Communication

Synthesis and Biological Evaluation of 3-Aryl Pyrazoles as CDK2/HDAC Inhibitor for Anticancer Agents

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Abstracts: A novel series of pyrazole derivatives containing hydroxamic acid group were designed and synthesized as multi-target inhibitors targeting CDK2 (cyclin-dependent kinases 2) and HDAC (histone deacetylase). Compounds 6e and 6f exhibited most potent CDK2 inhibition as well as HDAC inhibition. *In vitro* antiproliferative assay indicated that several compounds showed better antiproliferative potency compared to olomoucine and SAHA. Docking simulation suggested a common mode of interaction at the active binding sites of CDK2 and HDAC, which demonstrates that compound 6f is a potential agent for cancer therapy deserving further researching.

Keywords: Pyrazole, CDK2, HDAC, Inhibitor, Anticancer

1. Introduction

Protein kinases play pivotal roles in regulating aspects of metabolism, gene expression, cell growth, cell division and cell differentiation through exerting precise and reversible control on protein phosphorylation.[1, 2] The regulation of the cell cycle is a well orchestrated pairing of different family members of the Cyclin Dependent Kinases (CDKs) and cyclin regulatory subunits. [3] These kinases upon activation with their cyclins, phosphorylate and modulate the activity of many target substrates such as organizational proteins, transcription factors as well as proteins involved in the replication assembly and machinery of cells. Among these CDKs, CDK2 is especially important, which controls in the majority of cancer cases. [4] In complex with cyclin E, CDK2 plays a paramount role during the G1/S transition of the cell cycle while in complex with cyclin A it facilitates the progression of the S phase of the cell cycle. However, so far, the activity observed does not match the initial expectations

for CDK inhibitors. [5-7] No CDK inhibitor has been approved for commercial use until now, despite first-in-class clinical compounds, such as the pan-CDK inhibitors Flavopiridol and CY-202 have undergone numerous phase II and phase III clinical trials. [8]

There are several possible explanations for both the modest activity and the toxicity of the CDK inhibitory molecules observed in the clinical setting. Compounds with activity against CDKs and additional kinase targets that might be useful to enhance anti-tumor activity; this group features ZK-304709 (with additional VEGFR1-VEGFR3 and PDGFR- β activity), JNJ-7706621 (CDK1, CDK2 and CDK3 inhibitor with Aurora A/B activity) and GPC-286199 [CDK1, CDK2, CDK3, CDK5, CDK7 and CDK9 inhibitor with additional activity toward CRK (CDK-related kinase)]. [5-9] Among traditional CDK2 inhibitors, pyrazole ring is a promising skeleton that has shown the good anti-CDK2 activity with selective and low toxic properties, such as PNU-292137, 1H-indazole-3-carboxylic acid (4-sulfamoyl

phenyl)amide and 4-amino-1H-pyrazole-3-carboxylic acid ethyl Ester (Figure 1). [10-12] Actually, in our group a series of

N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)aniline derivatives have been designed and synthesized, and their biological activities were also evaluated as potential antitumor and cyclin dependent kinase 2 (CDK2) inhibitors. [13] Several compounds displayed high potent CDK2/cyclin E inhibitory activity *in vitro*.

As a continuation of our effort to obtain more potent anticancer agents, we tried to extend the CDK2 inhibitors

mutual interactions by introduction of additional kinase target (HDAC). HDAC inhibitors have also been shown to synergize with other agents, including RTK inhibitors, to suppress proliferation and induce apoptosis in tumor cells. [14-18] For example, two reference compounds, vorinostat and panobinostat, (Figure 1) were used to achieve HDAC and EGFR inhibition, respectively. Actually, in those reported literatures, it was a common strategy that a hydroxamic acid group was linked to another pharmacophore for obtaining multi-target anticancer agents.

Figure 1. Inhibitors of CDK2 with pyrazole core and HDAC.

We wondered whether there would be any advantage for a CDK2 inhibitor connected with a hydroxamic acid group could serve as both a CDK2 and HDAC inhibitor. Herein, a series of CDK/HDAC inhibitors containing pyrazole ring and hydroxamic acid group was synthesized. This work describes our efforts in exploring a CDK2/HDAC inhibitor for anticancer agents represented by structure1, which shows excellent activities against both CKD2 and HDAC.

Figure 2. General synthesis of 6a-6i.

2. Synthesis

The synthetic route of the 3-aryl pyrazole derivatives with hydroxamic acid group (6a-6i) are outlined in Figure 2. [13, 19] Firstly, acetophenone as starting material was converted to semicarbazone 2, which was formylated under the action of POCl_3 and DMF to form the 3-aryl pyrazole-4-carboxaldehydes 3. 4 was synthesized from 3 and

various amines by direct reductive amination using NaBH_4 as a reducing agent. Ethyl acetate was introduced through electrophilic substitution reaction by Ethyl bromoacetate in good yield. And the esters were replaced with NH_2OH to afford the target compounds. The synthetic compounds gave satisfactory elementary analytical and spectroscopic data. ^1H NMR and ESI-MS spectra were consistent with the assigned structures.

3. Biological Evaluation

Figure 3. The chemical structure of 6a-6i.

Table 1. Inhibition of CDK2/cyclinE and HDAC and antiproliferative activity towards MCF-7 and B16-F10 celllines of 6a-6i.

Compounds	n	R	IC ₅₀ (μM)			
			CDK2	HDAC	MCF-7	B16-F10
6a	1		6.79±0.09	38±0.9	17.86±0.19	8.24±0.6
6b	1		0.82±0.03	35±1.3	10.82±0.13	5.23±0.04
6c	1		1.86±0.03	29±0.8	8.88±0.41	39.2±0.9
6d	3		10.88±1.17	19.8±0.98	5.32±0.11	21.3±0.87
6e	3		4.63±0.06	3.7±0.53	1.12±0.05	3.90±0.35
6f	3		2.95±0.02	2.76±0.08	2.96±0.03	2.53±0.06
6g	6		36.79±1.88	0.62±0.03	16.79±1.28	3.23±0.03
6h	6		10.82±0.93	8.33±0.51	13.77±0.13	9.31±0.21
6i	6		17.86±0.92	10.3±0.12	6.96±0.03	5.13±0.01
Olomoucine			4.11±0.07	37±0.8	2.19±0.01	0.82±0.01
SAHA			26±0.5	0.95±0.02	10.55±0.17	7.33±0.07

The enzymatic inhibitory activities of the target compounds were evaluated in CDK2 and HDAC kinase activity assays. The results were summarized in Figure 3 and Table 1. As shown in the Table, all the target compounds showed moderate to potent CDK2 inhibitory activities, with IC_{50} values ranging from 0.82 to 36.79 μ M. Compounds 6b, 6c and 6f showed strong inhibitory effect (IC_{50} = 0.82 ± 0.03 μ M, 1.86 ± 0.03 μ M and 2.95 ± 0.02 μ M, respectively), which were better than that of Olomoucine (IC_{50} = 4.11 ± 0.07 μ M). For the HDAC inhibitory activities, it was marginally affected by the length of linker, as indicated by the comparison of three groups of compounds (compounds 6a-6c, compounds 6d-6f and compounds 6g-6i). The optimal carbon chain length seems to be six, which gives an IC_{50} of HDAC inhibition similar to that of SAHA. Compound 6g exhibited the most potent HDAC inhibition, with an IC_{50} value of 0.62 μ M. In contrast, the data suggested that the carbon chain length was not so critical for CDK2 inhibition. Even though, when the carbon chain was too long ($n = 6$), the CDK2 inhibitory activities decreased to a certain extent. Therefore, the chain length of 3 carbon atoms may be a suitable connecting point for CDK2 inhibitory activities, without sacrificing HDAC inhibitory activities. Just as that indicated in Table 1, only compounds 6e and 6f exhibited potent CDK2 inhibition as well as HDAC.

To further ascertain the utility of hetero aromatic HDAC inhibitors at the cellular level, MCF-7 and B16-F10 celllines were selected for evaluation, Table 1 showed their IC_{50} values. Part of the target compounds exhibited similar or superior antiproliferative effect to Olomoucine and SAHA. From the results, to a certain degree, the correlation between the *in vitro* and in cell IC_{50} values was observed. Compounds 6e and 6f exhibited the high potent antiproliferative activities against both MCF-7 and B16-F10 cells, which was similar to SAHA and SB1317. The greater cellular potency of 6e and 6f may be attributed to the synergistic effects resulting from simultaneous inhibition of HDAC and CDK2. This result indicated the anti-proliferative effect was produced partly by connection of CDK2/HDAC protein and the compounds.

Figure 4. Compound 6f (colored red) is bond into ATP binding sites of CDK2 (entry 1H0V in the Protein Data Bank). The blue lines show the hydrogen bonds.

Figure 5. Compound 6f (colored red) is bond into the active sites of HDLP (entry 1C3S in the Protein Data Bank). The blue lines show the hydrogen bonds.

To gain better understanding on the possible binding modes of the synthesized target compounds on CDK2 and HDAC, we proceeded to examine the interaction of these compounds with CDK2 and HDAC. All docking runs were applied CDOCKER protocol of Discovery Studio 2.5, and the compound 6f was selected as the representative compound. Fig.4 demonstrates the compound 6f docking into the ATP binding cavity of CDK2 (PDB code: 1H0V). In the binding mode, compound 6f is nicely bound to the ATP binding site of CDK2 *via* two hydrogen bonds. The nitrogen atom near to cyclopropyl formed one hydrogen bond with the amino group hydrogen of LYS 33 (bond length: 2.316 Å). The oxygen atom of the amide bond formed another hydrogen bond with the amino group hydrogen of LYS 89 (bond length: 2.478 Å). The binding model of compound 6f into the binding site of HDLP (Histone Deacetylase-Like Protein, PDB code: 1C3S) is depicted in Fig.5. In this binding model, the two hydrogen atoms of hydroxamic acid group of compound 6f forms two hydrogen bonds with the active hydrogen atom on imidazole ring of His 131 (bond length: 2.142 Å) and His 132 (bond length: 2.345 Å), respectively. In addition, it is obvious that the aromatic rings at the other end of the aliphatic chain is well tolerated. The nice binding model of compound 6f with CDK2 and HDLP indicates that compound 6f could be a potent dual CDK2/HDAC inhibitor.

4. Conclusion

In summary, several novel pyrazole derivatives containing an hydroxamic acid group simultaneously inhibiting CDK2 and HDAC were conceived, synthesized, and evaluated *in vitro*. Most of the compounds exhibited potent CDK2 or HDAC inhibitory activities and antiproliferative activities against MCF-7 and B16-F10 cells. Among all of the compounds, only compounds 6e and 6f exhibited potent

CDK2 and HDAC inhibition simultaneously. The two compounds may display promise as multi-target anticancer agent, and have been selected for further study as novel anticancer agents.

5. Experiments

5.1. Materials and Measurements

All the NMR spectra were recorded on a Bruker DRX 400 model Spectrometer in CDCl₃, and chemical shifts were reported in ppm(δ). ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. Elemental analyses were performed on a CHN-O-Rapid instrument. Melting points were measured on a Boetius micro melting point apparatus. All compounds were purified by recrystallization, column chromatography or reverse phase chromatography. Column chromatography was performed on silica gel (200-300 mesh). TLC was run on the silica gel coated aluminum sheets (Silica Gel 60 GF254, E. Merk, Germany) and visualized in UV light (254 nm).

5.2. Synthesis of (E)-1-(1-Phenylethylidene)Semicarbazide (2)

A thoroughly ground mixture of 100 g of semicarbazide hydrochloride and 100 g of anhydrous sodium acetate was suspended in 1 l of ethanol, and the suspension was refluxed for 45 min and filtered while hot. Acetophenone, 0.83mol, was added to the mother liquor under reflux, and the mixture was refluxed for 1 h, cooled to 20°C, allowed to stand for 10 h, and the precipitate that formed was filtered off, washed with a little ethanol cooled to 5°C, and dried to isolate semicarbazone.

Yield: 93%, white solid, MS (ESI): 178.3 (C₉H₁₂N₃O, [M+H]⁺). Anal. Calcd. for C₉H₁₁N₃O: C61.00, H6.26, N23.71%. Found: C61.63, H6.32, N23.62%. ¹H NMR (500MHz, DMSO-*d*₆, δ ppm): 2.18 (s, 3H), 6.47 (m, 2H), 7.35 (m, 3H), 7.83 (m, 2H), 9.31 (s, 1H).

5.3. 3-Phenyl-1H-Pyrazole-4-Carbaldehyde (3)

Freshly distilled POCl₃, 353g, was added dropwise at 0°C to 440 ml of dry DMF. The mixture was allowed to stand for 20 min at 20 °C and cooled to 0°C, after which 1 mol of semicarbazone was added so as the temperature of the reaction mixture did not rise over 40-50°C and uniform gas evolution was observed. After all semicarbazone had been added, the mixture was stirred for 1.5 h at 80°C and quickly poured onto 1.5 kg of ice. The mixture was treated with 30% aqueous NaOH to pH 8-9, allowed to stand for 30 min, and treated with conc. HCl until heterogenization (pH 5-7). After 18 h, the solid material was separated, washed with water, and dried at 50°C to obtain 3.

Yield 91%, yellow powders, MS (ESI): 173.5 (C₁₀H₉N₂O, [M+H]⁺). Anal. Calcd. For C₉H₈N₂O: C69.76, H4.68, N16.27%. Found: C69.08, H4.62, N16.39%. ¹H NMR (500 MHz, DMSO-*d*₆, δ ppm): 7.50 (m, 3H), 7.82 (m, 2H), 8.36 (s, 1H), 9.90 (s, 1H), 13.58 (m, 1H).

5.4. 3-Phenyl-1H-Pyrazole-4-Carbaldehyde (6a-6i)

Compound 3 (1 mmol) was dissolved in 5 ml of ethanol, and various amines was added to the solution. The reaction mixture was refluxed for 30 min, and cooled to room temperature. NaBH₄ of 0.5 mmol was added to the reaction solution slowly, and stirred at room temperature for 2 h. The mixture was evaporated under vacuum, and dissolved in dichloromethane (5 ml). The solution was washed with saturated NaCl solution and water, respectively, dried over anhydrous sodium sulfate, and evaporated to afford 4. Compound 4 could be used in the next step with no purification.

Compound 4 (0.256 g, 1 mmol), proportional ethyl bromoacetate, and K₂CO₃ in 50 ml of dry CH₃CN were stirred at reflux for 2-3 h. After the completion of reaction, the soln. was added to a mixture of ice and H₂O, and the yellow solution was extracted with three 10-ml portions of AcOEt. The combined organic layers were washed with H₂O, dried (Na₂SO₄), filtered, and evaporated to afford 5.

A solution of hydroxyl amine hydrochloride (271 mg, 3.9 mmol) in 10 mL of MeOH, KOH (219 mg, 3.9 mmol) was added and stirred at 40°C for 10 min. The reaction mixture was cooled to 0°C and filtered. Compound 5 (10 mg, 0.02 mmol) was added to the filtrate followed by KOH (0.04 mmol) at room temperature for 3 hours. The reaction mixture was extracted with EtOAc. The organic layer was washed with saturated NH₄Cl solution and brine, and dried over MgSO₄, filtered and concentrated. The residue was purified by reverse phase preparative HPLC.

2-(4-((benzylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)-N-hydroxyacetamide (6a)

Pale solid, m.p.: 251-253°C. MS(ESI): 337.2 (C₁₉H₂₁N₄O₂, [M+H]⁺). Anal. Calcd. for C₁₉H₂₀N₄O₂: C67.84, H5.99, N16.66%. Found: C67.72, H5.91, N16.58%. ¹H NMR (500 MHz, DMSO-*d*₆, δ ppm): 2.97 (s, 2H), 3.75 (m, 4H), 7.21-7.40 (m, 8H), 7.67-7.75 (m, 3H), 8.78 (s, 1H), 10.29 (s, 1H), 12.86 (s, 1H).

2-(4-((cyclohexylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)-N-hydroxyacetamide (6b)

White solid, m.p.: 222-223°C. MS (ESI): 329.6 (C₁₈H₂₅N₄O₂, [M+H]⁺). Anal. Calcd. for C₁₈H₂₄N₄O₂: C65.83, H7.37, N17.06%. Found: C65.26, H7.31, N17.19%. ¹H NMR (500 MHz, DMSO-*d*₆, δ ppm): 1.06-1.24 (m, 5H), 1.67-1.76 (m, 5H), 2.49 (m, 1H), 3.00 (s, 2H), 3.66 (s, 2H), 7.34 (m, 1H), 7.42 (m, 2H), 7.70 (m, 3H), 8.67 (s, 1H), 9.87 (s, 1H), 12.78 (s, 1H).

N-hydroxy-2-(4-((isobutylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)acetamide (6c)

White solid, m.p.: 245-247 °C. MS (ESI): 303.6 (C₁₆H₂₃N₄O₂, [M+H]⁺). Anal. Calcd. for C₁₆H₂₂N₄O₂: C63.55, H7.33, N18.53%. Found: C63.68, H7.39, N18.66%. ¹H NMR (500 MHz, DMSO-*d*₆, δ ppm): 0.77 (m, 6H), 1.72 (m, 1H), 2.25 (m, 2H), 2.98 (m, 2H), 3.67 (m, 2H), 7.31-7.52 (m, 3H), 7.74-7.81 (m, 3H), 8.75 (s, 1H), 10.20 (s, 1H), 12.77 (s, 1H).

4-(4-((benzylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)-N-hydroxybutanamide (6d)

Pale solid, m.p.: 215-217°C. MS (ESI): 365.8 (C₂₁H₂₅N₄O₂, [M+H]⁺). Anal. Calcd. for C₂₁H₂₄N₄O₂: C69.21, H6.64, N15.37%. Found: C68.65, H6.69, N15.21%. ¹H NMR (500 MHz, DMSO-*d*₆, δppm): 1.66 (m, 2H), 1.83 (m, 2H), 2.34 (m, 2H), 3.49 (m, 4H), 7.17-7.37 (m, 8H), 7.71 (m, 3H), 8.63 (s, 1H), 10.28 (s, 1H), 12.83 (s, 1H).

4-(4-((cyclohexylmethylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)-N-hydroxybutanamide (6e)

White solid, m.p.: 199-201°C. MS (ESI): 371.2 (C₂₁H₃₁N₄O₂, [M+H]⁺). Anal. Calcd. for C₂₁H₃₀N₄O₂: C68.08, H8.16, N15.12%. Found: C68.23, H8.19, N15.03%. ¹H NMR (500 MHz, DMSO-*d*₆, δppm): 1.03-1.22 (m, 8H), 1.53 (m, 2H), 1.67 (m, 2H), 1.89 (m, 2H), 2.39 (m, 3H), 3.53 (m, 2H), 7.40 (m, 4H), 7.81 (m, 2H), 8.62 (s, 1H), 10.28 (s, 1H), 12.98 (s, 1H).

N-hydroxy-4-(4-((isobutylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)butanamide (6f)

White solid, m.p.: 190-191°C. MS (ESI): 331.7 (C₁₈H₂₇N₄O₂, [M+H]⁺). Anal. Calcd. for C₁₈H₂₆N₄O₂: C65.43, H7.93, N16.96%. Found: C65.31, H7.99, N16.82%. ¹H NMR (500 MHz, DMSO-*d*₆, δppm): 0.76 (m, 6H), 1.60 (m, 3H), 2.14 (m, 2H), 2.27 (m, 2H), 2.40 (m, 2H), 3.48 (m, 2H), 7.35-7.70 (m, 3H), 7.72 (s, 1H), 7.91 (m, 2H), 8.61 (s, 1H), 10.43 (s, 1H), 12.87 (s, 1H).

7-(4-((benzylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)-N-hydroxyheptanamide (6g)

White solid, m.p.: 196-198°C. MS (ESI): 407.2 (C₂₄H₃₁N₄O₂, [M+H]⁺). Anal. Calcd. for C₂₄H₃₀N₄O₂: C70.91, H7.44, N13.78%. Found: C70.73, H7.52, N13.89%. ¹H NMR (500 MHz, DMSO-*d*₆, δppm): 1.71-1.74 (m, 2H), 1.39-1.58 (m, 4H), 1.27 (m, 2H), 2.24 (m, 2H), 2.40 (m, 2H), 3.54 (s, 2H), 3.57 (s, 2H), 7.23-7.37 (m, 8H), 7.61-7.73 (m, 3H), 8.63 (s, 1H), 10.42 (s, 1H), 12.93 (s, 1H).

7-(4-((cyclohexylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)-N-hydroxyheptanamide (6h)

White solid, m.p.: 160-162°C. MS (ESI): 399.7 (C₂₃H₃₅N₄O₂, [M+H]⁺). Anal. Calcd. for C₂₃H₃₄N₄O₂: C69.31, H8.60, N14.06%. Found: C69.59, H8.51, N14.18%. ¹H NMR (500 MHz, DMSO-*d*₆, δppm): 1.66-1.80 (m, 4H), 1.57 (m, 3H), 1.30-1.48 (m, 7H), 1.17 (m, 4H), 2.25 (m, 2H), 2.42 (m, 2H), 2.64 (m, 1H), 3.64 (s, 2H), 7.46 (m, 4H), 7.83 (m, 2H), 8.59 (s, 1H), 10.33 (s, 1H), 13.04 (s, 1H).

N-hydroxy-7-(4-((isobutylamino)methyl)-3-phenyl-1H-pyrazol-1-yl)heptanamide (6i)

White solid, m.p.: 187-189°C. MS (ESI): 373.2 (C₂₃H₃₅N₄O₂, [M+H]⁺). Anal. Calcd. for C₂₃H₃₄N₄O₂: C67.71, H8.66, N15.04%. Found: C67.23, H8.62, N15.17%. ¹H NMR (500 MHz, DMSO-*d*₆, δppm): 0.79 (m, 6H), 1.72-1.76 (m, 2H), 1.37-1.52 (m, 4H), 1.27-1.33 (m, 3H), 2.25 (m, 2H), 2.29 (m, 2H), 2.43 (m, 2H), 3.67 (s, 2H), 7.33-7.68 (m, 3H), 7.76-7.83 (m, 3H), 8.65 (s, 1H), 10.29 (s, 1H), 12.92 (s, 1H).

5.5. Antiproliferation Assay

The antiproliferative activities of the prepared compounds against MCF-7 and B16-F10 cells were evaluated as described elsewhere with some modifications. [20] Target

tumor celllines were grown to logphase in RPMI1640 medium supplemented with 10% fetal bovine serum. After diluting to 2 x 10⁴ cells mL⁻¹ with the complete medium, 100 μL of the obtained cell suspension was added to each well of 96-well culture plates. The subsequent incubation was permitted at 37°C, 5% CO₂ atmosphere for 24 h before the cytotoxicity assessments. Tested samples at pre-set concentrations were added to six wells with Olomoucine as positive references. After 48 h exposure period, 40 μL of PBS containing 0.5 mg/mL⁻¹ of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)) was added to each well. After 4 h incubation, the optical absorbance was measured at 570 nm on an ELISA microplate reader. In all experiments three replicate wells were used for each drug concentration. Each assay was carried out for at least three times.

5.6. CDK2/CyclinE and HDAC Inhibition Assay

The ability of the test compounds 6a-6i to inhibit CDK2/cyclinE was determined according to the reported method. [21] The HDAC inhibitory activities were assessed using the Biomol *FluorideLys* assaykit (AK-500, Enzo Life Sciences, Inc.), as previously described by us [20, 21]. [22, 23]

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Competing Interests

The authors declare that there is no conflict of interest regarding the publication of this article.

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