Synthesis, biological evaluation and molecular docking of N, N’-bis([1,2,4]triazole[4,3-b] [1,2,4,5]tetrazine-6-yl)alkylamine derivatives as potent c-Met antagonists

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Abstract

A series of N, N’-bis([1,2,4]triazole[4,3-b][1,2,4,5]tetrazine-6-yl)alkylamine derivatives is designed, synthesized and evaluated for their inhibition activities against three tumor cell lines and c-Met kinase activity in vitro. These compounds are fully characterized by 1H NMR, 13C NMR, MS, IR and elemental analysis. Antitumor experiments indicate that some of these compounds exhibit significant inhibition activities against A549, Bewo and MCF-7 cancer cell lines. Among them, the IC50 values of 4a indicate better antitumor activities against the A549 (1.21 μM), Bewo (0.68 μM) and MCF-7 (3.74 μM) cell lines than the positive agent cisplatin (9.97 μM for A549, 10.46 μM for Bewo, and 15.03 μM for MCF-7), respectively.

Keywords

anticancer, c-Met, molecular docking, synthesis, tetrazine, triazole

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A series of novel N, N’-bis([1,2,4]triazole[4,3-b][1,2,4,5]tetrazine-6-yl)alkylamine derivatives were synthesized and their anticancer activities against A549, Bewo and MCF-7 were tested. The results suggested that these compounds displayed potent antiproliferative activities.

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Introduction

Cancers are a class of diseases in which a group of cells display uncontrolled growth, invasion, and sometimes metastasis.\(^1,2\) It is major cause of morbidity and mortality worldwide, in every region of the world, irrespective of the level of human development.\(^3\) In preclinical and clinical trials, it has been demonstrated that c-Met inhibitors exhibit antitumor activity in the treatment of multiple types of cancers.\(^4\) Specific small-molecule inhibitors targeting the c-Met tyrosine kinase domain are one of the treatment strategies in clinical trials.\(^5\)

About 75% of Food and Drug Administration (FDA)-approved drugs are derivatives of nitrogen-containing heterocyclic compounds.\(^6\) These N-heterocyclic products exhibit anticancer effects in different types of cancers through inhibiting cell growth and induction of cell differentiation and apoptosis.\(^7\) The successful synthesis and antitumor activity of such nitrogen-containing heterocyclic moieties over the past few years are certainly a case for optimism.\(^7\)

Recently, a series of triazole tetrazine derivatives was synthesized and exhibited significant antitumor activities.\(^6\) 6-Alkylamino[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazine derivatives show strong antiproliferative activities against MCF-7, Bewo, and HL-60 cell lines (e.g. TZXU 1–2, 1.26-2.24 \(\mu M\), 1.09-1.90 \(\mu M\), and 1.16-1.61 \(\mu M\), respectively).\(^8,9\) Some \(\alpha\)-\(\omega\) alkenyl-bis-S-guanidine thiourea dihydrobromide derivatives have been found to exhibit good antiproliferative activities against HeLa, RKO and MCF-10A cell lines (e.g. CJ-2, 9.1 \(\mu M\), 18.7 \(\mu M\), 10.5 \(\mu M\)).\(^11\)

Based on the above information, we have synthesized a series of derivatives containing a bis-triazole tetrazine ring symmetrical structure by substitution reaction with alkyl diamines (Figure 1). Our goal was to improve the antitumor activity of the target compounds.

Results and discussion

Chemical synthesis

The synthetic route to the \(N, N'\)-bis[1,2,4]triazolo[4,3-b][1,2,4,5]tetrazine-6-yl]piperazin-1-yl)methyl]benzoyl]-2-substituted benzohydrazide derivatives show strong anticancer activities against A549, Bewo and HepG2 cell lines (e.g. TZXU-3: 8.4 \(\mu M\), 5.2 \(\mu M\) and 2.4 \(\mu M\), respectively).\(^10\) Some \(\alpha\)-\(\omega\) alkenyl-bis-S-guanidine thiourea dihydrobromide derivatives have been found to exhibit good antiproliferative activities against HeLa, RKO and MCF-10A cell lines (e.g. CJ-2: 9.1 \(\mu M\), 18.7 \(\mu M\), 10.5 \(\mu M\)).\(^11\)

Based on the above information, we have synthesized a series of derivatives containing a bis-triazole tetrazine ring symmetrical structure by substitution reaction with alkyl diamines (Figure 1). Our goal was to improve the antitumor activity of the target compounds.
Finally, the title compounds 4a-f were obtained from compound 3 by treatment with the corresponding alkyl diamine in dioxane or ethyl acetate. The reactions were monitored by thin-layer chromatography (TLC).

The chemical structures of synthesized compounds were elucidated on the basis of 1H NMR, 13C NMR and elemental analysis. In the 1H NMR spectra of compounds 4a-f, a characteristic signal due to the CH proton on the triazole ring appeared at 9.33–9.49 ppm, and the signal due to the NH protons of the alkylamine group appeared at 8.70–9.00 ppm. In the 13C NMR spectra for compounds 4a-f, the signals observed in the region of 125.95–166.94 ppm accounted for the carbons of the triazole-tetrazine rings.

Anticancer activity in vitro

To test the antitumor activities of the synthesized compounds 4a-f, we evaluated their antiproliferative activities against the A549, Bewo, and MCF-7 cell lines by employing the MTT assay. As shown in Table 1, the active analogues demonstrated remarkable cytotoxic activity. In particular, it should be noted that compound 4a (1.21 μM for A549, 0.68 μM for Bewo, and 3.74 μM for MCF-7) showed the strongest biological activity, being better than the positive control cisplatin (9.97 μM for A549, 10.46 μM for Bewo, and 15.03 μM for MCF-7). In addition, compound 4f (2.95 μM for MCF-7) showed better antitumor activity than the positive control (15.03 μM for MCF-7).

Binding mode analysis

To examine whether the compounds inhibit c-Met kinase, we screened products 4a, 4b and 4f against c-Met kinase via homogeneous time-resolved fluorescence immunoassays (HTRF). Compounds 4a, 4b and 4f displayed potent c-Met kinase inhibitory activity with IC50 values of 16.81, 16.34 and 10.77 μM (compared to the positive control staurosporine with an IC50 value of 0.042 μM for c-Met kinase) respectively, which indicated that the potent antitumor activities of these synthetic compounds probably correlated to their c-Met kinase inhibitory activities.

To gain a better understanding of the potency of the studied compounds and to guide further SAR studies, we proceeded to examine the interactions of compounds 4a, 4b and 4f with the c-Met crystal structure (3EFJ.pdb). The molecular docking was performed by inserting compounds 4a, 4b and 4f into the binding site of c-Met kinase. All docking runs were applied using the Sulfurex-Dock of Sybyl-X 2.0.

The binding modes of compounds 4a, 4b and 4f and c-Met kinase are depicted in Figure 2(a)–(c). Compound 4a was potently bound to the active site of c-Met kinase via hydrophobic interactions and the binding was stabilized by four hydrogen bonds and one C—H—π interaction, compound 4b was stabilized by four hydrogen bonds and one π—π interaction, while compound 4f was stabilized by four hydrogen bonds.

The geometries of the interactions are illustrated in Table 2. As can be seen, the order of the effect of molecular docking according to the Total-Score is 4f > 4b > 4a, which is in good agreement with the c-Met kinase inhibitory activity, indicating that compound 4a may have poor antitumor activity. However, 4a shows the best antitumor activities against the A549, Bewo, and MCF-7 cell lines in vitro (Table 1). The above results show that product 4a may possibly be a multi-target inhibitor and is worthy of further study.

The enzyme surface models are shown in Figure 2(d)–(f), which reveal that molecules 4a, 4b and 4f are well embedded in the active pocket of c-Met kinase. These molecular docking results, along with the biological assay data, suggest that compounds 4a, 4b and 4f are all inhibitors of c-Met kinase.

Conclusion

In summary, a series of N, N’-bis([1,2,4]triazole[4,3-b][1,2,4,5]tetrazine-6-yl)alkylamine derivatives have been designed, synthesized and evaluated for their inhibition activities against three tumor cell lines (A549, Bewo and MCF-7) in vitro. Several of the newly synthesized compounds demonstrate obvious antitumor activities. Among them, compound 4a (1.21 μM for A549, 0.68 μM for Bewo, and 3.74 μM for MCF-7) showed the strongest biological activity, being better than the positive control cisplatin (9.97 μM for A549, 10.46 μM for Bewo, and 15.03 μM for MCF-7), and is worthy of further study. The molecular docking result between 4a and c-Met kinase (3EFJ) suggests that this compound is a potential inhibitor of c-Met kinase.

Experimental

Materials and methods

Melting points were recorded on a XRC-1 apparatus and are uncorrected (Beijing Technical Instrument Co., Beijing, China). Infrared spectra were recorded as KBr disks for solid materials on a Nicolet FT-IR-170 spectrometer. The 1H NMR and 13C NMR spectra were run on a Bruker AC400 (400 MHz) spectrometer. Compounds were dissolved in DMSO-d6, and chemical shifts were referenced to TMS (tetramethylsilane). Mass spectra were obtained on an
Table 2. Geometries of interactions of compounds 4a, 4b and 4f.

| Compound | IC50 for c-Met (μM) | Total-scorea | D-H—A | D-H—π/π—π |
|----------|---------------------|---------------|--------|-------------|
|          |                     |               | D-H    | A^b         | H- A (Å) | C-H Cg | Cg | H- Cg (Å) |
| 4a       | 16.81               | 4.20          | N-H(LYS1110) | N^4     | 2.704 | C-H^c | Cg1 | 2.490 |
|          |                     |               | N-H(LYS1110) | N^6     | 1.986 |       |      |        |
|          |                     |               | N-H(ARG1114) | N^7     | 2.172 |       |      |        |
|          |                     |               | N-H(ARG1114) | N^8     | 2.224 |       |      |        |
| 4b       | 16.34               | 5.45          | N-H(MET1160) | N^7     | 1.867 | Cg2   | Cg3  | 3.796 |
|          |                     |               | N-H(MET1160) | N^8     | 2.691 |       |      |        |
|          |                     |               | N-H(PRO1158) | N^10    | 2.405 |       |      |        |
|          |                     |               | N-H(LYS1110) | N^1     | 2.203 |       |      |        |
| 4f       | 10.77               | 8.31          | N-H(LYS1110) | N^4     | 2.677 |       |      |        |
|          |                     |               | N-H(LYS1110) | N^6     | 1.960 |       |      |        |
|          |                     |               | N-H(MET1160) | N^4     | 2.651 |       |      |        |
|          |                     |               | N-H(MET1160) | N^5     | 2.049 |       |      |        |

aTotal-Score represents the overall effect of molecular docking. The higher the score, the better the docking effect between compounds and receptors.
bThe nitrogen atoms all belong to the tetrazine rings, N and N' belong to the different tetrazine rings in the same compound structure, and the numbers represent their positions on the compounds.
cThe chiral carbon atom in PHE1223.
dCg1 is the centroid of the tetrazine ring of 4a.
eCg2 is the centroid of benzene ring of Phe1223.
fCg3 is the centroid of the tetrazine ring of 4f. 

Figure 2. (a–c) Compounds 4a, 4b, and 4f (carbon: gray; nitrogen: blue; oxygen: red) are bonded into c-Met (entry 3EFJ in the Protein Data Bank). The dotted lines show the hydrogen bond interactions. (d–f) The surface model structures of compounds 4a, 4b, and 4f binding with the c-Met complex.
Agilent 1260 Ion Trap LC/MS 500 analysis system. Elemental analyses were performed on a Thermo-Finnigan Flash EA 1112 instrument. TLC was carried out on silica gel UV-254 plates.

**Synthesis of compound 3**

3-(3,5-dimethyl-1H-pyrazol-1-yl)-6-hydrazinyl-1,2,4,5-tetrazine (2) (2 g, 10 mmol) and a catalytic amount of p-toluenesulfonic acid (0.2 g, 1.2 mmol) was added to triethyl orthoformate (16 mL). The reaction mixture was stirred at 60 °C for 2 h. The reaction was monitored by TLC (ethyl acetate). After the reaction was complete, the mixture was cooled to room temperature, and the resulting orange solid was filtered off and washed with diethyl ether to give compound 3 (1.84 g, 85%).

**Synthesis of compounds 4a-f; general procedure**

Compound 3 (1 g, 4.6 mmol) and the corresponding alkylene diamine compound (2.3 mmol) were heated at reflux in ethyl acetate (30 mL). The reaction was monitored by TLC (ethyl acetate). After the reaction was complete, the solvent was evaporated and ice-cold absolute ethanol (5 mL) was added. The precipitated solid was filtered off, and the filter cake was recrystallized from absolute ethanol to afford the target compounds 4a-f.

1,4-Bis[(1,2,4)triazolo[4,3-b][1,2,4,5]tetrazin-6-yl]piperezine (4a): Red solid, 76% yield, mp 300-305 °C. 1H NMR (DMso-d6, 400 MHz): δ = 9.49 (s, 2H, CH), 8.94 (s, 2H, NH), 2.00 (br s, 4H, NHCH2). 13C NMR (100 MHz, DMSO-d6): δ = 166.94 (2C, C=N), 161.74 (2C, C=N), 144.55 (2C, C=N), 46.01 (4C, CH2). IR (KBr, cm-1): 1580 (C=N), 1474 (C=N), 1376 (C=N), 1023 (C=N), 950 (C=N). ESI-MS: m/z (%): 327.1 [(M+H) +, 100]. Anal. Calcd for C8H8N14: C, 34.40; H, 3.21; N, 65.39. Found C, 34.45; H, 3.20; N, 65.45.

N3, N4-Bis[(1,2,4)triazolo[4,3-b][1,2,4,5]tetrazin-6-yl]hexane-1,6-diamine (4b): Yellow solid, 80% yield, mp 189-194 °C. 1H NMR (DMso-d6, 400 MHz): δ = 9.37 (s, 2H, CH), 9.34 (s, 2H, NH), 4.10 (br s, 2H, CH2), 3.03 (br s, 1H, CH), 2.06 (d, J=6.8 Hz, 3H, CH3). 13C NMR (100 MHz, DMSO-d6): δ = 160.28 (2C, C=N), 143.66 (2C, C=N), 142.81 (2C, C=N), 141.26 (2C, C=N), 24.58 (2C, CH2). IR (KBr, cm-1): 1576 (C=N), 1465 (C=N), 1374 (C=N), 1025 (C=N), 971 (C=N), 700 (N-H). ESI-MS: m/z (%): 326.9 [(M+H) +, 100]. Anal. Calcd for C12H16N14: C, 36.58; H, 3.68; N, 59.73. Found C, 36.51; H, 3.67; N, 59.80.

**Biological evaluation**

In vitro cancer cell growth inhibition assay. The antiproliferative activities of compounds 4a-f against several human cancer cell lines were assayed by standard MTT assay procedures. Cells were cultured in DMEM medium at 37 °C with 5% CO2 and 95% air, supplemented with 10% (v/v) bovine calf serum. Cells were plated in 96-well plates at a density of 10,000 cells per well. After 24 h, the cells were treated with various concentrations of all compounds from 0.4 to 50 μM. Wells containing culture medium without cells were used as blanks and cisplatin was assayed over the same time as the positive control. The cells were further incubated for 72 h. The cytotoxicity was measured by adding 5 mg/mL of MTT to each well with incubation for another 4 h. The formazan crystals were dissolved by adding 150 μL of DMSO to each well. The optical density of each well was then measured on a microplate spectrophotometer at a wavelength of 570 nm. The IC50 values were determined from plots of % viability against the dose of each compound added. Each assay was performed in triplicate.

**Molecular docking**

Molecular docking was performed with the Surflex-Dock program interfaced with SybylX-2.0. The programs adapted an empirical scoring function and a patented searching engine. The ligand was locked into the corresponding protein binding site guided by protomol,
which is an idealized representation of a ligand that makes every potential interaction with a binding site. In this work, the crystal structure of c-Met complexed with 2-benzyl-5-(3-chloro-4-(6,7-dimethoxyquinolin-3-yl)oxy)phenyl)pyrimidine-4(3H)-one (PDB entry: 3EFl) was extracted from the Brookhaven Protein Database (PDB http://www.rcsb.org/pdb). At the beginning of docking, all the water and ligands were removed and random hydrogen atoms were added. Next, the receptor structure was minimized over 10,000 cycles using the Powell method in SybylX-2.0. All the compounds were constructed using a sketch molecular module. Hydrogen and Gasteiger-Hückel charges were added to every molecule. Next, their geometries were optimized by the conjugate gradient method in the TRIPPOS forcefield. The energy convergence criterion is 0.001 kcal/mol. Finally, the ligand-based mode was adopted to generate “protomol,” leaving the threshold at the default value of 1.

**c-Met kinase assay** in vitro. HTRF (Homogeneous Time-Resolved Fluorescence) uses two fluorescence labels, europium cryptate (fluorescence donor, EuK) and crosslinked allophycocyanin (fluorescence acceptor, XL665). When both fluorescence molecules are in proximity (<10 nm), the energy of EuK excited by a nitrogen laser (λ = 340 nm) is transferred nonradiatively to XL665, resulting in long-lived emission at λ = 665 nm. The nonspecific fluorescence from unbound XL665 and from some other components in the media or plastic have short decay times, and their interference of the detection signal can be delayed by delaying the detection time. On the other hand, the free EuK excited by a nitrogen laser (λ = 340 nm), resulting in long-lived emission at λ = 620 nm, is used as a background signal. These two specific signals at 665 and 620 nm were measured with a multifunctional microplate reader, and the strength of the detection signal from the reaction system reflects the activity of the tested compounds against c-Met kinase. The HTRF experimental method is described as follows: (1) tested compounds (4 μL, diluted with buffer solution to nine different concentrations: 100000, 20000, 4000, 800, 160, 32, 6.4, 1.28 and 0.256 nM) and c-Met kinase (2 μL) were added to each well, to which was added a mixture (4 μL, v/v = 1:1) of TK Substrate-biotin (1 μM) and ATP (3 μM). Each well was kept at room temperature for 40 min. (2) The mixture (10 μL, v/v = 1:1) of SA-XL665 (0.125 μM) and TK antibody-cryptate (diluted to 100 times) was added to the above wells. After keeping the mixture at room temperature for 1 h, the fluorescence signal was measured with a multifunctional microplate reader.

**Declaration of conflicting interests**

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