1 plays a crucial role in cell proliferation and cell survival (24), our results proved that the synthesized compounds did not show significant toxicity on HT-29 cell line. We found that **Ic**, **IIa** and **Ia** caused no discernible cytotoxicity on human colon carcinoma cell line (HT-29) while they were potent PIM-1 inhibitors.

Various studies have demonstrated that the therapeutic effects of PIM inhibitors are mediated through certain pathways since combinations of PIM blockers with individual signal transduction inhibitors did not exhibit the same effect (26).

We found that **Ic**, **IIa** and **Ia** alone caused no discernible cytotoxicity on human colon carcinoma cell line HT-29 and PIM-1 maybe the exclusive target known to be potently antagonized by these compounds. Further studies are required to search for appropriate anti-proliferative drugs which can be co-administered with our newly synthesized potent PIM-1 inhibitors for cancer chemotherapy.

The obtained results of RMSD showed that there is a good ability to reproduce the ligand binding mode. The suitable interactions between ligands and protein were demonstrated in docking studies. The interaction of **Ic**, **Ia** and **IIa** being the most potent inhibitors of PIM-1 kinase amongst studied compounds with protein revealed that hydrogen bonds between nitrogen atom in imidazole ring and Lys-169 was an important interaction in these chemicals.

Our results were in agreement with previous study (22) which showed that the benzodiazocine derivative of pyrrolo [2,3-*a*] carbazole potently inhibited PIM-1 (IC_{50} = 8 nM) whereas exhibited a negligible cytotoxicity for human colon carcinoma HCT116 cell line at concentrations >10 µM within 72 h of cell exposure (25). The synthetized compounds in our study may potentiate the effect of other chemotherapeutic agents like observed in the last report which in the presence of PIM-1 and 3 inhibitors, IC_{50} of doxorubicin in HCT116 cells significantly decreased from 200 nM to 25 nM (22).

Briefly, In comparison with pervious work on the same compounds (22) it seems the compounds were less active in inhibition of PIM-1 in comparison to phosphodiesterase-3 inhibition.

CONCLUSION

Altogether, our data indicated that synthesized PIM-1 inhibitors (**Ia, Ic**, **IIa**) could effectively decrease PIM-1 kinase activity in submicromolar concentrations < 150 nM while they exhibited low cytotoxicity in HT-29 cell line (IC_{50} > 130 µM). In fact, the synthesized compounds (**Ia, Ic**, **IIa**) could inhibit PIM-1 activity at non-toxic concentration. Overall, it seems hydrogen bonds through Lys-169 and Lys-67 are the most important interaction in PIM-1 kinase inhibition by the synthesized analogues. This new series of PIM-1 inhibitors could be implemented in combined cancer chemotherapy regimens along with routine antiproliferative drugs.

ACKNOWLEDGMENTS

The authors are thankful for financial support from the Research Council of Mashhad University of Medical Sciences (Grant No. 88515). This work was a part of Pharm. D thesis.

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