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Synthesis, biological evaluation, and molecular docking of ((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl) piperazin-1-yl)methyl) benzohydrazide derivatives

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Abstract

A series of ((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5] methyl) benzo-hydrazide derivatives was designed, synthesized, and evaluated for their inhibition activities against five tumor cells and c-Met kinase in vitro. These compounds were fully characterized by ^{1}H NMR, ^{13}C NMR, MS, and elemental analysis. Antitumor experiments indicated that some compounds exhibited significant inhibition activities against A549 and Bewo. Especially, the IC_{50} values of **5f** (12 μM), **5h** (7.1 μM), **6a** (8.4 μM), and **6d** (9.2 μM) demonstrated better antitumor activities against A549 than the positive agent cisplatin (13.3 μM), and the IC_{50} value of **6a** (5.2 μM) exhibited better antitumor activity against Bewo than cisplatin (7.7 μM).

Keywords

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

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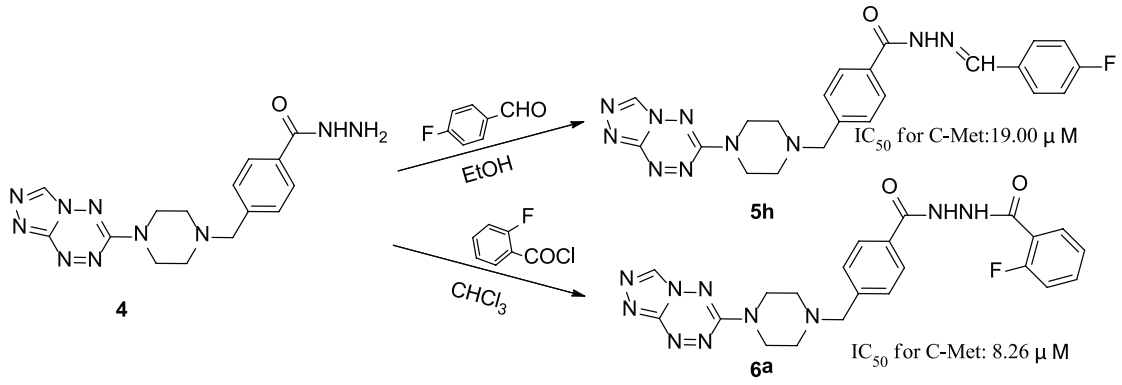

Introduction

Cancer is a class of diseases in which a group of cells display uncontrolled growth, invasion, and sometimes metastasis.[1,2] According to the World Health Organization (WHO), cancer is the second leading cause of death after cardiovascular disease all around the globe and accounted for 8.8 million deaths in 2015 worldwide.[3,4]

c-Met is a unique member of the receptor tyrosine kinases (RTKs) expressed in both normal and malignant

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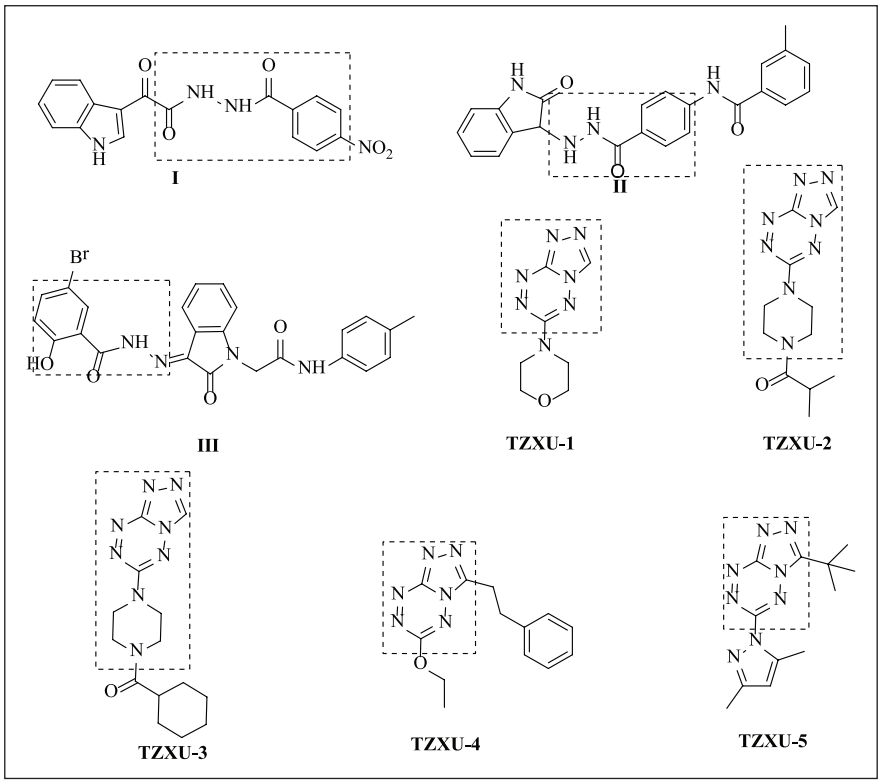


Figure 1. Examples of c-Met inhibitors: benzoyl hydrazide and triazolotetrazine derivatives.

cells. It is a cell-surface receptor for hepatocyte growth factor (HGF), a pleiotropic cytokine that conveys a unique combination of pro-migratory, anti-apoptotic, and mitogenic signals.[5,6] Under normal physiological conditions, the pleiotropic effects of the HGF/c-Met axis are essential for embryogenesis and tissue homeostasis.[7,8] Aberrant HGF/c-Met signaling has been identified in a wide range of human malignancies, including brain, colorectal, gastric, lung, stomach, breast, and head and neck cancers.[9,10] Moreover, both c-Met overexpression and MET amplification have been associated with poor clinical outcomes for cancer patients. Of particular note, amplification of the MET oncogene is observed in epidermal growth factor receptor (EGFR)-mutant non-small-cell lung carcinomas after tyrosine kinase inhibitor failure.[11–14] Therefore, c-Met has become one of the most promising therapeutic targets in anticancer drug discovery.[15]

There have been a number of studies on developing small molecule c-Met inhibitors, and various c-Met inhibitors have been reported.[16] Among these, benzoyl hydrazide derivatives **I**–**III** have been synthesized and found to display c-Met inhibitory activity with IC_{50} values of 69, 1.3, and 8.1 μM, respectively (Figure 1), which has led to them emerging as promising and attractive scaffolds in the anticancer field.[17]

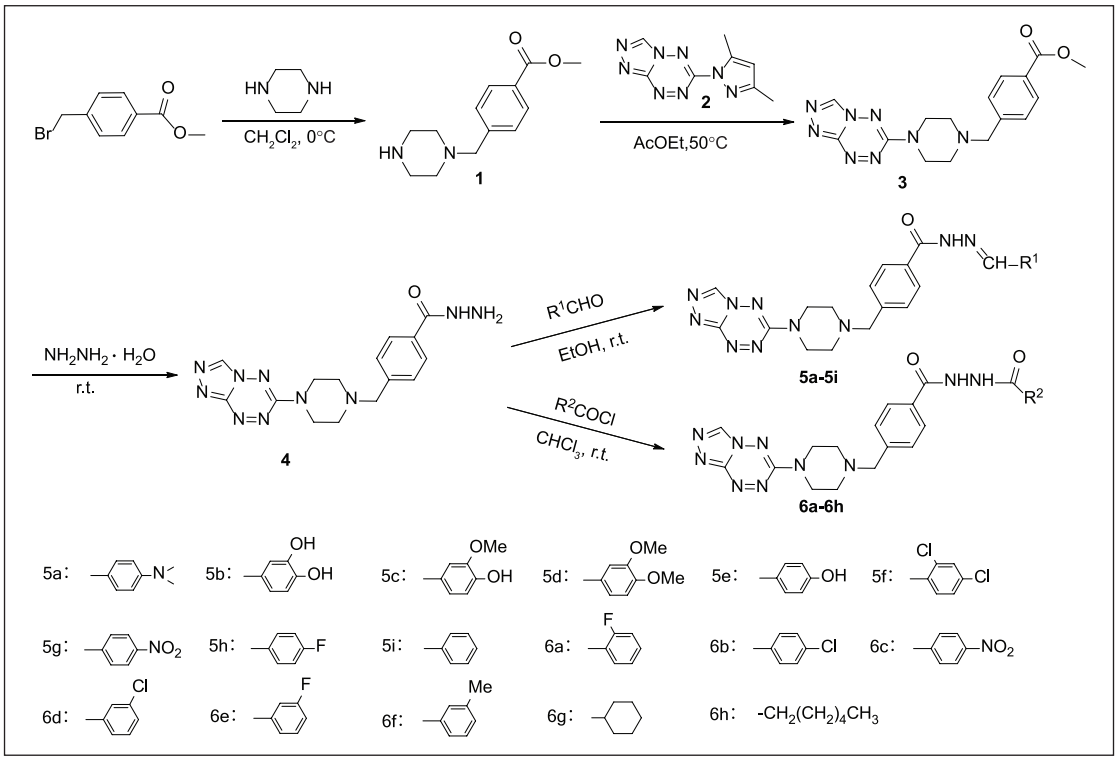
Recently, a series of triazolotetrazine derivatives has been synthesized and found to exhibit significant anticancer activities. 6-Alkylamino-[1,2,4]triazolo[4,3-*b*][1,2,4,5] tetrazine derivatives (**TZXU-1**–**3**) show strong antiproliferative activities against MCF-7, Bewo, and HL-60 cells (IC_{50} = 1.09–2.24 μM) and c-Met kinase inhibitory activities (IC_{50} = 7.9–23.70 μM);[18] 6-alkoxyl-[1,2,4]triazolo[4,3-*b*] [1,2,4,5] tetrazine derivatives exhibit significant anticancer activities against A549, Bewo, and MCF-7 (e.g. **TZXU-4**: IC_{50} = 21.68, 20.72, and 2.81 μM, respectively);[19] 3-alkyl(aryl)-[1,2,4]triazolo[4,3-*b*][1,2,4,5] tetrazine derivatives also show significant antiproliferative activities against MCF-7, Bewo, and HL-60 cells (e.g. **TZXU-5**: IC_{50} = 17.63, 0.73, and 0.73 μM, respectively) (Figure 1).[20]

Based on the above information, we designed a series of new triazolotetrazine derivatives with benzohydrazide groups at the 6-position to investigate further how these substituents located at the 6-position of the [1,2,4] triazolo[4,3-*b*][1,2,4,5]tetrazine ring influence antitumor activity.

Results and discussion

The synthetic routes to the 6-substituted-[1,2,4]triazolo[4,3-*b*][1,2,4,5] tetrazines are summarized in Scheme 1. Methyl 4-(piperazin-1-ylmethyl) benzoate **1** was prepared from methyl 4-(bromomethyl)benzoate and piperazine using dichloromethane as the solvent,[21] which was then reacted with 6-(3,5-dimethyl-*1H*-pyrazol-1-yl)-[1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazine **2** in ethyl acetate at 50 °C to yield compound **3**.[18] Compound **3** was then heated with 80% hydrazine hydrate to obtain compound **4**. Finally, the title compounds **5** and **6** were prepared from compound **4** by treatment with aromatic aldehydes and acyl chlorides, respectively. The reactions were monitored by thin layer chromatography (TLC) plates.

The chemical structures of the compounds synthesized were elucidated on the basis of ^{1}H NMR, ^{13}C NMR, and elemental analysis. In the ^{1}H NMR spectra of



Scheme 1. Synthetic routes to the target compounds **5** and **6**.

compounds **5** and **6**, the characteristic signal due to the CH proton on the triazole ring appeared at 9.46–9.48 ppm and 9.54–9.61 ppm, respectively, and the signal due to the CH_2 protons on the benzyl group appeared at 3.61–3.66 ppm and 4.11–4.66 ppm, respectively. In the ^{13}C NMR spectra for compounds **5** and **6**, the signals observed in the region of 163.0–163.7 ppm and 165.6–175.1 ppm accounted for the carbons at the 6-position of the triazolotetrazine ring, respectively, and the signals observed in the region of 61.8–62.5 ppm and 58.7–62.2 ppm accounted for the carbons (CH_2) on the benzyl group, respectively.

To test the antitumor activities of the compounds synthesized, we evaluated antiproliferative activities of compounds **3–6** against A549, Bewo, Hela, HepG2, and HT29 cells by the MTT assay. The results are summarized in Table 1. As illustrated in Table 1, the active analogues showed remarkable cytotoxic activity. Particularly, it should be noticed that compound **6a** (8.4 μM for A549, 5.2 μM for Bewo, 19.1 μM for Hela, 2.4 μM for HepG2, and 31.1 μM for HT29, respectively) showed the strongest biological activity, comparable to the positive control cisplatin (13.3 μM for A549, 7.7 μM for Bewo, 6.7 μM for Hela, 2.3 μM for HepG2, and 14.0 μM for HT29, respectively). Overall, the inhibitory activities of compound **6** are better than those of compound **5**. This is because the effect of the benzoyl group attached to the benzoyl hydrazide binding to the c-Met receptor is better than that of benzylidene group. For compound **5**, comparing **5a-5e** and **5f-5h**, shows that electron-withdrawing groups such as F, Cl on the phenyl ring enhance the inhibitory activities and electron-donating groups such as $N(CH_3)_2$, OCH_3, OH decrease the inhibitory activities. For A549 cell line, compounds **5f** (12 μM), **5h** (7.1 μM), **6a** (8.4 μM), and **6d** (9.2 μM) show better antitumor activity than the positive cisplatin (13.3 μM). For compounds **6a-6f**, the inhibitory activities of compounds with an *ortho*-substituent on the phenyl ring are superior to those

Table 1. Antitumor activities against A549, Bewo, Hela, HepG2, and HT29 cell lines in vitro (IC_{50} in μM)[a].

Compounds	IC_{50} (μM)				
	A549	Bewo	Hela	HepG2	HT29
3	21.2	18.5	42.2	2.1	45.4
4	13.3	30.8	32.9	6.7	48.0
5a	106.2	134.8	215.2	128.9	204.2
5b	102.1	92.7	186.7	47.8	85.6
5c	239.3	162.8	242.2	129.4	213.8
5d	150.6	98.2	92.7	23.8	174.9
5e	61.7	39.6	60.2	31.2	64.9
5f	12.0	48.8	88.2	53.0	69.4
5g	65.8	85.9	133.0	123.8	178.5
5h	7.1	19.3	16.0	11.9	28.7
5i	25.7	21.6	24.3	8.6	28.7
6a	8.4	5.2	19.1	2.4	31.1
6b	19.7	15.5	20.2	18.2	105.4
6c	49.2	68.5	51.4	28.5	78.9
6d	9.2	38.2	18.8	8.9	34.2
6e	38.5	37.9	28.4	6.5	31.3
6f	24.7	40.1	26.6	7.6	33.3
6g	27.8	37.9	11.3	7.9	39.1
6h	38.9	39.7	13.4	6.5	33.8
Cisplatin[b]	13.3	7.7	6.7	2.3	14.0

[a]Average of three independent experiments.
[b]Cisplatin was used as positive control.

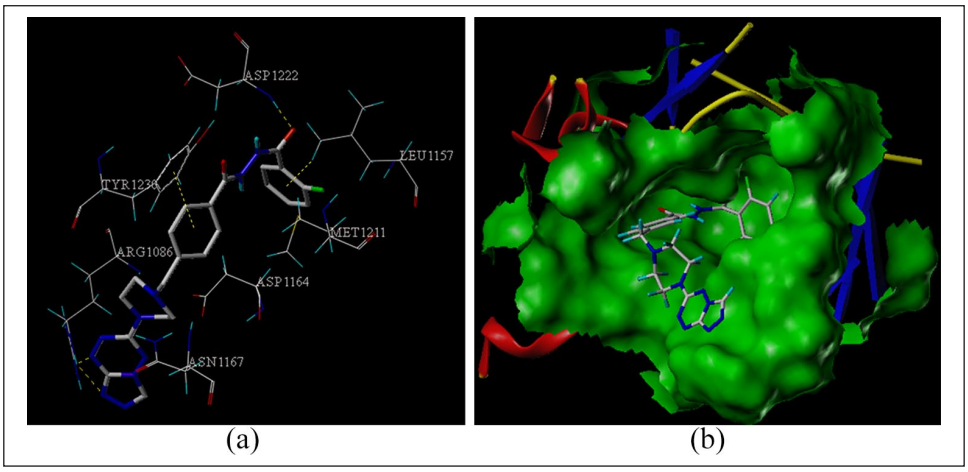


Figure 2. (a) Compound **6a** (colored by atom: carbons: gray, nitrogen: blue, oxygen: red) is bond into c-Met (entry 4DEH in the Protein Data Bank). The dotted lines show the hydrogen bonds, C–H—π and π—π interactions. (b) The surface model structure of compound **6a** binding model with c-Met complex.

with *para*- or *meta*-substitution. In addition, comparing **6a-6f** and **6g-6h**, the inhibitory activities of the latter with alkyl R^2 substitution are almost the same order of magnitude as those of former with phenyl substitution.

To examine whether the compounds inhibit c-Met kinase, we screened compounds **5h**, **6a**, and **6d** against c-Met kinase via homogeneous time-resolved fluorescence immunoassay (HTRF). Compounds **5h**, **6a**, and **6d** displayed strong c-Met kinase inhibitory activity with IC_{50} values of 19.00, 8.26, and 22.4 μM, respectively (the positive control staurosporine has an IC_{50} of 0.042 μM for c-Met kinase), which demonstrated that the antitumor activities of the synthetic compounds probably correlate to their c-Met kinase inhibitory activities.

To gain a better understanding of the potency of the compounds studied and to guide further SAR studies, we proceeded to examine the interaction of compound **6a** with the c-Met crystal structure (4DEH.pdb). The molecular docking was performed by inserting compound **6a** into the binding site of c-Met kinase. All docking runs were applied using the Surflex-Dock of Sybyl-X 2.0.

The binding modes of compound **6a** and c-Met kinase are depicted in Figure 2(a). As illustrated in this figure, compound **6a** is potently bound to the active site of c-Met kinase via hydrophobic interactions and the binding is stabilized by three hydrogen bonds, one C–H—π and one π—π interaction.

The two nitrogen atoms of the triazolotetrazine ring formed two hydrogen bonds with the same amido hydrogen of ARG1086 (bond length: 2.23 and 2.60 Å, respectively), and the oxygen atom of benzoyl group formed one hydrogen bond with the amido hydrogen of ASP1222 (bond length: 2.12 Å). A C–H—π interaction was predicted to form between the methyl hydrogen of LEU1157 and the *o*-chlorophenyl of **6a** (Cg1, Cg1 is centroid of *o*-chlorophenyl ring of **6a**) (C–H—Cg1 = 2.72 Å). A π—π interaction was predicted to form between the two phenyl rings in **6a** (Cg2, Cg2 is centroid of phenyl ring in **6a**) and TYR1236 (Cg3, Cg3 is centroid of phenyl ring in TYR1236) (Cg2—Cg3 = 4.06 Å) (Figure 2(a)). The enzyme surface model is shown in Figure 2(b), which reveals that the target molecule **6a** was well embedded in the active pocket of c-Met kinase. This molecular docking result, along with the biological assay data, suggests that compound **6a** is a potential inhibitor of c-Met kinase.

Conclusion

In summary, a series of ((4-([1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl) methyl) benzohydrazide derivatives has been prepared and evaluated for anticancer activity against five cancer cell lines (A549, Bewo, Hela, HepG2, and HT29). Compounds **5f** (12 μM), **5h** (7.1 μM), **6a** (8.4 μM), and **6d** (9.2 μM) demonstrated better antitumor activities against A549 than the positive agent cisplatin (13.3 μM); **6a** (5.2 μM) exhibited better antitumor activity against Bewo than cisplatin (7.7 μM). The molecular docking of **6a** and c-Met kinase (4DEH) suggested that compound **6a** was a potential inhibitor of c-Met kinase.

Experiment

Materials and methods

Melting points were carried out on a XRC-1 apparatus and are uncorrected (Beijing Technical Instrument Co., Beijing, China). Infrared spectra were recorded using KBr disks for solid materials on a Nicolex FI-IR-170 instrument. The ^1H NMR and ^{13}C NMR spectra were run on a Bruker AC400 (400 MHz). Compounds were dissolved in DMSO-d_6, and chemical shifts were referenced to TMS (tetramethylsilane). Mass spectra were obtained on an 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.

General procedure for the synthesis of Methyl 4-((4-([1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl) benzoate (3)

Step 1. Methyl 4-(bromomethyl)benzoate (7.3 g, 30 mmol) in dichloromethane (50 mL) was added dropwise to a stirred solution of anhydrous piperazine (12.9 g,

0.15 mol) and dichloromethane (150 mL) at 0 °C. The mixture was stirred at room temperature overnight. After the reaction was over (the reaction course was monitored by TLC (AcOEt)), the mixture was washed by a saturated aqueous $NaHCO_3$ solution (2 × 100 mL) and dried over Na_2SO_4. Finally, the solvent was evaporated under reduced pressure and the resulting product was used directly in the next step (yield: 5.6 g, 75.2%).

Step 2. 6-(3,5-dimethyl-*1H*-pyrazol-1-yl)-[1,2,4] triazolo[4,3-b][1,2,4,5]tetrazine **2** (13.5 g, 62.0 mmol) was added to a mixture of methyl 4-(piperazin-1-ylmethyl) benzoate **1** (14.6 g, 62.0 mmol) and ethyl acetate (300 mL). The reaction was carried out for 1 h at 50 °C. After the reaction was over (the reaction course was monitored by TLC (AcOEt/PE = 1/1)), the solvent was evaporated under reduced pressure, and 95% ethanol (50 mL) was added. The yellow solid sediment was filtered and recrystallized in 95% ethanol to yield orange solid (14.2 g, 62.0%).

General procedure for the synthesis of ((4-([1,2,4]triazolo [4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl) methyl) benzohy-drazide (4). Methyl 4-((4-([1,2,4]triazolo[4,3-b][1,2,4,5] piperazin-1-yl)methyl)benzoate **3** (2.0 g, 5.4 mmol) and 80% hydrazine hydrate (30 mL) in a 50-mL three-necked bottle and stirred at room temperature for 1 h. After the reaction was over (the reaction course was monitored by TLC (AcOEt)), the mixture was filtered, washed twice with water and recrystallized in 95% ethanol to yield an orange solid (1.25 g, 65.2%). CAUTION: Safety precautions must be taken when the reaction solution is filtered, as 80% hydrazine hydrate may release a lot of heat during filtration.

General procedure for the synthesis of compounds 5a-5i. Intermediate **4** (1.0 g, 2.7 mmol), the aromatic aldehyde (2.7 mmol), and 95% ethanol (20 mL) were added to a 50-mL single-necked bottle and stirred at room temperature for 2–5 h. After the reaction was over (the reaction course was monitored by TLC (AcOEt/EtOH = 10/1)), the mixture was filtered and recrystallized in 95% ethanol to obtain an orange solid.

General procedure for the synthesis of compounds 6a-6h. Intermediate **4** (1.0 g, 2.7 mmol), the acyl chloride (2.7 mmol), and chloroform (20 mL) were added to a 50-mL single-necked bottle and stirred at room temperature for 5–15 h. After the reaction was over (the reaction course was monitored by TLC (AcOEt/EtOH = 10/1)), the mixture was filtered and recrystallized in 95% ethanol to obtain a yellow solid.

Methyl 4-((4-([1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)benzoate (3). Orange solid, m.p. 141–143 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.48 (s, 1H, CH), 7.95 (d, 2H, *J* = 8.0 Hz, Ar), 7.52 (d, 2H, *J* = 8.0 Hz, Ar), 3.85 (brs, 7H, $2CH_2 + CH_3$), 3.65 (s, 2H, CH_2), 2.58 (t, 4H, *J* = 6.0 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d6): δ 166.6, 155.6, 150.1, 144.2, 136.9, 129.7(2C), 129.5(2C), 128.9, 61.8, 52.6(2C), 52.3(2C), 44.7. ESI-MS *m/z* (%): 377 [$(M+Na)^+$, 100]. Anal. calcd (%) for $C_{16}H_{18}N_8O_2$: C, 54.23; H, 5.12; N, 31.62; found: C, 54.10; H, 5.11; N, 31.70.

((4-([1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl) methyl)benzohydrazide (4). Orange solid, m.p. 176–178 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.77 (s, 1H, NH), 9.48 (s, 1H, CH), 7.82 (d, 2H, *J* = 7.8 Hz, Ar), 7.43 (d, 2H, *J* = 7.8 Hz, Ar), 3.84 (t, 4H, *J* = 6.2 Hz, $2CH_2$), 3.61(s, 2H, CH_2), 2.57(t, 4H, *J* = 6.2 Hz, $2CH_2$), 2.38(brs, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.2, 156.6, 136.9, 132.6, 129.6, 129.4, 129.2 (2C), 127.5 (2C), 61.9, 53.1 (2C), 52.3 (2C). ESI-MS: *m/z* (%): 377 [$(M+Na)^+$, 100]. Anal. calcd (%) for $C_{15}H_{18}N_{10}O$: C, 50.84; H, 5.12; N, 39.53; found: C, 50.95; H, 5.10; N, 39.44.

4-((4-([1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-N′-(4-(dimethylamino)benzylidene) benzohydrazide (5a). Yield: 78%. Orange solid, m.p. 149–151 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.57 (s, 1H, NH), 9.46 (s, 1H, CH), 8.30 (s, 1H, N=CH), 7.88 (d, 2H, *J* = 8.0 Hz, Ar), 7.51 (m, 4H, Ar), 6.75 (d, 2H, *J* = 7.8 Hz, Ar), 3.86 (t, 4H, *J* = 5.8 Hz, $2CH_2$), 3.63 (s, 2H, CH_2), 2.97 (s, 6H, $2CH_3$), 2.58 (t, 4H, *J* = 5.8 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.0, 155.6, 151.9, 150.1, 149.0, 142.0, 136.9, 133.1, 129.3(2C), 128.8(2C), 128.0(2C), 122.1, 112.3(2C), 61.9, 52.3(2C), 44.7(2C), 40.2(2C). ESI-MS: *m/z* (%): 486 [$(M+H)^+$, 40], 508 [$(M+Na)^+$, 100]. Anal. calcd (%) for $C_{24}H_{27}N_{11}O$: C, 59.37; H, 5.60; N, 31.73; found: C, 59.51; H, 5.57; N, 31.63.

4-((4-([1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-N′-(3,4-dihydroxybenzylidene)benzohydrazide (5b). Yield: 85%. Orange solid, m.p. 268–270 °C. ^{1}H NMR (400 MHz, DMSO-d_6):δ 11.61 (s, 1H, NH), 9.47 (brs, 3H, CH+2OH), 8.27 (s, 1H, N=CH), 7.88 (d, 2H, *J* = 7.1 Hz, Ar), 7.49 (d, 2H, *J* = 7.1 Hz, Ar), 7.25 (s, 1H, Ar), 6.92(d, 1H, *J* = 7.3 Hz, Ar), 6.79 (d, 1H, *J* = 7.3 Hz, Ar), 3.87 (t, 4H, *J* = 5.9 Hz, $2CH_2$), 3.64 (s, 2H, CH_2), 2.59 (t, 4H, *J* = 5.9 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.1, 155.5, 150.1, 148.7, 148.4, 146.2, 142.1, 136.8, 132.9, 129.3 (2C), 128.0 (2C), 126.2, 121.1, 116.0. 113.1, 61.8, 52.2 (2C), 44.7 (2C). ESI-MS: *m/z* (%): 497 [$(M+Na)^+$, 100]. Anal. calcd (%) for $C_{22}H_{22}N_{10}O_3$: C, 55.69; H, 4.67; N, 29.52; found: C, 55.81; H, 4.63; N, 29.44.

4-((4-([1,2,4]triazolo[4,3-b][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-N′-(3-hydroxy-4-methoxybenzylidene) benzohydrazide (5c). Yield: 82%. Orange solid, m.p. 210–212 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.68 (s, 1H, NH), 9.48 (s, 1H, CH), 9.37 (s, 1H, OH), 8.30 (s, 1H, N=CH), 7.89 (d, 2H, *J* = 8.1 Hz, Ar), 7.51 (d, 2H, *J* = 8.1 Hz, Ar), 7.28 (s, 1H, Ar), 7.06 (d, 1H, *J* = 8.4 Hz, Ar), 6.98 (d, 1H, *J* = 8.4 Hz, Ar), 3.87 (t, 4H, *J* = 6.1 Hz, $2CH_2$), 3.81 (s, 3H, OCH_3), 3.65 (s, 2H, CH_2), 2.59 (t, 4H, *J* = 6.1 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.2, 155.6, 150.2, 150.1, 148.3, 147.3, 142.2, 136.9, 132.9, 129.3 (2C), 128.1 (2C), 127.6, 120.8, 112.7. 112.3, 61.9, 56.0, 52.3 (2C), 44.8 (2C). ESI-MS: *m/z* (%): 511 [$(M+Na)^+$, 100]. Anal. calcd (%) for

$C_{23}H_{24}N_{10}O_3$: C, 56.55; H, 4.95; N, 28.67; found: C, 56.40; H, 4.91; N, 28.78.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(3,4-dimethoxybenzylidene)benzohydrazide (5d).* Yield: 88%. Orange solid, m.p. 185–187 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.75 (s, 1H, NH), 9.48 (s, 1H, CH), 8.39 (s, 1H, N=CH), 7.90 (d, 2H, *J* = 8.0 Hz, Ar), 7.51 (d, 2H, *J* = 8.0 Hz, Ar), 7.35 (s, 1H, Ar), 7.20 (d, 1H, *J* = 8.3 Hz, Ar), 7.03 (d, 1H, *J* = 8.3 Hz, Ar), 3.87 (t, 4H, *J* = 6.0 Hz, $2CH_2$), 3.83 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.66 (s, 2H, CH_2), 2.60 (t, 4H, *J* = 6.0 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.3, 155.6, 151.2, 150.2, 149.5, 148.4, 142.2, 136.9, 133.4, 129.4 (2C), 128.1 (2C), 127.5, 122.4, 111.9. 108.5, 61.8, 56.0, 55.9, 52.3 (2C), 44.7 (2C). ESI-MS: *m*/*z* (%): 503 [$(M+H)^+$, 100], 525 [$(M+Na)^+$, 80]. Anal. calcd (%) for $C_{24}H_{26}N_{10}O_3$: C, 57.36; H, 5.21; N, 27.87; found: C, 57.47; H, 5.24; N, 27.80.

*5-4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(4-hydroxybenzylidene)benzohydrazide (5e).* Yield: 75%. Orange solid, m.p. 148 °C (dec.). ^{1}H NMR (400 MHz, DMSO-d_6):δ 11.66 (s, 1H, NH), 9.99 (brs, 1H, OH), 9.48 (s, 1H, CH), 8.35 (s, 1H, N=CH), 7.89 (d, 2H, *J* = 8.1 Hz, Ar), 7.56 (d, 2H, *J* = 8.5 Hz, Ar), 7.50 (d, 2H, *J* = 8.1 Hz, Ar), 6.84 (d, 2H, *J* = 8.5 Hz, Ar), 3.87 (t, 4H, *J* = 5.9 Hz, $2CH_2$), 3.65 (s, 2H, CH_2), 2.59 (t, 4H, *J* = 5.9 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.1, 159.9, 155.6, 150.2, 148.5, 142.2, 136.9, 133.0, 129.3 (2C), 128.0 (2C), 125.8 (2C), 119.6, 116.2 (2C), 62.1, 52.2 (2C), 44.8 (2C). ESI-MS: *m*/*z* (%): 481 [$(M+Na)^+$, 100]. Anal. calcd (%) for $C_{22}H_{22}N_{10}O_2$: C, 57.63; H, 4.84; N, 30.55; found: C, 57.75; H, 4.80; N, 30.48.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(2,4-dichlorobenzylidene)benzohydrazide (5f).* Yield: 94%. Orange solid, m.p. 147–149 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.75 (s, 1H, NH), 9.48 (s, 1H, CH), 8.39 (s, 1H, N=CH), 7.90 (d, 2H, *J* = 8.0 Hz, Ar), 7.51 (d, 2H, *J* = 8.0 Hz, Ar), 7.35 (s, 1H, Ar), 7.20 (d, 1H, *J* = 8.3 Hz, Ar), 7.03 (d, 1H, *J* = 8.3 Hz, Ar), 3.87 (t, 4H, *J* = 5.7 Hz, $2CH_2$), 3.66 (s, 2H, CH_2), 2.60 (t, 4H, *J* = 5.7 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.6, 155.5, 150.3, 149.9, 143.1, 136.9, 135.6, 134.4, 129.9 (2C), 128.6 (2C), 128.3, 126.0, 120.9, 111.7. 100.0, 62.5, 52.2 (2C), 44.8 (2C). ESI-MS: *m*/*z* (%): 511 [$(M+H)^+$, 100]. Anal. calcd (%) for $C_{22}H_{20}Cl_2N_{10}O$: C, 51.67; H, 3.94; N, 27.39; found: C, 51.79; H, 3.93; N, 27.32.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(4-nitrobenzylidene)benzohydrazide (5g).* Yield: 90%. Orange solid, m.p. 153–155 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.19 (s, 1H, NH), 9.48 (s, 1H, CH), 8.56 (s, 1H, N=CH), 8.32 (d, 2H, *J* = 8.5 Hz, Ar), 8.01 (d, 2H, *J* = 8.5 Hz, Ar), 7.93 (d, 2H, *J* = 8.0 Hz, Ar), 7.54 (d, 2H, *J* = 8.0 Hz, Ar), 3.88 (t, 4H, *J* = 6.1 Hz, $2CH_2$), 3.66 (s, 2H, CH_2), 2.60 (t, 4H, *J* = 6.1 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.7, 155.6, 150.2, 148.3, 145.6, 141.1, 136.9, 132.3, 129.4 (2C), 128.5 (2C), 128.3, 124.6 (4C), 61.9, 52.3 (2C), 44.7 (2C). ESI-MS: *m*/*z* (%): 488 [$(M+H)^+$, 100]. Anal. calcd (%) for $C_{22}H_{21}N_{11}O_3$: C, 54.21; H, 4.34; N, 31.61; found: C, 54.03; H, 4.36; N, 31.69.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(4-fluorobenzylidene)benzohydrazide (5h).* Yield: 91%. Orange solid, m.p. 182–184 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.89 (s, 1H, NH), 9.48 (s, 1H, CH), 8.46 (s, 1H, N=CH), 7.90 (d, 2H, *J* = 8.0 Hz, Ar), 7.80 (dd, 2H, J_1 = 8.4 Hz, J_2 = 5.6 Hz, Ar), 7.52 (d, 2H, *J* = 8.0 Hz, Ar), 7.32 (t, 2H, *J* = 8.8 Hz, Ar), 3.88 (t, 4H, *J* = 5.8 Hz, $2CH_2$), 3.65 (s, 2H, CH_2), 2.59 (t, 4H, *J* = 5.8 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.6 (*J* = 241 Hz), 163.4, 155.6, 150.2, 147.0, 142.4, 136.9, 132.7, 131.4, 129.7 (2C) (*J* = 15 Hz), 129.4(2C), 128.2 (2C), 116.4 (2C) (*J* = 22 Hz), 61.9, 52.3 (2C), 44.8 (2C). ESI-MS: *m*/*z* (%): 461 [$(M+H)^+$, 5], 483 [$(M+Na)^+$, 100]. Anal. calcd (%) for $C_{22}H_{21}FN_{10}O$: C, 57.38; H, 4.60; N, 30.42; found: C, 57.52; H, 4.62; N, 30.32.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-benzylidenebenzohydrazide (5i).* Yield: 87%. Orange solid, m.p. 213–215 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.86 (s, 1H, NH), 9.47 (s, 1H, CH), 8.47 (s, 1H, N=CH), 7.92 (d, 2H, *J* = 7.0 Hz, Ar), 7.73 (d, 2H, *J* = 7.0 Hz, Ar), 7.46–7.53 (m, 5H, Ar), 3.88 (t, 4H, *J* = 5.9 Hz, $2CH_2$), 3.66 (s, 2H, CH_2), 2.60 (t, 4H, *J* = 5.9 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.4, 155.6, 150.2, 148.1, 142.4, 136.9, 134.8, 132.7, 130.6, 129.3(4C), 128.2(2C), 127.6(2C), 61.9, 52.3(2C), 44.8(2C). ESI-MS: *m*/*z* (%): 465 [$(M+Na)^+$, 100]. Anal. calcd (%) for $C_{22}H_{22}N_{10}O$: C, 59.72; H, 5.01; N, 31.66; found: C, 59.85; H, 4.98; N, 31.57.

N′-*(4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6yl)piperazin-1-yl)methyl)benzoyl)-2-fluorobenzohydrazide (6a).* Yield: 75%. Yellow solid, m.p. 184–186 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.67 (s, 1H, NH), 10.42 (s, 1H, NH), 9.54(s, 1H, CH), 7.36–8.31 (m, 8H, Ar), 4.11(s, 2H, CH_2), 3.86 (t, 4H, *J* = 6.2 Hz, $2CH_2$), 2.98 (t, 4H, *J* = 6.2 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.6, 164.0, 162.1 (*J* = 253 Hz), 155.4, 150.2, 148.3, 137.0, 133.6 (*J* = 6.5 Hz), 130.6 (*J* = 2.4 Hz), 128.5 (2C), 128.2 (2C), 125.1 (*J* = 2.8 Hz), 124.6, 122.7 (*J* = 15.3 Hz), 116.8 (*J* = 32.3 Hz), 58.7, 50.1(2C), 41.9(2C). ESI-MS: *m*/*z* (%): 477 [$(M+H)^+$, 100]. Anal. calcd (%) for $C_{22}H_{21}FN_{10}O_2$: C, 55.46; H, 4.44; N, 29.40; found: C, 55.35; H, 4.46; N, 29.45.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(4-chlorobenzoyl)benzohydrazide (6b).* Yield: 70%. Yellow solid, m.p. 178–180 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.69 (s, 1H, NH), 10.66 (s, 1H, NH), 9.60 (s, 1H, CH), 8.02 (d, 2H, *J* = 7.0 Hz, Ar), 7.95 (d, 2H, *J* = 7.9 Hz, Ar), 7.79 (d, 2H, *J* = 7.0 Hz, Ar), 7.62 (d, 2H, *J* = 7.9 Hz, Ar), 4.66 (s, 2H, CH_2), 3.86 (t, 4H, *J* = 6.1 Hz, $2CH_2$), 3.25 (t, 4H, *J* = 6.1 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.7, 165.3, 155.3, 150.2, 137.3, 137.1, 132.0, 131.7, 129.9 (3C), 129.2 (4C), 128.3 (2C), 58.9, 50.3 (2C), 42.0

(2C). ESI-MS: *m/z* (%): 515 [(M+Na)$^+$, 100]. Anal. calcd (%) for $C_{22}H_{21}ClN_{10}O_2$: C, 53.61; H, 4.29; N, 28.42; found: C, 53.70; H, 4.26; N, 28.35.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(4-Nitrobenzoyl)benzohydrazide (6c).* Yield: 70%. Yellow solid, m.p. 178–180 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.98 (s, 1H, NH), 10.76 (s, 1H, NH), 9.57 (s, 1H, CH), 8.33 (d, 2H, *J*=8.6 Hz, Ar), 8.17 (d, 2H, *J*=8.4 Hz, Ar), 8.00 (d, 2H, *J*=7.4 Hz, Ar), 7.74 (d, 2H, *J*=7.4 Hz, Ar), 4.11(s, 2H, CH_2), 3.86 (t, 4H, *J*=6.3 Hz, $2CH_2$), 3.14 (t, 4H, *J*=6.3 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.3, 165.7, 155.4, 150.5, 150.2, 137.0, 136.8, 131.2 (2C), 129.8, 129.5 (2C), 128.2 (2C), 128.0, 124.2 (2C), 62.2, 50.7 (2C), 42.8 (2C). ESI-MS: *m/z* (%): 504 [(M+H)$^+$, 100]. Anal. calcd (%) for $C_{22}H_{21}N_{11}O_4$: C, 52.48; H, 4.20; N, 30.60; found: C, 52.61; H, 4.18; N, 30.52.

N′*-(4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl) piperazin-1-yl)methyl)benzoyl)-3-chlorobenzohydrazide (6d).* Yield: 83%. Yellow solid, m.p. 181–183 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.74 (s, 1H, NH), 10.69 (s, 1H, NH), 9.58(s, 1H, CH), 8.01 (s, 1H, Ar), 7.98 (d, 2H, *J*=7.5 Hz, Ar), 7.90 (d, 1H, *J*=7.8 Hz, Ar), 7.75 (d, 2H, *J*=7.5 Hz, Ar), 7.70 (d, 1H, *J*=7.8 Hz, Ar), 7.59 (t, 1H, *J*=7.8 Hz, Ar), 4.27 (s, 2H, CH_2), 3.86 (t, 4H, *J*=6.2 Hz, $2CH_2$), 3.19 (t, 4H, *J*=6.2 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.7, 165.0, 155.4, 150.2, 137.0, 134.9, 133.9, 133.3, 132.3, 131.5, 131.2, 129.8 (2C), 128.2 (2C), 127.7, 126.7, 59.4, 50.6(2C), 42.5(2C). ESI-MS: *m/z* (%): 493 [(M+H) $^+$, 20], 515 [(M+Na)$^+$, 30]. Anal. calcd (%) for $C_{22}H_{21}ClN_{10}O_2$: C, 53.61; H, 4.29; N, 28.42; found: C, 53.72; H, 4.25; N, 28.36.

N′*-(4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)benzoyl)-3-fluorobenzohydrazide (6e).* Yield: 85%. Yellow solid, m.p. 207–209 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.72 (s, 1H, NH), 10.70 (s, 1H, NH), 9.60(s, 1H, CH), 8.02 (d, 2H, *J*=8.2 Hz, Ar), 7.81 (d, 3H, *J*=8.2 Hz, Ar), 7.72 (d, 1H, *J*=8.3 Hz, Ar), 7.46–7.67 (m, 2H, Ar), 4.47 (s, 2H, CH_2), 3.87 (t, 4H, *J*=6.0 Hz, $2CH_2$), 3.51 (t, 4H, *J*=6.0 Hz, $2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 165.6, 165.0, 162.4 (*J*=242 Hz), 155.3, 150.2, 137.1, 135.1 (*J*=6.9 Hz), 133.8, 132.2, 131.4 (*J*=7.0 Hz), 129.9(2C), 128.3(2C), 124.1 (*J*=2.7 Hz), 116.2 (*J*=21.8 Hz), 114.7 (*J*=22.7 Hz), 58.7, 50.2(2C), 41.9(2C). ESI-MS: *m/z* (%): 477 [(M+H)$^+$, 20]. Anal. calcd (%) for $C_{22}H_{21}FN_{10}O_2$: C, 55.46; H, 4.44; N, 29.40; found: C, 55.59; H, 4.40; N, 29.29.

N′*-(4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin -1-yl)methyl)benzoyl)-3-methylbenzohydrazide (6f).* Yield: 65%. Yellow solid, m.p. 174–176 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.63 (s, 1H, NH), 10.54 (s, 1H, NH), 9.61 (s, 1H, CH), 8.02 (d, 2H, *J*=8.2 Hz, Ar), 7.81 (d, 1H, *J*=8.2 Hz, Ar), 7.73–7.75 (m, 3H, Ar), 7.38–7.42 (m, 2H, Ar), 4.47 (s, 2H, CH_2), 3.87 (t, 4H, *J*=5.9 Hz, $2CH_2$), 3.52 (t, 4H, *J*=5.9 Hz, $2CH_2$), 2.37 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.4, 165.6, 155.3, 150.2, 138.3, 137.1, 134.0, 132.9, 132.2, 130.2, 128.9 (2C), 128.5, 128.3 (2C), 126.9, 125.0, 58.7, 50.1 (2C), 41.9 (2C), 21.4. ESI-MS: *m/z* (%): 473 [(M+H)$^+$, 7], 495 [(M+Na)$^+$, 6]. Anal. calcd (%) for $C_{23}H_{24}N_{10}O_2$: C, 58.46; H, 5.12; N, 29.64; found: C, 58.61; H, 5.10; N, 29.43.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-(cyclohexanecarbonyl)benzohydrazide (6g).* Yield: 54%. Yellow solid, m.p. 185–187 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.36 (s, 1H, NH), 9.85 (s, 1H, NH), 9.56 (s, 1H, CH), 7.92 (d, 2H, *J*=7.6 Hz, Ar), 7.69 (d, 2H, *J*=7.6 Hz, Ar), 4.20 (s, 2H, CH_2), 3.85 (t, 4H, *J*=5.8 Hz, $2CH_2$), 3.12 (t, 4H, *J*=5.8 Hz, $2CH_2$), 2.24–2.29 (m, 1H, CH), 1.63–1.74 (m, 4H, $2CH_2$), 1.15–1.40 (m, 6H, $3CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.1, 165.4, 155.4, 150.1, 137.0, 133.3, 131.2, 129.7 (2C), 128.1 (2C), 59.6, 52.7, 50.8 (2C), 42.5 (2C), 29.5 (2C), 25.6 (3C). ESI-MS: *m/z* (%): 465 [(M+H)$^+$, 60]. Anal. calcd (%) for $C_{22}H_{28}N_{10}O_2$: C, 56.88; H, 6.08; N, 30.15; found: C, 57.98; H, 6.06; N, 30.11.

*4-((4-([1,2,4]triazolo[4,3-*b*][1,2,4,5]tetrazin-6-yl)piperazin-1-yl)methyl)-*N*′-hexanoylbenzohydrazide (6h).* Yield: 61%. Yellow solid, m.p. 185–187 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.42 (s, 1H, NH), 9.94 (s, 1H, NH), 9.58 (s, 1H, CH), 7.96 (d, 2H, *J*=7.7 Hz, Ar), 7.78 (d, 2H, *J*=7.7 Hz, Ar), 4.41 (s, 2H, CH_2), 3.86 (t, 4H, *J*=5.6 Hz, $2CH_2$),3.56 (t, 4H, *J*=5.6 Hz, $2CH_2$), 2.19 (t, 2H, *J*=7.3 Hz, CH_2), 1.49–1.56 (m, 2H, CH_2), 1.25–1.32 (m, 4H, $2CH_2$), 0.84–0.90 (m, 3H, CH_3). ^{13}C NMR (DMSO-d_6, ppm): δ 175.0, 172.1, 165.3, 155.3, 150.1, 137.0, 133.7, 131.9 (2C), 128.3 (2C), 58.9, 50.2 (2C), 42.0 (2C), 34.2, 31.2, 25.2, 22.4, 14.4. ESI-MS: *m/z* (%): 453 [(M+H)$^+$, 85], 475 [(M+Na)$^+$, 27]. Anal. calcd (%) for $C_{21}H_{28}N_{10}O_2$: C, 55.74; H, 6.24; N, 30.95; found: C, 55.57; H, 6.26; N, 31.07.

In vitro cancer cell growth inhibition assay. The antiproliferative activities of the compounds **3–6** against several human cancer cell lines were assayed by standard MTT assay procedures. Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) medium at 37 °C with 5% CO_2 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 compounds from 0.4 to 500 μM. Wells containing culture medium without cells were used as blanks and cisplatin was assayed over the same time as a 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 IC_{50} value was determined from plots of % viability against the dose of each compound added. Each assay was performed in triplicate. Original data can be obtained from Supplemental material.

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.[22] The ligand was docked 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 5-phenyl-3-(quinolin-6-ylmethyl)-3,5,6,7-tetrahydro-*4H*-[1,2,3]triazolo[4,5-c] pyridin-4-one (PDB entry: 4DEH) 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. Then their geometries were optimized by the conjugate gradient method in the TRIPOS force field. The energy convergence criterion is 0.001 kcal/mol. Finally, ligand-based mode was adopted to generate the "protomol," leaving the threshold at their default value of 1.

c-Met kinase assay in vitro. HTRF 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 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 media or plastic have short decay times and can exclude their interference of the detection signal by delaying detected time. On the other hand, the free EuK excited by nitrogen laser (λ=340 nm), resulting in long-lived emission at λ=620 nm, which is used as a background signal. These two specific signals at 665 and 620 nm were measured by multifunctional microplate reader, and the strength of detection signal from the reaction system can reflect the activity of tested compounds against c-Met kinase. The experimental method of HTRF was described as follows: (1) tested compounds (4 μL, diluted with buffer solution to nine different concentrations: 100,000, 20,000, 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 the mixture (4 μL, V/V=1/1) of TK Substrate-biotin (1 μM) and ATP (3 μM) was added. Then 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 by multifunctional microplate reader. Original data can be obtained from Supplemental material.

Declaration of conflicting interests

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Supplemental material

Supplemental material for this article is available online.

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