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Identification and biological evaluation of novel benzothiazole derivatives bearing a pyridine-semicarbazone moiety as apoptosis inducers *via* activation of procaspase-3 to caspase-3†

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Three series of compounds were designed, synthesized and evaluated for their *in vitro* anticancer activity against a procaspase-3 over-expression cancer cell line (U937) and a procaspase-3 no-expression cancer cell line (MCF-7) to rule out off-target effects. Biological evaluation led to the identification of a series of benzothiazole derivatives bearing a pyridine-semicarbazone moiety, **8j** and **8k**, with promising anticancer activity and remarkable selectivity. Further mechanism studies revealed that compounds **8j** and **8k** could induce apoptosis of cancer cells by activating procaspase-3 to caspase-3, and compound **8k** exhibited the strongest procaspase-3 activation activity. Structure–activity relationships (SARs) revealed that the presence of benzothiazole and an *N,N,O*-donor set is crucial for the anticancer activity and selectivity, and reducing the electron density of the *N,N,O*-donor set results in a dramatic decline in the anticancer activity and selectivity. Furthermore, toxicity evaluation (zebrafish) *in vivo* and metabolic stability studies (human, rat and mouse liver microsomes) were performed to provide reliable guidance for further PK/PD studies *in vivo*.

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1. Introduction

Caspase-3 plays a significant role in apoptotic cascades as the most common and important downstream effector protein. The conversion of zymogen procaspase-3 to caspase-3 can catalyze the hydrolysis of hundreds of protein substrates, leading to apoptosis.^{1–3} One of the hallmarks of cancer is the ability to avoid apoptosis.^{4,5} Studies have shown that zymogen procaspase-3 is overexpressed in a variety of human tumors owing to inhibition of apoptosis.^{6–9} Therefore, identification of small molecules that induce the apoptosis of tumor cells by directly activating downstream over-expression of procaspase-3 to caspase-3 has become an effective strategy for improving selectivity and overcoming the mutation of the middle/upstream apoptotic protein.

PAC-1 (Fig. 1) was reported as the first procaspase-3 activator that induced apoptosis by selectively activating procaspase-3 to caspase-3 *via* chelating the inhibitory endogenous metal ion zinc, which inhibited the activation of procaspase-3.^{10,11} Structure–activity relationships (SARs) revealed that the *ortho*-hydroxy *N*-acylhydrazone moiety of PAC-1 is responsible for its strong procaspase-3 activity and participates in chelation with zinc.¹² In our previous studies, a series of benzothiazole derivatives bearing an *ortho*-hydroxy *N*-carbamoylhydrazone moiety were reported as procaspase-3 activators;^{13,14} however, due to the presence of lipophilic groups (*e.g.* substituted benzyloxy or heteroaryloxy groups), these compounds exhibited high lipophilicity (*clogP* = 6.13–9.67) and were not suitable for further development (Fig. 1). Pyridine-semicarbazone derivatives (Fig. 1) have long attracted much attention as an important class of compounds due to their remarkable biological and pharmacological properties, including antibacterial, antiviral, antineoplastic and antimalarial properties.^{15–17} Additionally, the pyridine-semicarbazone moiety could chelate with metal ions (Zn, Pd, Fe, Co, *etc.*) based on the *N,N,O*-donor set, which have been widely used as important structural components in modern drug design for the treatment of cancer.^{18–21}

Inspired by the above findings, we designed a series of benzothiazole derivatives bearing a pyridine-semicarbazone moiety (series A). In addition, the importance of the

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Fig. 1 Procaspase-3 activators and pyridine-semicarbazone derivatives.

benzothiazole and N,N,O -donor set was addressed with the replacement of a benzene group to obtain series B and series C, respectively (Fig. 2). All target compounds were evaluated for their *in vitro* anticancer activities against a procaspase-3 over-expression cancer cell line (U937, human lymphoma) and a procaspase-3 no-expression cancer cell line (MCF-7, human breast adenocarcinoma) to rule out off-target effects.²² In addition, these compounds with high selectivity and promising anticancer activity were selected for further mechanism studies, including a flow cytometric assay for apoptosis and caspase-3 and procaspase-3 activity evaluation. Furthermore,

the promising compounds were further selected for *in vivo* toxicity evaluation and apoptotic study in zebrafish embryos. Finally, a metabolic stability study was performed to assess the half-life *in vitro* and provide guidance for further PK/PD studies *in vivo*.

2. Results and discussion

2.1 Chemistry

The preparation of the target compounds is illustrated in Scheme 1. The commercially available starting material

Fig. 2 Design of target compounds.

Scheme 1 Reagents and conditions: (i) acetonitrile, amine, rt, 3 h; (ii) 80% hydrazine monohydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, activated carbon, ethanol, 65 °C to 78 °C, 5 h; (iii) Br_2 , NH_4SCN , HOAc, 10 °C to rt, 4–6 h; (iv) phenyl chloroformate, pyridine, CH_2Cl_2 , 0 °C to rt, 4–6 h; (v) 80% hydrazine monohydrate, 1,4-dioxane, 80 °C, 6 h; (vi) HOAc, ethanol, reflux.

4-nitrobenzyl bromide was reacted with various small molecule secondary amines (4-methylpiperidine, dimethylamine and diethylamine) *via* a nucleophilic substitution reaction to provide intermediates 1a–c, which were reduced in the presence of 80% hydrazine and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ to obtain the substituted anilines 2a–c. 2a–c were further reacted with ammonium thiocyanate and bromine to produce 3a–c in satisfactory yield. Treatment of 3a–c or 2a–c with phenyl chloroformate through an *N*-acylation reaction yielded 4a–c or 5a–c. The key intermediate semicarbazides 6a–c or 7a–c were prepared *via* hydrazinolysis of 4a–c or 5a–c with 80% hydrazine monohydrate. Subsequently, 6a–c or 7a–c were reacted with corresponding aldehydes or ketones *via* a condensation reaction in the presence of catalytic amounts of acetic acid to obtain the target compounds 8a–l, 9a–h and 10a–e.

2.2 *In vitro* anticancer activity and SARs

All the target compounds were evaluated for their *in vitro* anticancer activities against a procaspase-3 over-expression cancer cell line (U937) and a procaspase-3 no-expression cancer cell line (MCF-7) by using PAC-1 as a positive control. As shown in Table 1, all the target compounds showed more preferable *clogP* values ranging from 1.18 to 4.35, compared to reported benzothiazole derivatives in our previous studies. In addition, most of the compounds possessing benzothiazole (series A) showed potent anticancer activity and high selectivity against U937; however, no obvious anticancer activity and selectivity were observed in these compounds

possessing a benzene group (series B), indicating that the benzothiazole was more helpful for anticancer activity and selectivity than benzene. Among them, the promising compounds 8j and 8k showed the highest selectivity and potency with IC_{50} values of 5.2 μM and 6.6 μM as compared to other compounds.

Due to the potent anticancer activity and high selectivity of compounds 8a–l (series A), further investigations were carried out. The results revealed that compounds possessing a 4-methylpiperidine group (8i–k) showed enhanced activity compared to compounds possessing a dimethylamino or diethylamino group (8a–g). Furthermore, the IC_{50} values of 8a–c, 8e–g and 8i–k revealed that the introduction of methyl or 2-pyridyl in the R group had a stronger contribution to the potency than hydrogen. It was worth noting that replacement of the 2-pyridyl (8b, 8f, and 8j) with 2-pyrazinyl (8d, 8h, and 8l) led to a dramatic decline in selectivity. This may be due to the fact that the electron density of pyrazine is lower than that of pyridine, which reduces the electron density of the *N*, *N*, *O*-donor set, and further weakens the ability to chelate the zinc ion, leading to a remarkable change in selectivity. Thus, it was concluded that the electron density of the *N*, *N*, *O*-donor set was crucial for the selectivity.

In order to address the importance of the *N*, *N*, *O*-donor set, the anticancer activity and selectivity of compounds 10a–e (series C) were determined. As shown in Table 2, due to the absence of the *N*, *N*, *O*-donor set, compounds 10a–e showed insufficient anticancer activity (>40 μM) against the procaspase-3 over-expression cancer cell line (U937), whereas,

Table 1 Anticancer activity and selectivity of target compounds **8a-l** and **9a-h** (series A and series B)

Comp.		X	R	IC ₅₀ ^a (μM)		Selectivity ^c	clog P ^d
				U937 ^b	MCF-7 ^b		
8a		C	H	23.2 ± 0.6	>40	1.6	2.82
8b		C	-CH ₃	20.1 ± 0.4	>40	2.0	2.14
8c		C		7.8 ± 0.5	>40	5.1	2.64
8d		N	-CH ₃	>40	>40	—	1.18
8e		C	-H	24.8 ± 0.8	>40	1.6	3.88
8f		C	-CH ₃	15.6 ± 0.5	>40	2.6	3.19
8g		C		9.1 ± 0.2	>40	4.3	3.70
8h		N	-CH ₃	>40	>40	—	2.24
8i		C	-H	21.5 ± 0.9	>40	1.6	4.53
8j		C	-CH ₃	5.2 ± 0.7	12.4 ± 0.6	2.3	3.85
8k		C		6.6 ± 0.2	>40	6.0	4.35
8l		N	-CH ₃	>40	11.8 ± 0.3	<1	2.89
9a		C	-H	>40	>40	—	1.92
9b		C		>40	>40	—	2.70
9c		C	-CH ₃	>40	>40	—	3.26
9d		C		>40	>40	—	3.76
9e		C	-H	>40	>40	—	3.62
9f		C	-CH ₃	>40	>40	—	3.91
9g		C		>40	>40	—	4.41
9h		N	-CH ₃	>40	>40	—	2.95
PAC-1				6.0 ± 0.5	>40	6.6	5.20

^a IC₅₀: the biological data are generated from three independent experiments. ^b U937: procaspase-3 over-expression cancer cells; MCF-7: procaspase-3 no-expression cancer cells. ^c Selectivity is expressed as a ratio of MCF-7/U937. ^d clog P values were calculated by using Chemdraw software.

Table 2 Anticancer activity of target compounds **10a–e** (series C)

10a–e
Series C

Compd.	R ₁ N R ₂	Ar	X	R	R ₃	IC ₅₀ ± SD ^a (μM)			clog P ^d
						U937 ^b	MCF-7 ^b	Selectivity ^c	
10a			C	–CH ₃	4-F	>40	12.3 ± 0.7	<1	4.83
10b			C	–CH ₃	—	>40	>40	—	5.35
10c			C		—	>25	12.1 ± 0.6	<1	5.69
10d			C		—	>25	13.1 ± 0.8	<1	6.75
10e			C		—	>25	13.3 ± 0.9	<1	7.41
PAC-1						6.0 ± 0.5	>40	6.6	5.20

^a IC₅₀: the biological data are generated from three independent experiments. ^b U937: procaspase-3 over-expression cancer cell line; MCF-7: procaspase-3 no-expression cancer cell line. ^c Selectivity is expressed as a ratio of MCF-7/U937. ^d clog P values were calculated by using Chemdraw software.

compounds **10a**, **10c**, **10d** and **10e** displayed more potent activities with IC₅₀ values of 12.3 μM, 12.1 μM, 13.1 μM and 13.3 μM against the procaspase-3 no-expression cancer cell line (MCF-7). These results indicated that the presence of the *N,N,O*-donor set is crucial for the selectivity.

2.3 Apoptosis study

In order to verify whether compounds **8j** and **8k** could induce apoptosis of cancer cells, treatment of DMSO as a negative control was carried out, U937 cells were incubated with different concentrations of **8j** and **8k** for 24 h and 48 h, and the percentages of apoptotic cells were determined by FITC-Annexin V/PI staining and flow cytometry. As shown as Fig. 3, the treatment of U937 cells with different concentrations of **8j** and **8k** for 24 h and 48 h caused a significant dose- and time-dependent increase in the population of both early and late apoptotic cells compared to the control cells, suggesting that **8j** and **8k** could induce cancer cell apoptosis in a dose- and time-dependent manner.

2.4 Caspase-3 activation study

Because of their high selectivity and potent anticancer activity, compounds **8b**, **8c**, **8g**, **8j**, **8k**, **10a**, **10c**, **10d** and **10e** were selected for further investigation. By using PAC-1 and DMSO as positive and negative controls, the ability of these com-

pounds to activate executioner caspase-3 in whole cells was evaluated. Cells were treated with these compounds for 0 or 16 h and lysed, and the cleavage of the fluorescent caspase-3 substrate Ac-DEVD-AFC was analyzed *via* kinetic reads. As shown in Table 3 and Fig. 4, compared to PAC-1 (100%), compounds **8b**, **8c**, **8g**, **8j** and **8k** showed strong caspase-3 activation activity (>60%). Among them, compound **8j** showed a comparable activation activity (99%) with PAC-1, while compounds **8c**, **8g** and **8k** induced stronger caspase-3 activation activity than PAC-1 with degrees of 104%, 119% and 114%, respectively. Compounds **10a**, **10c**, **10d** and **10e** induced lower than 60% caspase-3 activation activity due to the absence of the *N,N,O*-donor set.

2.5 *In vitro* procaspase-3 activation assay

In order to further verify that the strong caspase-3 activation activity was caused by the activation of procaspase-3, by using PAC-1 and DMSO as positive and negative controls, the promising compounds **8j** and **8k** were selected to determine procaspase-3 activation activity. As shown in Table 4, compared to PAC-1, compounds **8j** and **8k** displayed potent procaspase-3 activation activities at 10 μM with degrees of 77.8% and 92.1%, respectively, suggesting that apoptosis of cancer cells induced by activating procaspase-3 to caspase-3 might be a potential mechanism for the antitumor effects of these target compounds.

Fig. 3 Apoptosis in U937 cells by the treatment with **8j** and **8k** for 24 h and 48 h.

Taken together, these above findings identified that compounds **8j** and **8k** could be developed as promising candidates for procaspase-3 activators.

2.6 *In vivo* toxicity evaluation in zebrafish

Zebrafish (*Danio rerio*) have been considered as a suitable animal model for drug discovery due to their high fertility, high homology with human DNA, rapid development, and transparency,²³ and have been widely used in high-quality *in vivo* validation of drug targets and toxicological studies.²⁴ In this study, the survival rates of zebrafish embryos exposed to various concentrations (5, 10, 20 and 40 μ M) of compounds **8j** and **8k** were determined at 24, 48 and 72 h post fertilization (hpf), and a no-treatment control was also included. As shown in Fig. 5(A and B), no obvious toxic effects were observed for **8j** and **8k** at the concentrations of 5, 10 and 20 μ M for 24, 48, and 72 hpf, indicating that **8j** and **8k** have low toxicity at these

concentrations, which are far greater than the half maximal inhibitory concentration (IC_{50}) of **8j** and **8k** in the anticancer effect. After 48 hpf exposure of zebrafish to 40 μ M **8k**, moderate toxic effects were observed; however, after a longer exposure (72 hpf), 40 μ M **8j** led to the death of most embryos, and 40 μ M **8k** led to the death of almost all embryos.

2.7 **8j** and **8k** induced apoptosis in zebrafish embryos

In order to further study whether compounds **8j** and **8k** could induce apoptosis through other pro-apoptotic proteins, we

Fig. 4 Caspase-3 activation activity of PAC-1 (control) and selected compounds in U937 at 30 μ M.

Table 3 Caspase-3 activation activity of PAC-1 and selected compounds in U937 at 30 μ M

Compd.	Caspase-3 activation ^a
PAC-1	100 $\pm$ 4
8b	68 $\pm$ 4
8c	104 $\pm$ 12
8g	119 $\pm$ 6
8j	99 $\pm$ 10
8k	114 $\pm$ 10
10a	40 $\pm$ 9
10c	28 $\pm$ 2
10d	20 $\pm$ 3
10e	58 $\pm$ 8
DMSO	7 $\pm$ 1

^a The biological data are generated from three independent experiments.

Table 4 Procaspase-3 activation activity of selected compounds **8j**, **8k** and PAC-1 at 10 μ M *in vitro*

Compd.	Procaspase-3 activation activity (%)
8j	77.8 $\pm$ 5.0
8k	92.1 $\pm$ 3.5
PAC-1	100 $\pm$ 7.6

Fig. 5 (A) Survival rates of zebrafish embryos exposed to various concentrations of **8j** and **8k** over 3 days of post-fertilization (72 hpf). Data were expressed as means $\pm$ standard deviation from three independent experiments (twenty-four embryos for each group). (B) Gross morphological phenotypes in zebrafish embryos at 72 hpf: treated with 20 μ M **8j** (left), untreated (middle), and treated with 20 μ M **8k** (right).

firstly used *in vivo* labeling with acridine orange to visualize apoptotic cells in live zebrafish embryos. As shown in Fig. 6A, we observed a very specific accumulation of dying cells in the head and cranial neural crest at 24 hpf. Meanwhile, treatment of zebrafish with **8j** and **8k** significantly increased the mRNA expression of proapoptotic protein, *bax* (Fig. 6B, $p < 0.01$); β -actin was detected as an internal control.

2.8 *In vitro* metabolic stability study

Using ketanserin as a reference compound, the metabolic stability of compounds **8j** and **8k** was evaluated *in vitro* by using the liver microsome assay against human, rat and mouse liver microsomes. As shown in Table 5, compounds **8j** and **8k** exhibited moderate metabolism ($T_{1/2} = 30$ –120 min) in human and rat liver microsomes with $T_{1/2}$ values of 77.93 min,

Fig. 6 **8j** and **8k** induced apoptosis in the zebrafish. (A) Acridine orange staining for apoptotic cells at 24 hpf on the control, **8j** and **8k**-treated embryos. (B) The expression of pro-apoptotic (*bax*) gene analyzed by real-time quantitative RT-PCR.

Table 5 *In vitro* metabolic half-life ($T_{1/2}$) [min] and intrinsic clearance (Cl_{int}) of **8j** and **8k**

Compd.	Index	Species		
		Human	Rat	Mouse
Ketanserin	$T_{1/2}$ (min)	36.07	14.40	20.46
	Cl_{int} (mL min ⁻¹ kg ⁻¹)	48.19	172.52	266.76
8j	$T_{1/2}$ (min)	77.93	95.55	53.63
	Cl_{int} (mL min ⁻¹ kg ⁻¹)	22.31	25.99	101.76
8k	$T_{1/2}$ (min)	79.06	73.87	16.72
	Cl_{int} (mL min ⁻¹ kg ⁻¹)	21.99	33.62	326.43

95.55 min, 79.06 min, and 73.87 min, respectively. Compound **8j** displayed better stability in mouse microsomes than **8k**, which was susceptible to metabolism with a $T_{1/2}$ of 16.72 min ($T_{1/2} < 30$ min).

3. Conclusions

In summary, a novel series of benzothiazole derivatives bearing a pyridine-semicarbazone moiety were identified as apoptosis inducers. Among them, compounds **8j** and **8k** displayed excellent anticancer activity against procaspase-3 over-expression cancer cells (U937) with IC_{50} values of 5.2 μ M and 6.6 μ M. Further mechanism studies showed that **8j** and **8k** could inhibit proliferation of cancer cells by inducing apoptosis by activating procaspase-3 to caspase-3, and compound **8k** exhibited the strongest procaspase-3 activation activity with a degree of 92.1% at 10 μ M. Toxicity evaluation in zebrafish embryos suggested that **8j** and **8k** showed weak toxicity. Furthermore, the metabolic stability study indicated that **8j** and **8k** exhibited moderate metabolism *in vitro* against human and rat liver microsomes. The analysis of SARs indicated that the presence of the benzothiazole and *N,N,O*-donor set are crucial for anticancer activity and selectivity, and reducing the electron density of the *N,N,O*-donor set causes a dramatic decline in selectivity. In addition, incorporation of a 4-methylpiperidine group and introduction of either a methyl or pyridyl in the R group can enhance the anticancer activity. The novel chemical structures of this series of compounds provide a new template for the further design of potential procaspase-3 activators, and the more preferable $clogP$ values bring a promising future for further development. Further investigations on the PK/PD *in vivo* are ongoing.

4. Experimental section

4.1 Chemistry

The 1H and ^{13}C NMR spectroscopy was performed using Bruker ARX-400, 400 MHz spectrometers (Bruker Bioscience, Billerica, MA, USA) with tetramethylsilane (TMS) as an internal standard. High resolution mass spectrometry was performed on an Agilent Accurate-Mass UHPLC-QTOF 1290–6545 in ESI mode or EI mode. Melting points were determined on a Melting Point XT5B apparatus (KeYi, Beijing).

Column chromatography was run on silica gel (200–300 mesh) from Qingdao Ocean Chemicals (Qingdao, Shandong, China). Unless otherwise noted, all materials were obtained from commercially available sources and were used without further purification.

4.1.1. Synthesis of key intermediate semicarbazides 6a–c or 7a–c. The preparation of key intermediate semicarbazides 7a–c has been illustrated in detail in our previous work,^{14,25} and the synthesis methods would not be listed here. The 6a–c were prepared by referring to synthesis methods of 7a–c.

4.1.2. General procedure for preparation of 8a–l, 9a–h and 10a–e. A mixture of compounds 6a–c or 7a–c (0.001 mol), appropriate aldehydes or ketones (0.0011 mol) and a drop of glacial acetic acid in 5 mL absolute ethanol was heated at reflux for 6–12 h and cooled to room temperature. The mixture was concentrated to obtain the crude product, which was purified by chromatography on silica gel using MeOH/ CH_2Cl_2 to afford the compounds 8a–k, 9a–h and 10a–e.

4.1.2.1. (E)-N-(6-((Dimethylamino)methyl)benzo[d]thiazol-2-yl)-2-(pyridin-2-ylmethylene)hydrazine-1-carboxamide (8a). Light yellow solid; yield: 52%; mp: 142–144 °C; HRMS (ESI) for $C_{17}H_{18}N_6OS$ [$M + H$]⁺. Calcd: 354.1263, found: 355.1339; 1H NMR (400 MHz, DMSO) δ 11.48 (s, 2H), 8.58 (d, $J = 4.4$ Hz, 1H), 8.56–8.46 (m, 1H), 8.06 (s, 1H), 7.87 (t, $J = 7.8$ Hz, 1H), 7.82 (s, 1H), 7.63 (d, $J = 8.2$ Hz, 1H), 7.42–7.36 (m, 1H), 7.34 (d, $J = 8.2$ Hz, 1H), 3.47 (s, 2H), 2.17 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 159.71, 153.62, 153.34, 149.65, 148.08, 144.16, 136.94, 134.59, 132.02, 127.37, 124.59, 121.79, 121.25, 119.79, 63.76, 45.40 (2C).

4.1.2.2. (E)-N-(6-((Dimethylamino)methyl)benzo[d]thiazol-2-yl)-2-(1-(pyridin-2-yl)ethylidene)hydrazine-1-carboxamide (8b). Light yellow solid; yield: 63%; mp: 147–149 °C; HRMS (ESI) for $C_{18}H_{20}N_6OS$ [$M + H$]⁺. Calcd: 368.1419, found: 369.1493; 1H NMR (400 MHz, DMSO) δ 11.09 (s, 1H), 10.47 (s, 1H), 8.59 (d, $J = 4.6$ Hz, 1H), 8.50 (s, 1H), 7.90–7.80 (m, 2H), 7.64 (d, $J = 8.4$ Hz, 1H), 7.44–7.38 (m, 1H), 7.34 (d, $J = 8.4$ Hz, 1H), 3.47 (s, 2H), 2.38 (s, 3H), 2.17 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 159.39, 155.12, 149.89, 148.86, 148.49, 140.26, 136.88, 134.60, 132.12, 127.41, 124.40, 121.83, 121.49, 120.01, 63.76, 45.40 (2C), 12.58.

4.1.2.3. 2-(Di(pyridin-2-yl)methylene)-N-(6-((dimethylamino)methyl)benzo[d]thiazol-2-yl)hydrazine-1-carboxamide (8c). Light yellow solid; yield: 71%; mp: 156–158 °C; HRMS (ESI) for $C_{22}H_{21}N_7OS$ [$M + H$]⁺. Calcd: 431.1528, found: 432.1609; 1H NMR (400 MHz, DMSO) δ 12.89 (s, 1H), 11.78 (s, 1H), 8.84 (d, $J = 4.7$ Hz, 1H), 8.56 (d, $J = 4.6$ Hz, 1H), 8.25–8.08 (m, 1H), 7.99 (q, $J = 7.8$ Hz, 2H), 7.84 (s, 1H), 7.64–7.55 (m, 2H), 7.53–7.43 (m, 2H), 7.34 (d, $J = 8.2$ Hz, 1H), 3.49 (s, 2H), 2.18 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 159.91, 155.79, 152.40, 151.72, 148.96, 148.83, 148.13, 145.35, 137.89, 137.50, 134.34, 131.81, 127.49, 126.79, 124.90, 124.28, 123.72, 121.97, 119.77, 63.65, 45.30 (2C).

4.1.2.4. (E)-N-(6-((Dimethylamino)methyl)benzo[d]thiazol-2-yl)-2-(1-(pyrazin-2-yl)ethylidene)hydrazine-1-carboxamide (8d). Light white solid; yield: 68%; mp: 234–236 °C; HRMS (ESI) for $C_{17}H_{19}N_7OS$ [$M + H$]⁺. Calcd: 369.1372, found: 370.1446;

^1H NMR (400 MHz, DMSO) δ 11.40 (s, 1H), 10.67 (s, 1H), 9.80 (s, 1H), 8.63 (s, 2H), 7.84 (s, 1H), 7.65 (d, J = 8.2 Hz, 1H), 7.34 (d, J = 8.2 Hz, 1H), 3.48 (s, 2H), 2.37 (s, 3H), 2.17 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 159.49, 153.40, 150.48, 148.50, 147.88, 144.45, 144.04, 143.47, 134.67, 132.14, 127.39, 121.79, 120.03, 63.78, 45.42 (2C), 12.33.

4.1.2.5. (*E*)-*N*-(6-((Diethylamino)methyl)benzo[d]thiazol-2-yl)-2-(pyridin-2-ylmethylene)hydrazine-1-carboxamide (**8e**). Light white solid; yield: 48%; mp: 164–166 °C; HRMS (ESI) for $\text{C}_{19}\text{H}_{22}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 382.1576, found: 383.1650; ^1H NMR (400 MHz, DMSO) δ 11.49 (s, 2H), 8.58 (d, J = 4.6 Hz, 1H), 8.57–8.47 (m, 1H), 8.07 (s, 1H), 7.90–7.81 (m, 2H), 7.63 (d, J = 8.2 Hz, 1H), 7.42–7.33 (m, 2H), 3.61 (s, 2H), 2.50–2.43 (m, 4H), 0.99 (t, J = 7.2 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 159.58, 153.64, 153.34, 149.64, 148.03, 144.13, 136.91, 135.63, 132.00, 127.16, 124.56, 121.44, 121.22, 119.80, 57.31, 46.52 (2C), 12.08 (2C).

4.1.2.6. (*E*)-*N*-(6-((Diethylamino)methyl)benzo[d]thiazol-2-yl)-2-(1-(pyridin-2-yl)ethylidene)hydrazine-1-carboxamide (**8f**). Light yellow solid; yield: 58%; mp: 187–189 °C; HRMS (ESI) for $\text{C}_{20}\text{H}_{24}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 396.1732, found: 397.1806; ^1H NMR (400 MHz, DMSO) δ 11.07 (s, 1H), 10.47 (s, 1H), 8.60 (d, J = 4.5 Hz, 1H), 8.54–8.41 (m, 1H), 7.90–7.82 (m, 2H), 7.64 (d, J = 8.1 Hz, 1H), 7.44–7.38 (m, 1H), 7.36 (d, J = 8.1 Hz, 1H), 3.62 (s, 2H), 2.49–2.44 (m, 4H), 2.38 (s, 3H), 0.99 (t, J = 6.8 Hz, 6H).

4.1.2.7. 2-(Di(pyridin-2-yl)methylene)-*N*-(6-((diethylamino)methyl)benzo[d]thiazol-2-yl)hydrazine-1-carboxamide (**8g**). Light yellow solid; yield: 69%; mp: 178–180 °C; HRMS (ESI) for $\text{C}_{24}\text{H}_{25}\text{N}_7\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 459.1841, found: 460.1917; ^1H NMR (400 MHz, DMSO- d_6) δ 12.95 (s, 1H), 11.84 (s, 1H), 8.84 (m, 1H), 8.61–8.50 (m, 1H), 8.12 (m, 2H), 7.99 (m, 2H), 7.69 (m, 1H), 7.64–7.54 (m, 2H), 7.53–7.44 (m, 2H), 4.12 (s, 2H), 2.87 (q, 4H), 1.18 (t, J = 7.3 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 160.62, 155.76, 151.67 (2C), 149.00 (2C), 148.85 (2C), 145.64, 137.91 (2C), 137.51 (2C), 132.06, 128.72, 126.77, 124.93, 124.31, 123.70, 120.13, 55.78, 46.21 (2C), 9.83 (2C).

4.1.2.8. (*E*)-*N*-(6-((Diethylamino)methyl)benzo[d]thiazol-2-yl)-2-(1-(pyrazin-2-yl)ethylidene)hydrazine-1-carboxamide (**8h**). Light white solid; yield: 61%; mp: 190–192 °C; HRMS (ESI) for $\text{C}_{19}\text{H}_{23}\text{N}_7\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 397.1685, found: 398.1760. ^1H NMR (400 MHz, DMSO) δ 11.72 (s, 1H), 10.89 (s, 1H), 10.11 (s, 1H), 8.67–8.61 (m, 2H), 8.18 (s, 1H), 7.78 (d, J = 8.2 Hz, 1H), 7.64 (d, J = 8.2 Hz, 1H), 4.38 (s, 2H), 3.20–2.97 (m, 4H), 2.39 (s, 3H), 1.26 (t, J = 7.2 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 160.81, 150.51, 150.17, 148.30, 144.50, 143.86, 143.55, 140.27, 132.54, 129.44, 125.39, 124.79, 120.62, 55.27, 46.12 (2C), 12.54, 8.84 (2C).

4.1.2.9. (*E*)-*N*-(6-((4-Methylpiperidin-1-yl)methyl)benzo[d]thiazol-2-yl)-2-(pyridin-2-ylmethylene)hydrazine-1-carboxamide (**8i**). Light yellow solid; yield: 49%; mp: 155–157 °C; HRMS (ESI) for $\text{C}_{21}\text{H}_{24}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 408.1732, found: 409.1809; ^1H NMR (400 MHz, DMSO) δ 11.57 (s, 1H), 8.59 (d, J = 4.6 Hz, 1H), 8.55 (s, 1H), 8.08 (s, 1H), 7.97–7.91 (m, 1H), 7.91–7.84 (m, 1H), 7.70 (d, J = 8.2 Hz, 1H), 7.47–7.37 (m, 2H), 3.89 (s, 3H), 3.09–2.95 (m,

2H), 2.47–2.18 (m, 2H), 1.66 (d, J = 12.6 Hz, 2H), 1.52–1.38 (m, 1H), 1.24 (d, J = 10.0 Hz, 2H), 0.90 (d, J = 6.4 Hz, 3H).

4.1.2.10. (*E*)-*N*-(6-((4-Methylpiperidin-1-yl)methyl)benzo[d]thiazol-2-yl)-2-(1-(pyridin-2-yl)ethylidene)hydrazine-1-carboxamide (**8j**). Light yellow solid; yield: 61%; mp: 162–164 °C; HRMS (ESI) for $\text{C}_{22}\text{H}_{26}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 422.1889, found: 423.1967; ^1H NMR (400 MHz, DMSO) δ 11.21 (s, 1H), 10.52 (s, 1H), 8.60 (s, 1H), 8.56–8.42 (m, 1H), 8.04–7.92 (m, 1H), 7.92–7.81 (m, 1H), 7.80–7.67 (m, 1H), 7.55–7.36 (m, 2H), 4.08 (s, 2H), 3.23–2.93 (m, 2H), 2.40–2.38 (m, 2H), 2.09 (s, 3H), 1.78–1.64 (m, 2H), 1.56–1.41 (m, 1H), 1.32–1.21 (m, 2H), 0.96–0.81 (m, 3H); ^{13}C NMR (101 MHz, DMSO) δ 160.36, 155.07, 153.20, 150.12, 149.57, 148.87, 136.88, 132.36, 130.23, 128.93, 124.45, 123.93, 121.50, 120.35, 60.58, 52.44 (2C), 32.10 (2C), 29.21, 21.63, 12.64.

4.1.2.11. 2-(Di(pyridin-2-yl)methylene)-*N*-(6-((4-methylpiperidin-1-yl)methyl)benzo[d]thiazol-2-yl)hydrazine-1-carboxamide (**8k**). Yellow solid; yield: 61%; mp: 134–136 °C; HRMS (ESI) for $\text{C}_{26}\text{H}_{27}\text{N}_7\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 485.1998, found: 486.2074; ^1H NMR (400 MHz, DMSO) δ 12.88 (s, 1H), 11.79 (s, 1H), 8.83 (s, 1H), 8.56 (s, 1H), 8.21–8.10 (m, 1H), 8.03–7.92 (m, 2H), 7.82 (s, 1H), 7.63–7.54 (m, 2H), 7.53–7.43 (m, 2H), 7.36–7.29 (m, 1H), 3.52 (s, 2H), 2.84–2.71 (m, 2H), 2.02–1.86 (m, 2H), 1.61–1.50 (m, 2H), 1.37–1.30 (m, 1H), 1.20–1.06 (m, 2H), 0.94–0.81 (m, 3H); ^{13}C NMR (101 MHz, DMSO) δ 159.86, 155.78 (2C), 151.71, 148.97 (2C), 148.83 (2C), 137.89 (2C), 137.50 (2C), 131.79, 127.62, 126.78, 124.90, 124.28, 122.08, 119.76, 62.65, 53.60 (2C), 34.22 (2C), 30.65, 22.24.

4.1.2.12. (*E*)-*N*-(6-((4-Methylpiperidin-1-yl)methyl)benzo[d]thiazol-2-yl)-2-(1-(pyrazin-2-yl)ethylidene)hydrazine-1-carboxamide (**8l**). Light white solid; yield: 61%; mp: 235–237 °C; HRMS (ESI) for $\text{C}_{21}\text{H}_{25}\text{N}_7\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 423.1841, found: 424.1918; ^1H NMR (400 MHz, DMSO) δ 11.73 (s, 1H), 10.92 (s, 1H), 9.75 (s, 1H), 8.67–8.61 (m, 2H), 8.15 (s, 1H), 7.78 (d, J = 8.2 Hz, 1H), 7.63 (d, J = 8.4 Hz, 1H), 4.42–4.24 (m, 2H), 3.37–3.34 (m, 2H), 3.02–2.84 (m, 2H), 2.39 (s, 3H), 1.84–1.70 (m, 2H), 1.67–1.54 (m, 1H), 1.52–1.34 (m, 2H), 0.91 (d, J = 6.2 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 160.71, 153.12, 150.53, 150.15, 148.30, 144.45, 143.54, 132.37, 129.82, 125.09, 124.98, 124.73, 120.43, 59.47, 51.88 (2C), 31.02 (2C), 28.70, 21.55, 12.61.

4.1.2.13. (*E*)-*N*-(4-((Dimethylamino)methyl)phenyl)-2-(pyridin-2-ylmethylene)hydrazine-1-carboxamide (**9a**). Light yellow solid; yield: 43%; mp: 102–104 °C; HRMS (ESI) for $\text{C}_{16}\text{H}_{19}\text{N}_5\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 297.1590, found: 298.1662; ^1H NMR (400 MHz, DMSO) δ 10.98 (s, 1H), 8.99 (s, 1H), 8.57 (d, J = 4.6 Hz, 1H), 8.34 (d, J = 8.0 Hz, 1H), 8.00 (s, 1H), 7.86 (m, 1H), 7.60 (d, J = 8.4 Hz, 2H), 7.40–7.35 (m, 1H), 7.21 (d, J = 8.4 Hz, 2H), 3.34 (s, 2H), 2.14 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 153.86, 153.34, 149.68, 141.63, 138.12, 136.93, 133.58, 129.33 (2C), 124.26, 120.62, 120.35 (2C), 63.45, 45.33 (2C).

4.1.2.14. 2-(Di(pyridin-2-yl)methylene)-*N*-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide (**9b**). Yellow solid; yield: 53%; mp: 110–112 °C; HRMS (ESI) for $\text{C}_{21}\text{H}_{22}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 374.1855, found: 375.1929; ^1H NMR (400 MHz, DMSO) δ 12.30 (s, 1H), 9.27 (s, 1H), 8.83 (d,

$J = 4.2$ Hz, 1H), 8.55 (d, $J = 4.1$ Hz, 1H), 8.22 (d, $J = 7.9$ Hz, 1H), 8.03–7.91 (m, 2H), 7.58–7.51 (m, 3H), 7.49 (d, $J = 8.0$ Hz, 1H), 7.47–7.42 (m, 1H), 7.23 (d, $J = 8.4$ Hz, 2H), 3.37 (s, 2H), 2.15 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 155.96, 152.74, 151.98, 148.81, 148.71, 142.43, 138.09, 137.79, 137.47, 133.43, 129.56 (2C), 126.87, 124.72, 124.04, 123.78, 120.11 (2C), 63.31, 45.23 (2C).

4.1.2.15. (*E*)-*N*-(4-((Diethylamino)methyl)phenyl)-2-(1-(pyridin-2-yl)ethylidene)hydrazine-1-carboxamide (**9c**). Yellow solid; yield: 57%; mp: 106–108 °C; HRMS (ESI) for $\text{C}_{19}\text{H}_{25}\text{N}_5\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 339.2059, found: 340.2133. ^1H NMR (400 MHz, DMSO) δ 9.92 (s, 1H), 8.93 (s, 1H), 8.58 (d, $J = 4.2$ Hz, 1H), 8.40 (d, $J = 8.2$ Hz, 1H), 7.86–7.80 (m, 1H), 7.56 (d, $J = 8.4$ Hz, 2H), 7.41–7.35 (m, 1H), 7.24 (d, $J = 8.4$ Hz, 2H), 3.48 (s, 2H), 2.46 (q, $J = 7.2$ Hz, 4H), 2.36 (s, 3H), 0.98 (t, $J = 7.2$ Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 155.43, 153.75, 148.82, 146.99, 137.86, 136.83, 134.58, 129.12 (2C), 124.02, 121.06, 120.45 (2C), 56.95, 46.46 (2C), 12.34, 12.14 (2C).

4.1.2.16. 2-(Di(pyridin-2-yl)methylene)-*N*-(4-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide (**9d**). Yellow solid; yield: 51%; mp: 118–120 °C; HRMS (ESI) for $\text{C}_{23}\text{H}_{26}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 402.2168, found: 403.2244; ^1H NMR (400 MHz, DMSO) δ 12.32 (s, 1H), 9.23 (s, 1H), 8.83 (d, $J = 4.2$ Hz, 1H), 8.55 (d, $J = 4.2$ Hz, 1H), 8.22 (d, $J = 8.0$ Hz, 1H), 8.02–7.91 (m, 2H), 7.59–7.55 (m, 1H), 7.53 (d, $J = 8.4$ Hz, 2H), 7.49 (d, $J = 8.0$ Hz, 1H), 7.47–7.42 (m, 1H), 7.24 (d, $J = 8.4$ Hz, 2H), 3.47 (s, 2H), 2.44 (q, $J = 7.2$ Hz, 4H), 0.97 (t, $J = 7.2$ Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 155.98, 152.76, 151.99, 148.78, 148.70, 142.33, 137.77, 137.73, 137.45, 134.75, 129.21 (2C), 126.87, 124.70, 124.01, 123.78, 120.20 (2C), 56.92, 46.47 (2C), 12.13 (2C).

4.1.2.17. (*E*)-*N*-(4-((4-methylpiperidin-1-yl)methyl)phenyl)-2-(pyridin-2-ylmethylene)hydrazine-1-carboxamide (**9e**). Yellow solid; yield: 64%; mp: 166–168 °C; HRMS (ESI) for $\text{C}_{20}\text{H}_{25}\text{N}_5\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 351.2059, found: 352.2133; ^1H NMR (400 MHz, DMSO) δ 11.01 (s, 1H), 9.01 (s, 1H), 8.57 (d, $J = 4.5$ Hz, 1H), 8.34 (d, $J = 8.0$ Hz, 1H), 8.01 (s, 1H), 7.86 (t, $J = 7.1$ Hz, 1H), 7.60 (d, $J = 8.3$ Hz, 2H), 7.42–7.31 (m, 1H), 7.24 (d, $J = 8.1$ Hz, 2H), 3.47 (s, 2H), 2.89–2.72 (m, 2H), 2.09–1.88 (m, 2H), 1.62–1.50 (m, 2H), 1.38–1.28 (m, 1H), 1.23–1.11 (m, 2H), 0.88 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 153.88, 153.32, 149.68, 141.69, 138.34, 136.92, 129.94, 129.70 (2C), 124.26, 120.59, 120.34 (2C), 62.12, 53.40 (2C), 34.00 (2C), 30.54, 22.18.

4.1.2.18. (*E*)-*N*-(4-((4-Methylpiperidin-1-yl)methyl)phenyl)-2-(1-(pyridin-2-yl)ethylidene)hydrazine-1-carboxamide (**9f**). Yellow solid; yield: 72%; mp: 170–172 °C; HRMS (ESI) for $\text{C}_{21}\text{H}_{27}\text{N}_5\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 365.2166, found: 366.2290; ^1H NMR (400 MHz, DMSO) δ 9.94 (s, 1H), 8.95 (s, 1H), 8.58 (d, $J = 4.2$ Hz, 1H), 8.40 (d, $J = 8.1$ Hz, 1H), 7.86–7.79 (m, 1H), 7.56 (d, $J = 8.4$ Hz, 2H), 7.41–7.33 (m, 1H), 7.21 (d, $J = 8.4$ Hz, 2H), 3.38 (s, 2H), 2.76 (d, $J = 11.4$ Hz, 2H), 2.36 (s, 3H), 1.93–1.82 (m, 2H), 1.56 (d, $J = 11.7$ Hz, 2H), 1.38–1.27 (m, 1H), 1.19–1.06 (m, 2H), 0.89 (d, $J = 6.5$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 155.42, 153.73, 148.82, 147.01, 138.03, 136.82, 133.40, 129.41 (2C), 124.02, 121.06, 120.39 (2C), 62.59, 53.69 (2C), 34.47 (2C), 30.85, 22.32, 12.35.

4.1.2.19. 2-(Di(pyridin-2-yl)methylene)-*N*-(4-((4-methylpiperidin-1-yl)methyl)phenyl)hydrazine-1-carboxamide (**9g**). Yellow solid; yield: 76%; mp: 110–112 °C; HRMS (ESI) for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 428.2325, found: 429.2398; ^1H NMR (400 MHz, DMSO) δ 12.31 (s, 1H), 9.25 (s, 1H), 8.83 (d, $J = 4.0$ Hz, 1H), 8.55 (d, $J = 4.0$ Hz, 1H), 8.21 (d, $J = 7.8$ Hz, 1H), 8.04–7.92 (m, 2H), 7.59–7.42 (m, 5H), 7.21 (d, $J = 8.0$ Hz, 2H), 3.38 (s, 2H), 2.76 (d, $J = 10.8$ Hz, 2H), 1.88 (t, $J = 10.8$ Hz, 2H), 1.55 (d, $J = 11.6$ Hz, 2H), 1.38–1.24 (m, 1H), 1.19–1.04 (m, 2H), 0.88 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 155.97, 152.74, 151.99, 148.80, 148.71, 142.38, 137.92, 137.78, 137.46, 133.47, 129.52 (2C), 126.86, 124.71, 124.02, 123.78, 120.15 (2C), 62.53, 53.67 (2C), 34.43 (2C), 30.81, 22.30.

4.1.2.20. (*E*)-*N*-(4-((4-Methylpiperidin-1-yl)methyl)phenyl)-2-(1-(pyrazin-2-yl)ethylidene)hydrazine-1-carboxamide (**9h**). Light white solid; yield: 61%; mp: 212–214 °C; HRMS (ESI) for $\text{C}_{20}\text{H}_{26}\text{N}_6\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 366.2168, found: 367.2245; ^1H NMR (400 MHz, DMSO) δ 10.12 (s, 1H), 9.69 (d, $J = 1.4$ Hz, 1H), 9.05 (d, $J = 11.9$ Hz, 1H), 8.65–8.56 (m, 2H), 7.58 (d, $J = 8.4$ Hz, 2H), 7.22 (d, $J = 8.4$ Hz, 2H), 3.39 (s, 2H), 2.77 (d, $J = 11.4$ Hz, 2H), 2.34 (s, 3H), 1.88 (t, $J = 11.0$ Hz, 2H), 1.56 (d, $J = 11.0$ Hz, 2H), 1.38–1.25 (m, 1H), 1.20–1.06 (m, 2H), 0.89 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 153.69, 150.76, 145.05, 144.03, 143.56, 143.45, 137.93, 133.60, 129.33 (2C), 120.88 (2C), 62.58, 53.69 (2C), 34.46 (2C), 30.84, 22.32, 12.05.

4.1.2.21. (*E*)-*N*-(6-((Diethylamino)methyl)benzo[d]thiazol-2-yl)-2-(1-(4-fluorophenyl)ethylidene)hydrazine-1-carboxamide (**10a**). Light yellow solid; yield: 81%; mp: 171–173 °C; HRMS (ESI) for $\text{C}_{21}\text{H}_{24}\text{FN}_5\text{OS}$ $[\text{M} + \text{H}]^+$. Calcd: 413.1686, found: 414.1762; ^1H NMR (400 MHz, DMSO) δ 10.90 (s, 1H), 10.38 (s, 1H), 8.10–7.99 (m, 2H), 7.85 (s, 1H), 7.63 (d, $J = 8.4$ Hz, 1H), 7.36 (dd, $J = 8.4, 1.3$ Hz, 1H), 7.26 (t, $J = 8.8$ Hz, 2H), 3.62 (s, 2H), 2.51–2.43 (m, 4H), 2.30 (s, 3H), 0.99 (t, $J = 7.2$ Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 164.47, 162.01, 159.18, 148.29, 135.56, 134.73, 134.70, 132.09, 129.35, 129.27, 127.18, 121.48, 119.96, 115.61, 115.40, 57.30, 46.53 (2C), 14.50 (2C), 12.07.

4.1.2.22. (*E*)-*N*-(6-((4-Methylpiperidin-1-yl)methyl)benzo[d]thiazol-2-yl)-2-(1-phenylethylidene)hydrazine-1-carboxamide (**10b**). Light yellow solid; yield: 76%; mp: 232–234 °C; HRMS (ESI) for $\text{C}_{23}\text{H}_{27}\text{N}_5\text{OS}$ $[\text{M} + \text{H}]^+$. Calcd: 421.1936, found: 422.2012; ^1H NMR (400 MHz, DMSO) δ 11.07 (s, 1H), 10.47 (s, 1H), 8.08 (s, 1H), 7.96 (d, $J = 5.4$ Hz, 2H), 7.78 (d, $J = 8.2$ Hz, 1H), 7.55 (d, $J = 8.2$ Hz, 1H), 7.48–7.38 (m, 3H), 4.36 (s, 2H), 3.43–3.34 (m, 2H), 3.02–2.85 (m, 2H), 2.32 (s, 3H), 1.79 (d, $J = 13.6$ Hz, 2H), 1.59 (s, 1H), 1.42–1.27 (m, 2H), 0.91 (d, $J = 6.4$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 160.76, 153.12, 150.22, 149.69, 138.17, 132.48, 130.10, 129.67, 128.72 (2C), 127.00 (2C), 125.03, 124.73, 120.50, 59.75, 52.12 (2C), 31.26 (2C), 28.63, 21.57, 14.56.

4.1.2.23. *N*-(4-((Dimethylamino)methyl)phenyl)-2-(diphenylmethylene)hydrazine-1-carboxamide (**10c**). Light white solid; yield: 38%; mp: 123–125 °C; HRMS (ESI) for $\text{C}_{23}\text{H}_{24}\text{N}_4\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 372.1950, found: 373.2027; ^1H NMR (400 MHz, DMSO) δ 9.10 (s, 1H), 8.68 (s, 1H), 7.66–7.61 (m, 2H), 7.61–7.58 (m, 1H), 7.58–7.53 (m, 2H), 7.48 (d, $J = 8.4$ Hz, 2H), 7.42–7.36 (m, 3H), 7.35–7.30 (m, 2H), 7.19 (d, $J = 8.4$ Hz, 2H),

3.32 (s, 2H), 2.13 (s, 6H); ^{13}C NMR (101 MHz, DMSO) δ 152.54, 148.55, 138.28, 137.82, 133.38, 132.73, 130.10 (2C), 129.99 (2C), 129.51 (2C), 128.97 (2C), 128.75 (2C), 127.51 (2C), 119.56 (2C), 63.45, 45.34 (2C).

4.1.2.24. *N*-(4-((Diethylamino)methyl)phenyl)-2-(diphenylmethylene)hydrazine-1-carboxamide (**10d**). Light white solid; yield: 41%; mp: 125–127 °C; HRMS (ESI) for $\text{C}_{25}\text{H}_{28}\text{N}_4\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 400.2263, found: 401.2339; ^1H NMR (400 MHz, DMSO) δ 9.08 (s, 1H), 8.67 (s, 1H), 7.67–7.61 (m, 2H), 7.61–7.58 (m, 1H), 7.58–7.53 (m, 2H), 7.46 (d, J = 8.4 Hz, 2H), 7.41–7.36 (m, 3H), 7.35–7.30 (m, 2H), 7.22 (d, J = 8.5 Hz, 2H), 3.46 (s, 2H), 2.44 (q, J = 7.2 Hz, 4H), 0.97 (t, J = 7.2 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 152.55, 148.50, 138.03, 137.83, 134.30, 132.73, 130.10 (2C), 129.98, 129.49, 129.25 (2C), 128.97 (2C), 128.74 (2C), 127.51 (2C), 119.63 (2C), 56.90, 46.45 (2C), 12.12 (2C).

4.1.2.25. 2-(Diphenylmethylene)-*N*-(4-(4-methylpiperidin-1-yl)methyl)phenyl)hydrazine-1-carboxamide (**10e**). Light white solid; yield: 43%; mp: 126–128 °C; HRMS (ESI) for $\text{C}_{27}\text{H}_{30}\text{N}_4\text{O}$ $[\text{M} + \text{H}]^+$. Calcd: 426.2420, found: 427.2493; ^1H NMR (400 MHz, DMSO) δ 9.08 (s, 1H), 8.67 (s, 1H), 7.67–7.61 (m, 2H), 7.61–7.58 (m, 1H), 7.58–7.50 (m, 2H), 7.47 (d, J = 8.4 Hz, 2H), 7.41–7.36 (m, 3H), 7.35–7.29 (m, 2H), 7.19 (d, J = 8.4 Hz, 2H), 3.37 (s, 2H), 2.75 (d, J = 11.4 Hz, 2H), 1.87 (t, J = 10.8 Hz, 2H), 1.55 (d, J = 11.4 Hz, 2H), 1.38–1.23 (m, 1H), 1.17–1.05 (m, 2H), 0.88 (d, J = 6.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO) δ 152.53, 148.52, 138.20, 137.83, 133.12, 132.73, 130.09 (2C), 129.99, 129.54 (2C), 129.50, 128.97 (2C), 128.74 (2C), 127.51 (2C), 119.59 (2C), 62.55, 53.68 (2C), 34.45 (2C), 30.82, 22.31.

4.2 CCK-8 assay

U937 and MCF-7 cell lines were purchased from the cell bank of Chinese Academy of Sciences, Shanghai, China. U937 cells were grown in Roswell Park Memorial Institute 1640 medium mixed with 10% fetal bovine serum, and MCF-7 cells were grown in Dulbecco's modified Eagle's medium mixed with 10% fetal bovine serum; all the cells were cultured in a 5% CO_2 incubator at 37 °C. The anticancer activity was evaluated by the CCK-8 assay. A stock solution of each test compound was prepared in DMSO at a concentration of 1 mM. Prior to use, the stock solution was further diluted to a final concentration with the culture medium. The concentration of DMSO was less than 0.01%, and the amount was determined by preliminary testing. Cells were seeded at a density of 5×10^4 cells per well in a 96-well plate and incubated for 12 h, followed by the treatment with the test compounds. Control culture received the carrier solvent (0.01% DMSO). After 24 h, the viability of U937 and MCF7 cells was measured by a cell counting kit 8 (CCK-8) assay. 10 μL of CCK-8 solution was added into each well and incubated at 37 °C for 2 h. The optical density at 450 nm was determined with a microplate reader.

4.3 Apoptosis study

Apoptosis of U937 cells was detected using a flow cytometric assay. Briefly, cells were seeded in 6-well plates and incu-

bated overnight. The following day, cells were treated with different concentrations of compounds **8j** and **8k** for 24 h and 48 h. The cells and supernatants were harvested and washed twice with cold PBS and then resuspended in 100 μL $1\times$ binding buffer. 5 μL FITC Annexin V and 5 μL PI were added into each tube and the cells were then gently vortexed and incubated for 15 min at rt (25 °C) in the dark. 400 μL of $1\times$ binding buffer was then added to each tube. The stained cells were analyzed using a flow cytometer (FACS Calibur; BD).

4.4 Caspase activity in cell lysate

To each inner well of two 96-well plates was added 100 μL of a suspension of U937 cells in phenol red-free RPMI-1640 complete growth media at 500 000 cells per mL (50 000 cells per well). Inner wells without compounds were filled with 200 μL of media, and outer wells were filled with 200 μL of sterile PBS. The plates were incubated at 37 °C with 5% CO_2 for 15 hours. Solutions of compounds (selected compounds, PAC-1 and DMSO alone) in media were prepared at $3\times$ final concentrations (3% DMSO), and 50 μL of each solution was added to the appropriate wells. Each compound was tested in six wells per plate. In the first plate, 50 μL of bifunctional cell lysis/caspase activity buffer was added to each treatment well. Fluorescence (ex. 400 nm, em. 505 nm) was monitored on an Analyst AD 96-384 plate reader *via* a 60 minute kinetic read, and the slope was used to determine the caspase activity. The second plate was incubated at 37 °C with 5% CO_2 for 16 hours, at which time 50 μL of bifunctional cell lysis/caspase activity buffer was added to each treatment well, and fluorescence was monitored as above. The slopes at 16 hours were normalized to the slopes of each compound at 0 hours (0% activity) and the PAC-1-treated samples at 16 hours (100% activity) to give the percent caspase activity.

4.5 Procaspace-3 activation assay

Procaspace-3 was purchased from R&D Systems. Procaspace-3 was incubated at 100 nM, in the presence of selected compounds at 10 μM in a reaction buffer consisting of 20 mM Tris, 300 mM NaCl, 5 mM dithiothreitol, 5% sucrose and 0.05% CHAPS, pH 8.0. The mixtures were assayed for kinetic activity by incubation with 20 μM Ac-DEVD-AFC for 5 minutes after 4 hours of incubation at 37 °C. The fluorescence value was read at excitation and emission wavelengths of 400 nm and 505 nm. The activation rate (%) was calculated using the following equation: $(F_{\text{compound}} - F_{\text{DMSO}})/(F_{\text{PAC-1}} - F_{\text{DMSO}}) \times 100$.

4.6 Toxicity and morphological observation in zebrafish embryos

Wild type zebrafish (AB strain) was raised and maintained at 28.5 °C as described previously.²⁶ The developmental stages were staged in hours post-fertilization according to morphological criteria described by Kimmel *et al.* Embryos were exposed to different concentrations of **8j** and **8k** (5, 10, 20 and 40 μM) prepared by diluting the stock solution of each

compound in the fish medium. A no-treatment control was also included. Experiments were performed in triplicates and a total of 24 embryos for each group have been used per treatment. The survival rate and gross morphology of the embryos in each system were observed every 24 h for a period of 72 h under a stereo microscope (Olympus, Japan). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Huaqiao University and approved by the Animal Ethics Committee of School of Medicine.

4.7 Acridine orange staining in zebrafish embryos

Embryos that developed normally and reached the blastula stage (2 h post fertilization [hpf]) were exposed to 8j and 8k (40 μM and 60 μM) in a culture dish until 24 hpf. Live embryos were placed in 50 $\mu\text{g mL}^{-1}$ acridine orange (Sigma-Aldrich) diluted in egg water for 30 minutes at room temperature in the dark. The embryos were then washed in egg water and analyzed using a fluorescence microscope.

4.8 RNA isolation and real-time PCR

Total RNAs were isolated from zebrafish embryos or tissues of adults using an SV total RNA isolation system (Promega), and the quantity and quality were determined by agarose electrophoresis and spectrophotometry. The isolated RNAs were then reverse-transcribed by using an M-MLV reverse transcriptase kit (Takara Biochemicals, Dalian, China) following the manufacturer's recommendation. The real-time SYBR Green primers for zebrafish *bax* and β -actin were chosen as follows: *bax* forward: 5'-TAAACAACCTCTAATCTTCAGCCGAC-3' and reverse: 5'-AGCTTATGATGGTTCTGATGATGTC-3'; β -actin forward: 5'-AGCACGGTATTGTGACTAACTG-3' and reverse: 5'-TCGAACATGATCTGTGTCATC-3'. β -actin was detected as an internal control. Real-time PCR analyses were performed as described previously. The probability (*P*) of <0.01 was considered statistically significant.

4.9 Metabolic stability

Experimental procedure: 1. Buffer A: 1.0 L of 0.1 M monobasic potassium phosphate buffer containing 1.0 mM EDTA, buffer B: 1.0 L of 0.1 M dibasic potassium phosphate buffer containing 1.0 mM EDTA, buffer C: 0.1 M potassium phosphate buffer, 1.0 mM EDTA, pH 7.4 by titrating 700 mL of buffer B with buffer A while monitoring with the pH meter. 2. Reference compound (ketanserin) and test compound spike solution: 500 μM spike solution: add 10 μL of 10 mM DMSO stock solution into 190 μL ACN. 1.5 μM spike solution in microsomes (0.75 mg mL^{-1}): add 1.5 μL of 500 μM spike solution and 18.75 μL of 20 mg mL^{-1} liver microsomes into 479.75 μL of buffer C on ice. 3. Prepare NADPH stock solution (6 mM) by dissolving NADPH in buffer C. 4. Dispense 30 μL of 1.5 μM spike solution containing 0.75 mg mL^{-1} microsome solution to the assay plates designated for different time points (0, 5, 15, 30, and 45 min) on ice. 5. For 0 min, add 135 μL of ACN containing IS to the wells of the 0 min

plate and then add 15 μL of NADPH stock solution (6 mM). 6. Pre-incubate all other plates at 37 $^{\circ}\text{C}$ for 5 minutes. 7. Add 15 μL of NADPH stock solution (6 mM) to the plates to start the reaction and timing. 8. At 5 min, 15 min, 30 min, and 45 min, add 135 μL of ACN containing IS to the wells of the corresponding plates, respectively, to stop the reaction. 9. After quenching, shake the plates at the vibrator (IKA, MTS 2/4) for 10 min (600 rpm) and then centrifuge at 5594g for 15 min (Thermo Multifuge $\times 3\text{R}$). 10. Transfer 50 μL of the supernatant from each well into a 96-well sample plate containing 50 μL of ultrapure water (Millipore, ZMQS50F01) for LC/MS analysis.

Conflicts of interest

There are no conflicts to declare.

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