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Semicarbazone derivatives bearing phenyl moiety: synthesis, anticancer activity, cell cycle, apoptosis-inducing and metabolic stability study

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A series of semicarbazone derivatives bearing phenyl moiety were synthesized and evaluated for the *vitro* anticancer activities in four human cancer cell lines (HT29, SK-N-SH, MDA-MB-231 and MKN45). Biological evaluation led to the identification of 11q and 11s, which showed excellent anticancer activities against tested cancer cell lines with IC_{50} values ranging from 0.32 to 1.57 μM, respectively, while exhibiting weak cytotoxicity on the normal cells (HUVEC). Flow cytometric assay for cell cycle and apoptosis revealed that 11q and 11s caused an arrest in the Sub-G1 cell cycle and inhibited proliferation of cancer cells by inducing apoptosis in a dose-dependent manner. Further enzymatic assay suggested that 11q and 11s could significantly activated procaspase-3 to caspase-3. Metabolic stability study indicated that 11q and 11s showed moderate stability *in vitro* in human and rat liver microsomes. In view of promising pharmacological activities of 11q and 11s, which had emerged as the valuable lead for further development in the treatment for cancer.

Key words anticancer activity; semicarbazone; sub-G1; apoptosis; metabolic stability.

Cancer is one of the most death-defying health hazards distressing a greater part of the world population. As evasion of apoptosis is a hallmark of cancer[1, 2], thus, it has been an effective approach to explore novel apoptosis-inducing compounds for the treatment of cancer. Procaspase-3 is the most common and key proapoptotic protein in the downstream apoptotic cascade, playing a significant role in the cancer development and progression. Procaspase-3 activators, which can directly induce apoptosis by activating over-expression procaspase-3 to caspase-3[3-8], show greater advantages over anticancer agents targeting early or intermediate positions in the apoptotic cascade, such as p53 disruptors (CBL-0137 and COTI-2)[9-11], XIAP inhibitors (AZD5582 and GDC-0152)[12, 13], MDM2 inhibitors(HDM201 and RG7112) [14, 15], and Bcl-2 inhibitors (GDC-0199 and ABT-737)[16, 17], which likely resist the anticancer effects due to mutations of downstream apoptotic proteins.

PAC-1 (**Fig. 1**) was reported as the first procaspase-3 activator that induced apoptosis by selectively activating procaspase-3 to caspase-3. Structure−activity relationships (SARs) revealed that ortho-hydroxy *N*-acylhydrazone moiety of PAC-1 was responsible for strong caspase-3 activation activity. In our previous study, based on the SARs, inspired by semicarbazone moiety widely used as building block in design of different potential anticancer agents due to the presence of several hydrogen donors and acceptors as well as its flexible skeleton[18-20], a series of semicarbazone derivatives containing benzothiazole had been independently reported as procaspase-3 activators[21, 22], and the compounds **1** and **2** (**Fig. 1**) showed promising procaspase-3 activation activities and anticancer activities, further SARs of which indicated that introduction of lipophilic groups (eg. substituted benzyloxy or heteroaryloxy groups) in the 2-hydroxy phenyl ring could enhance the anticancer activity.
(**Fig. 1** should be listed here)

In this manuscript, we described a series of optimized semicarbazone derivatives (**Fig. 1**), in which we reserved these lipophilic groups that were beneficial for anticancer activity, while we replaced benzothiazole with phenyl to reduce lipophilicity. Furthermore, all the designed compounds were salified with hydrogen chloride (HCl) to increase the solubility. All the target compounds were evaluated for their *in vitro* anticancer activities against four cancer cell lines (HT29, SK-N-SH, MDA-MB-231 and MKN45). Compounds with promising anticancer activities were selected to further determine the cytotoxicity on the normal cells (HUVEC). Cell cycle and apoptosis studies were carried out to explore their mechanism of action. Metabolic stability studies were also performed to assess $T_{1/2}$ *in vitro* and provided a reliable guide for further PK/PD *in vivo*.

Results and Discussion

Chemistry The synthesis of target compounds is illustrated in **Chart 1**. 4-nitrobenzyl bromide reacted with excessive secondary amines (dimethylamine and diethylamine) in acetonitrile *via* a nucleophilic substitution reaction for 3 h to produce intermediates **3a-b**, which were further reduced in the presence of $FeCl_3 \cdot 6H_2O$ and 80 % hydrazine to obtain the substituted anilines **4a-b**. Treatment of **4a-b** with phenyl chloroformate though *N*-acylation reaction yielded **5a-b**. The key semicarbazides **6a-b** were synthesized *via* hydrazinolysis of **5a-b** with 80% hydrazine in 1,4-dioxane at 80 °C for 6 h. In addition, the key intermediates **7a–j** and **10** were prepared by using known preparation methods in our previous work[21]. Subsequently, **6a-b** reacted with appropriate 2-hydroxy aromatic aldehydes (**7a–j** and **10)** *via*

condensation reaction in ethanol at 78 °C in the presence of catalytic amounts of acetic acid, then reaction mixtures were further salified with hydrogen chloride (HCl) in ethanol to get target compounds **11a-v**. Target compounds **11a-v** might exist as either the *E* or *Z* isomeric form due to presence of imino bond. Accordingly, compound **11s** was selected to determine the stereochemistry *via* undergoing NOESY-NMR. Our results (**Fig. 2**) indicated that an obvious NOE signal was observed between the H_1 (-N**H**-N=, 10.99 ppm) and H_2 (-N=C**H**-, 8.24 ppm), which existed only in the *E* isomer due to the appropriate intramolecular H_1–H_2 distance. No NOE signal would be observed when compound existed in the *Z* isomer. Thus, based on the above results, target compounds **11a-v** were confirmed as the *E* isomer.

(**Chart 1** should be listed here)

(**Fig. 2** should be listed here)

***In vitro* anticancer activity** All the target compounds (**11a-v**) were evaluated for their *in vitro* anticancer activities against four human cancer cell lines, including human colon cancer(HT29), human neuroblastoma(SK-N-SH), human breast cancer(MDA-MB-231) and human gastric cancer(MKN45). The results were summarized in Table 1. As shown in Table 1, compared to the positive control PAC-1, most compounds displayed moderate to prominent anticancer activities against these tested cancer cell lines. Among them, the promising compounds **11q** and **11s** showed excellent activities against four cancer cell lines with IC_{50} values ranging from 0.32 to 1.57 μM.

Further investigations were carried out to study the effect of different substituents of benzyloxyl group on the activity (compounds **11a-p**). The results revealed that incorporation of bulk substituent at the 4-position of the benzyloxyl group could enhance anticancer activity (**11b** *vs.* **11c**, **11h** *vs.* **11i**), indicating that increasing steric hindrance of the group at this region of benzyloxyl group exhibited a positive effect on the activity. Moreover, chlorine (**11d**, **11j**, R_3=4-Cl) at the 4-position of benzyloxyl group was more preferable than the fluorine (**11e**, **11m**, R_3=4-F), and the anticancer activities of compounds **11j**, **11k** and **11l** revealed that the 4-position of benzyl group did a better contribution to the potency than the 2 or 3-position. In addition, introduction of mono-electron-withdrawing group (**11d**, **11j**, R_3=4-Cl) or weak mono-electron-donating group (**11b**, **11h**, R=4-CH_3) caused no remarkable and regular alteration in activity, whereas introduction of two-EWGs such as 2,4-di-Cl or 2,3-di-Cl (**11f**, **11g**, **11n** and **11o**) on the benzyl group result in a dramatic decrease in activity, implying that greatly reducing the electron density of benzyl group was not beneficial for the anticancer activity.

(**Table 1** should be listed here)

To exclude the effect of cytotoxicity, compounds (**11q** and **11s**) with promising anticancer activities were carried out to evaluate their inhibitory activities for 48 h against normal cells derived from Human Umbilical Vein Endothelial Cells (HUVEC). The results (**Fig. 3**) revealed that no significant inhibition of proliferation was observed for treatment with different concentrations (0.25, 0.5, 1, 2, 4, 8 μM) of **11q** and **11s,** suggesting that **11q** and **11s** exhibited weak cytotoxicity on the normal cells. In addition, we also tested the anticancer activities of **11q** and **11s** against three other tumor cell lines (U937, MCF-7 and H226) in our laboratory, as show in **Table 2**, **11q** and **11s** showed potent activities against these three cancer cell lines with IC_{50} values ranging from 1.60 to 3.85 μM, which were more

active than PAC-1. Taken together, these above findings identified compound **11q** and **11s** as the promising lead compounds for subsequent biological assessment.

(**Fig.3** should be listed here)

(**Table 2** should be listed here)

Cell cycle Encouraged by the anticancer activities of compounds **11q** and **11s**, we conducted a series of cell-based assays to study their mechanism in depth. The assessment of cell cycle of HT-29 cells treated with different concentrations (0, 2, 4, 8 μM) of **11q** and **11s** was performed. **Fig. 4** and **Fig. 5** showed the distribution of HT-29 cells in different phases of the cell cycle. It was observed that the treatment with **11q** lead to a remarkable increase in the sub-G1 area from 10.9% to 26.7% and 94.1% in the concentrations of 2, 4 and 8 μM, respectively, while the treatment with **11s** lead to a more obvious increase in the sub-G1 area from 19.6% to 35.0% and 94.3% in the same concentrations. These results indicated that **11q** and **11s** inhibited cancer cells proliferation by arresting the Sub-G1 phase (indicative of apoptosis) in a dose-dependent manner.

(**Fig. 4** should be listed here)

(**Fig. 5** should be listed here)

Apoptosis study In order to determine whether the inhibitory effects of **11q** and **11s** were dependent on inducing cancer cells apoptosis, HT29 cells were incubated with different concentrations (0, 2, 4, and 8 μM) of **11q** and **11s** for 48 h and the percentages of apoptotic cells were determined by FITC-Annexin V/PI staining and flow cytometry. As shown **Fig. 6**, treatment HT-29 cells with different concentrations of **11q** and **11s** for 48 h caused a significant dose-dependent increase in the population of both early and late apoptotic cells compared to the control cells, suggesting that **11q** and **11s** could induce cancer cells apoptosis in a dose-dependent manner.

(**Fig. 6** should be listed here)

In vitro procaspase-3 activation assay In order to explore further mechanism, compounds **11q** and **11s** were selected for the enzymatic assay to determine procaspase-3 activation activity. As shown in Table 3, compared to PAC-1, compounds **11q** and **11s** displayed potent procaspase-3 activation activities at 10 μM with degrees of 68.4% and 76.3%, respectively, suggesting that apoptosis of cancer cells induced by activation of procaspase-3 might be a potential mechanism for antitumor effects of these target compounds.

(**Table 3** should be listed here)

Metabolic stability study Compounds **11q** and **11s** were submitted for *in vitro* metabolic stability study in the presence of human, rat and mouse liver microsomes and NADPH. The metabolic half-time($T_{1/2}$) and intrinsic clearance(Cl_{int}) were determined by using LC-MS analysis. As shown in **Table 4**, compounds **11q** and **11s** showed moderate metabolism in human and rat liver microsomes *in vitro* with suitable $T_{1/2}$ values of 72.19 min, 57.17 min and 57.01 min, 47.69 min($T_{1/2}$ = 30-120 min), respectively. However, compounds **11q** and **11s** were susceptible to metabolism in mouse microsomes, with $T_{1/2}$ values of 16.72 and 4.04 min($T_{1/2}$ < 30 min). These above findings provided a reliable guide for further PK/PD study *in vivo*.

(**Table 4**. should be listed here)

Conclusions

In conclusion, we synthesized a series of semicarbazone derivatives bearing phenyl moiety. All the target compounds were evaluated for their *in vitro* anticancer activities in four human cancer cell lines (HT29, SK-N-SH, MDA-MB-231 and MKN45). Most of them exhibited moderate to prominent activities against all the tested cancer cell lines. We identified 2 promising compounds (**11q** and **11s)**, which showed excellent anticancer activities with IC_{50} values ranging from 0.32 to 1.57 μM, and exhibited weak cytotoxicity on the normal cells (HUVEC). Cell cycle analysis revealed that **11q** and **11s** caused an arrest in the Sub-G1 (indicative of apoptosis) cell cycle. Apoptosis induction study indicated that **11q** and **11s** could inhibit proliferation of HT29 cells by inducing apoptosis in a dose-dependent manner. Procaspase-3 activation assay suggested that **11q** and **11s** could activate procaspase-3 by 68.4% and 76.3% at a concentration of 10 μM comparing to PAC-1. Metabolic stability study revealed that **11q** and **11s** showed moderate stability *in vitro* in human and rat liver microsomes. All these findings demonstrated that compounds **11q** and **11s** have the potential to be developed as valuable lead compounds. Studies on the mechanism of action and *in vivo* PK/PD are in progress and will be reported in future.

Experimental section

Chemistry Reagents and solvents were obtained from commercial sources and used without further purification. All the reactions were monitored by TLC using silica gel GF/UV 254. Flash chromatography was performed using silica gel (300–400mesh). The ^{1}H and ^{13}C NMR spectra were recorded on Bruker AV-400 spectrometer, with TMS as an internal standard. Mass spectra (MS) were taken in ESI mode on Agilent 1100 LC-MS (Agilent, Palo Alto, CA, U.S.A.). The elemental analysis of compounds were performed on a Perkin Elmer 2400 Elemental Analyser.

General procedure for preparation of compounds 3a–b and 4a-b The preparation methods of **3a–b** and **4a-b** has been illustrated in detail in previous study[21, 22], and so the synthesis method would not be listed here.

General procedure for preparation of compounds 5a-b To the mixture of compounds **4a-b** (0.01 mol), DCM (20 mL) and pyridine (0.8 ml,0.02 mol) was added at room temperature, then the mixture was cooled to 0 °C in an ice-salt bath, phenyl chloroformate was added drop wise at such a rate to keep the temperature below 10 °C. The reaction mixture was stirred at room temperature for 4-6 h and filtered. The white to light yellow solid was collected and washed with DCM to obtain the compounds **5a-b**.

Phenyl (4-((dimethylamino)methyl)phenyl)carbamate (5a) White solid; Yield: 82 %; MS (ESI) m/z: 270.7 $[M+H]^+$.

Phenyl (4-((diethylamino)methyl)phenyl)carbamate (5b) White solid; Yield: 88 %; MS (ESI) m/z: 298.8 $[M+H]^+$.

General procedure for preparation of 6a-b To the mixture of the **5a-b** (0.01 mol), 1,4-dioxane (20 mL) and 80% hydrazine hydrate (1.29 ml, 0.02 mol)was added at room temperature. The mixture was heated to 80 °C for 6 h, then the reaction mixture was cooled to room temperature and concentrated, diethyl ether was added and stirred for 0.5 h and filtered, a white solid was collected to get the compounds **6a-b**.

***N*-(4-((dimethylamino)methyl)phenyl)hydrazinecarboxamide (6a)** White solid; Yield:

54 %; MS (ESI) m/z: 208.4 $[M+H]^+$.

***N*-(4-((diethylamino)methyl)phenyl)hydrazinecarboxamide (6b)** White solid; Yield: 62%; MS (ESI) m/z: 236.7 $[M+H]^+$.

General procedure for preparation of 7a-j, 8, 9 and 10 The intermidiates **7a-j**, **8**, **9** and **10** were prepared according to a known procedure[21, 22].

General procedure for preparation of 11a-v A mixture of the compounds **6a-b** (0.001 mol), appropriate 2-hydroxy aromatic aldehydes or **7a-j** or **10** (0.0011 mol) and a drop of glacial acetic acid in 10 mL absolute ethanol was heated at reflux for 6 h and cooled to room temperature, HCl in absolute ethanol (3 mL) was added, and then stirred at room temperature for 4-6 h and filtered to get a light yellow solid, the crude product was washed with 5 mL diethyl ether to afford compounds **11a-v**.

(*E*)-2-(4-(benzyloxy)-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methyl)phenyl) hydrazine-1-carboxamide hydrochloride (11a) Yellow solid; yield: 47%; MS (ESI) m/z: 418.8 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (s, 1H), 10.64 (s, 1H), 10.44 (s, 1H), 9.14 (s, 1H), 8.19 (s, 1H), 7.81 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.50 – 7.43 (m, 4H), 7.40 (t, J = 7.2 Hz, 2H), 7.35 (d, J = 7.2 Hz, 1H), 6.59 – 6.52 (m, 2H), 5.10 (s, 2H), 4.19 (d, J = 4.4 Hz, 2H), 2.67 (d, J = 4.4 Hz, 6H); Anal. Calcd. for $C_{24}H_{27}ClN_4O_3$ (%):C, 63.36; H, 5.98; N, 12.32. Found (%):C, 63.44; H, 5.92; N, 12.44.

(*E*)-2-(2-hydroxy-4-((4-methylbenzyl)oxy)benzylidene)-*N*-(4-((dimethylamino)meth yl)phenyl)hydrazine-1-carboxamide hydrochloride (11b) Yellow solid; yield: 52%; MS (ESI) m/z: 432.5 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.65 (s, 1H), 10.36 (s, 2H), 9.10 (s, 1H), 8.18 (s, 1H), 7.81 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 8.4 Hz, 2H), 7.45 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 7.8 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 6.57 – 6.48 (m, 2H), 5.04 (s, 2H), 4.19 (d, J = 4.8 Hz, 2H), 2.68 (d, J = 4.8 Hz, 6H), 2.31 (s, 3H); Anal. Calcd. for $C_{25}H_{29}ClN_4O_3$ (%):C, 64.03; H, 6.23; N, 11.95. Found (%):C, 64.13; H, 6.28; N, 11.86.

(*E*)-2-(4-((4-(tert-butyl)benzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((dimethylamino) methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11c) Yellow solid; yield: 56%; MS (ESI) m/z: 474.5 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 1H), 10.31 (s, 2H), 9.08 (s, 1H), 8.18 (s, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 8.4 Hz, 2H), 7.47 – 7.39 (m, 4H), 7.37 (d, J = 8.4 Hz, 2H), 6.57 – 6.49 (m, 2H), 5.05 (s, 2H), 4.19 (s, 2H), 2.68 (d, J = 4.0 Hz, 6H), 1.28 (s, 9H); Anal. Calcd. for $C_{28}H_{35}ClN_4O_3$ (%):C, 65.81; H, 6.90; N, 10.96. Found (%):C, 65.88; H, 6.92; N, 10.86.

(*E*)-2-(4-((4-chlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methy l)phenyl) hydrazine-1-carboxamide hydrochloride (11d) Yellow solid; yield: 54%; MS (ESI) m/z: 452.1 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (s, 1H), 10.59 (s, 1H), 10.44 (s, 1H), 9.14 (s, 1H), 8.19 (s, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.48 (s, 5H), 7.45 (s, 1H), 6.57 – 6.51 (m, 2H), 5.10 (s, 2H), 4.19 (d, J = 4.8 Hz, 2H), 2.67 (d, J = 4.8 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 160.74, 158.23, 153.20, 140.95, 136.38, 132.92, 131.98, 130.00, 128.92, 124.07, 119.54, 114.02, 107.18, 102.42, 68.83, 59.53, 41.69. Anal. Calcd. for $C_{24}H_{22}Cl_2N_4O_3$ (%):C, 58.90; H, 5.36; N, 11.45. Found (%):C, 58.81; H, 5.42; N, 11.52.

(*E*)-2-(4-((4-fluorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methy l)phenyl) hydrazine-1-carboxamide hydrochloride (11e) Yellow solid; yield: 54%; MS

(ESI) m/z: 436.1 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 10.68 (s, 1H), 10.49 (s, 2H), 9.12 (s, 1H), 8.19 (s, 1H), 7.82 (d, J = 8.0 Hz, 1H), 7.71 (d, J = 8.4 Hz, 2H), 7.54 – 7.48 (m, 2H), 7.46 (d, J = 8.4 Hz, 2H), 7.23 (t, J = 8.8 Hz, 2H), 6.59 – 6.50 (m, 2H), 5.08 (s, 2H), 4.19 (d, J = 4.8 Hz, 2H), 2.67 (d, J = 4.8 Hz, 6H); Anal. Calcd. for $C_{24}H_{26}ClFN_4O_3$ (%):C, 60.95; H, 5.54; N, 11.85. Found (%):C, 60.86; H, 5.59; N, 11.78.

(*E*)-2-(4-((2,4-dichlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11f) Yellow solid; yield: 59%; MS (ESI) m/z: 486.0 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 10.61 (s, 1H), 10.37 (s, 1H), 9.01 (s, 1H), 8.19 (s, 1H), 7.85 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 2.0 Hz, 1H), 7.66 (d, J = 8.4 Hz, 2H), 7.61 (d, J = 8.4 Hz, 1H), 7.49 (dd, J = 8.4, 2.0 Hz, 1H), 7.36 (d, J = 8.4 Hz, 2H), 6.58 – 6.53 (m, 2H), 5.15 (s, 2H), 3.90 (s, 2H), 2.50 (s, 6H); Anal. Calcd. for $C_{24}H_{25}Cl_3N_4O_3$ (%):C, 55.03; H, 4.81; N, 10.70. Found (%):C, 55.22; H, 4.73; N, 10.75.

(*E*)-2-(4-((2,3-dichlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11g) Yellow solid; yield: 55%; MS (ESI) m/z: 486.1 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 10.70 (s, 1H), 10.49 (s, 1H), 9.12 (s, 1H), 8.20 (s, 1H), 7.82 (d, J = 8.1 Hz, 1H), 7.67 (t, J = 8.1 Hz, 3H), 7.57 (d, J = 7.2 Hz, 1H), 7.48 – 7.39 (m, 3H), 6.60 – 6.54 (m, 2H), 5.20 (s, 2H), 4.10 (s, 2H), 2.61 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 160.51, 158.22, 153.21, 140.65, 137.31, 132.41, 131.67, 130.82, 130.61, 129.11, 128.87, 128.77, 119.57, 114.36, 114.03, 107.15, 102.34, 67.62, 60.07, 42.20. Anal. Calcd. for $C_{24}H_{25}Cl_3N_4O_3$ (%):C, 55.03; H, 4.81; N, 10.70. Found (%):C, 55.12; H, 4.77; N, 10.53.

(*E*)-2-(2-hydroxy-4-((4-methylbenzyl)oxy)benzylidene)-*N*-(4-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11h) Yellow solid; yield: 62%; MS (ESI) m/z: 460.5 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 10.66 (s, 1H), 10.39 (s, 1H), 10.18 (s, 1H), 9.10 (s, 1H), 8.18 (s, 1H), 7.80 (d, J = 8.2 Hz, 1H), 7.70 (d, J = 8.3 Hz, 2H), 7.50 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 7.8 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 6.59 – 6.47 (m, 2H), 5.04 (s, 2H), 4.21 (d, J = 5.0 Hz, 2H), 3.03 (dd, J = 12.3, 7.4 Hz, 4H), 2.31 (s, 3H), 1.24 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{26}H_{31}ClN_4O_3$ (%):C, 64.65; H, 6.47; N, 11.60. Found (%):C, 64.72; H, 6.58; N, 11.51.

(*E*)-2-(4-((4-(tert-butyl)benzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11i) Yellow solid; yield: 63%; MS (ESI) m/z: 502.5 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 10.66 (s, 1H), 10.39 (s, 1H), 10.18 (s, 1H), 9.10 (s, 1H), 8.18 (s, 1H), 7.80 (d, J = 8.2 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.50 (d, J = 8.4 Hz, 2H), 7.33 (d, J = 7.8 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 6.59 – 6.47 (m, 2H), 5.04 (s, 2H), 4.21 (d, J = 5.0 Hz, 2H), 3.03 (dd, J = 12.4, 7.2 Hz, 4H), 2.31 (s, 3H), 1.24 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{30}H_{39}ClN_4O_3$ (%):C, 66.84; H, 7.29; N, 10.39. Found (%):C, 66.92; H, 7.42; N, 10.31.

(*E*)-2-(4-((4-chlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11j) Yellow solid; yield: 55%; MS (ESI) m/z: 480.2 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 10.68 (s, 1H), 10.42 (s, 1H), 10.26 (s, 1H), 9.12 (s, 1H), 8.19 (s, 1H), 7.81 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 8.8 Hz, 2H), 7.47 (s, 4H), 6.59 – 6.50 (m, 2H), 5.10 (s, 2H), 4.21 (d, J = 4.8 Hz, 2H), 3.02 (dd, J = 12.4, 7.2 Hz, 4H), 1.24 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{26}H_{30}Cl_2N_4O_3$ (%):C, 60.35;

H, 5.84; N, 10.83. Found (%):C, 60.48; H, 5.88; N, 10.89.

(*E*)-2-(4-((3-chlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl) phenyl)hydrazine-1-carboxamide hydrochloride (11k) Yellow solid; yield: 58%; MS (ESI) m/z: 480.5 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.64 (s, 1H), 10.38 (s, 1H), 10.15 (s, 1H), 9.08 (s, 1H), 8.17 (s, 1H), 7.80 (d, J = 8.4 Hz, 1H), 7.68 (d, J = 8.4 Hz, 2H), 7.50 (s, 2H), 7.47 (s, 1H), 7.43 – 7.36 (m, 3H), 6.56 – 6.50 (m, 2H), 5.10 (s, 2H), 4.19 (d, J = 5.2 Hz, 2H), 3.10 – 2.92 (m, 4H), 1.23 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{26}H_{30}Cl_2N_4O_3$ (%):C, 60.35; H, 5.84; N, 10.83. Found (%):C, 60.45; H, 5.83; N, 10.79.

(*E*)-2-(4-((2-chlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl) phenyl)hydrazine-1-carboxamide hydrochloride (11l) Yellow solid; yield: 65%; MS (ESI) m/z: 480.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.67 (s, 1H), 10.40 (s, 1H), 10.19 (s, 1H), 9.11 (s, 1H), 8.20 (s, 1H), 7.84 (d, J = 8.9 Hz, 1H), 7.71 (d, J = 8.5 Hz, 2H), 7.56 (dd, J = 8.4, 6.8 Hz, 1H), 7.51 (d, J = 8.6 Hz, 2H), 7.44 (ddd, J = 9.5, 7.4, 1.7 Hz, 1H), 7.26 (dd, J = 15.8, 7.9 Hz, 2H), 6.57 (dd, J = 5.7, 2.3 Hz, 2H), 5.14 (s, 2H), 4.21 (d, J = 5.3 Hz, 2H), 3.57 (s, 4H), 3.03 (dd, J = 12.5, 7.6 Hz, 4H), 1.25 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{26}H_{30}Cl_2N_4O_3$ (%):C, 60.35; H, 5.84; N, 10.83. Found (%):C, 60.30; H, 5.81; N, 10.88.

(*E*)-2-(4-((4-fluorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl) phenyl)hydrazine-1-carboxamide hydrochloride (11m) Yellow solid; yield: 48%; MS (ESI) m/z: 464.5 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.68 (s, 1H), 10.42 (s, 1H), 10.26 (s, 1H), 9.12 (s, 1H), 8.19 (s, 1H), 7.81 (d, J = 8.4 Hz, 1H), 7.70 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 8.8 Hz, 2H), 7.47 (s, 4H), 6.59 – 6.50 (m, 2H), 5.10 (s, 2H), 4.21 (d, J = 4.8 Hz, 2H), 3.02 (dd, J = 12.4, 7.2 Hz, 4H), 1.24 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{26}H_{30}ClFN_4O_3$ (%):C, 62.33; H, 6.04; N, 11.18. Found (%):C, 62.38; H, 6.14; N, 11.07.

(E)-2-(4-((2,4-dichlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)met hyl)phenyl)hydrazine-1-carboxamide hydrochloride (11n) Yellow solid; yield: 65%; MS (ESI) m/z: 514.4 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.65 (s, 1H), 10.38 (s, 1H), 10.15 (s, 1H), 9.08 (s, 1H), 8.17 (s, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.72 – 7.66 (m, 3H), 7.59 (d, J = 8.4 Hz, 1H), 7.52 – 7.45 (m, 3H), 6.57 – 6.51 (m, 2H), 5.13 (s, 2H), 4.19 (d, J = 5.2 Hz, 2H), 3.10 – 2.92 (m, 4H), 1.23 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{26}H_{29}Cl_3N_4O_3$ (%):C, 56.58; H, 5.30; N, 10.15. Found (%):C, 56.42; H, 5.39; N, 10.33.

(*E*)-2-(4-((2,3-dichlorobenzyl)oxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)met hyl)phenyl)hydrazine-1-carboxamide hydrochloride (11o) Yellow solid; yield: 55%; MS (ESI) m/z: 514.3 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 10.28 (s, 1H), 8.98 (s, 1H), 8.20 (s, 1H), 7.87 (d, J = 8.4 Hz, 1H), 7.71 (d, J = 7.9 Hz, 2H), 7.66 (d, J = 7.6 Hz, 1H), 7.56 (d, J = 7.1 Hz, 1H), 7.49 – 7.29 (m, 3H), 6.57 (d, J = 8.5 Hz, 1H), 6.52 (s, 1H), 5.21 (s, 2H), 4.15 (s, 2H), 3.08 – 2.90 (m, 4H), 1.20 (t, J = 6.4 Hz, 6H); ^{13}C NMR (101 MHz, DMSO) δ 160.56, 158.04, 153.31, 137.30, 132.42, 131.53, 130.79, 130.61, 128.99, 128.89, 128.73, 119.90, 114.44, 107.25, 102.24, 67.62, 65.37, 46.32, 15.63; Anal. Calcd. for $C_{26}H_{29}Cl_3N_4O_3$ (%):C, 56.58; H, 5.30; N, 10.15. Found (%):C, 56.50; H, 5.41; N, 10.23.

(*E*)-2-(2-hydroxy-4-((4-(trifluoromethyl)benzyl)oxy)benzylidene)-*N*-(4-((diethylami no)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11p) Yellow solid; yield: 52%; MS (ESI) m/z: 514.0 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.68 (s, 1H), 10.43 (s, 1H), 10.25 (s, 1H), 9.12 (s, 1H), 8.19 (s, 1H), 7.83 (d, J = 9.2 Hz, 1H), 7.78 (d, J = 8.4 Hz,

2H), 7.69 (t, J = 9.2 Hz, 4H), 7.51 (d, J = 8.4 Hz, 2H), 6.59 – 6.52 (m, 2H), 5.23 (s, 2H), 4.21 (d, J = 5.2 Hz, 2H), 3.12 – 2.94 (m, 4H), 1.24 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{27}H_{30}ClF_3N_4O_3$ (%):C, 58.86; H, 5.49; N, 10.17. Found (%):C, 58.93; H, 5.41; N, 10.28.

(*E*)-2-(3,5-di-tert-butyl-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11q) Yellow solid; yield: 61%; MS (ESI) m/z: 424.5 $[M+H]^+$ ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.57 (s, 1H), 10.99 (s, 1H), 10.54 (s, 1H), 9.63 (s, 1H), 8.24 (s, 1H), 7.60 (d, J = 8.6 Hz, 2H), 7.48 (d, J = 8.6 Hz, 2H), 7.26 (d, J = 2.4 Hz, 1H), 7.24 (d, J = 2.4 Hz, 1H), 4.20 (s, 2H), 2.68 (s, 6H), 1.41 (s, 9H), 1.28 (s, 9H); ^{13}C NMR (101 MHz, DMSO) δ 154.23, 152.37, 146.84, 141.07, 140.85, 135.89, 132.21, 125.80, 125.17, 124.03, 118.91, 118.07, 59.57, 41.74, 35.10, 34.36, 31.80, 29.83; Anal. Calcd. for $C_{25}H_{37}ClN_4O_2$ (%):C, 65.13; H, 8.09; N, 12.15. Found (%):C, 65.28; H, 8.19; N, 12.10.

(*E*)-2-(3-allyl-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11r) Yellow solid; yield: 44%; MS (ESI) m/z: 352.5 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.95 (s, 1H), 10.57 (s, 1H), 9.52 (s, 1H), 8.24 (s, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.4 Hz, 2H), 7.37 (d, J = 6.4 Hz, 1H), 7.14 (d, J = 6.4 Hz, 1H), 6.87 (t, J = 7.2 Hz, 1H), 6.07 – 5.91 (m, 1H), 5.10 – 5.01 (m, 2H), 4.19 (d, J = 4.8 Hz, 2H), 3.38 (d, J = 6.8 Hz, 2H), 2.68 (s, 3H), 2.66 (s, 3H); Anal. Calcd. for $C_{20}H_{25}ClN_4O_2$ (%):C, 61.77; H, 6.48; N, 14.41. Found (%):C, 61.89; H, 6.42; N, 14.55.

(*E*)-2-(3,5-di-tert-butyl-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11s) Yellow solid; yield: 57%; MS (ESI) m/z: 452.5 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.57 (s, 1H), 10.99 (s, 1H), 10.31 (s, 1H), 9.63 (s, 1H), 8.24 (s, 1H), 7.59 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 8.6 Hz, 2H), 7.26 (d, J = 2.4 Hz, 1H), 7.24 (d, J = 2.4 Hz, 1H), 4.22 (s, 2H), 3.16 – 2.92 (m, 4H), 1.41 (s, 9H), 1.28 (s, 9H), 1.27 – 1.20 (m, 6H); ^{13}C NMR (101 MHz, DMSO) δ 154.23, 152.38, 146.84, 140.96, 140.85, 135.89, 132.24, 125.80, 125.17, 123.80, 118.94, 118.07, 54.78, 45.93, 35.10, 34.36, 31.80, 29.82, 8.79; Anal. Calcd. for $C_{27}H_{41}ClN_4O_2$ (%):C, 66.30; H, 8.45; N, 11.46. Found (%):C, 66.41; H, 8.40; N, 11.33.

(*E*)-2-(3-allyl-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11t) Yellow solid; yield: 46%; MS (ESI) m/z: 380.5 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.81 (s, 1H), 10.75 (s, 1H), 9.96 (s, 1H), 9.39 (s, 1H), 8.22 (s, 1H), 7.61 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 7.36 (d, J = 6.4 Hz, 1H), 7.12 (d, J = 7.2 Hz, 1H), 6.85 (t, J = 7.5 Hz, 1H), 6.04 – 5.90 (m, 1H), 5.09 – 4.98 (m, 2H), 4.20 (d, J = 4.2 Hz, 2H), 3.36 (d, J = 6.4 Hz, 2H), 3.31 (s, 1H), 3.08 – 2.96 (m, 4H), 1.22 (t, J = 7.2 Hz, 6H); Anal. Calcd. for $C_{22}H_{29}ClN_4O_2$ (%):C, 63.37; H, 7.01; N, 13.44. Found (%):C, 63.45; H, 7.09; N, 13.34.

(*E*)-2-(4-((2-(benzo[d][1,3]dioxol-5-ylmethyl)thiazol-4-yl)methoxy)-2-hydroxybenzylidene)-*N*-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11u) Yellow solid; yield: 67%; MS (ESI) m/z: 559.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 10.36 (s, 1H), 8.99 (s, 1H), 8.18 (s, 1H), 7.83 (d, J = 7.8 Hz, 1H), 7.64 (d, J = 8.2 Hz, 2H), 7.58 (s, 1H), 7.33 (d, J = 8.2 Hz, 2H), 6.93 (s, 1H), 6.89 (d, J = 7.8 Hz, 1H), 6.83 (d, J = 7.8 Hz, 1H), 6.62 – 6.51 (m, 2H), 6.00 (s, 2H), 5.11 (s, 2H), 4.24 (s, 2H), 3.80 (s, 2H), 2.43 (s, 6H); Anal. Calcd. for $C_{28}H_{28}ClN_5O_5S$ (%):C, 57.78; H, 4.85; N, 12.03. Found (%):C, 57.92; H, 4.89; N, 11.93.

(*E*)-2-(4-((2-(benzo[d][1,3]dioxol-5-ylmethyl)thiazol-4-yl)methoxy)-2-hydroxybenzylidene)-*N*-(4-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride(11v) Yellow solid; yield: 71%; MS (ESI) m/z (%): 587.1 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 10.49 (s, 1H), 10.29 (s, 1H), 8.83 (s, 1H), 8.17 (s, 1H), 7.83 (d, *J* = 8.4 Hz, 1H), 7.57 (s, 1H), 7.54 (d, *J* = 7.2 Hz, 2H), 7.21 (d, *J* = 7.2 Hz, 2H), 6.93 (s, 1H), 6.89 (d, *J* = 8.0 Hz, 1H), 6.83 (d, *J* = 8.0 Hz, 1H), 6.60 – 6.49 (m, 2H), 6.00 (s, 2H), 5.11 (s, 2H), 4.24 (s, 2H), 3.46 (s, 2H), 2.49 – 2.34 (m, 4H), 1.07 – 0.89 (m, 6H); Anal. Calcd. for $C_{30}H_{32}ClN_5O_5S$ (%):C, 59.06; H, 5.29; N, 11.48. Found (%):C, 59.16; H, 5.23; 11.35.

MTT assay The anticancer activities of compounds **11a-v** were evaluated against tested cell lines using the standard MTT assay *in vitro*, with **PAC-1** as the positive control. Cancer cell lines were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Approximate 4×10^3 cells, suspended in MEM medium, were plated onto each well of a 96-well plate and incubated in 5% CO_2 at 37 °C for 24 h. The compounds tested at the indicated final concentrations were added to the culture medium and the cell cultures were continued for 72 h. Fresh MTT was added to each well at a terminal concentration of 5 mg/mL, and incubated with cells at 37 °C for 4 h. The formazan crystals were dissolved in 100 mL of DMSO each well, and the absorbance at 492 nm (for absorbance of MTT formazan) and 490 nm (for the reference wavelength) was measured with an ELISA reader. All compounds were tested three times in each cell line. The results expressed as IC_{50} (inhibitory concentration 50%) were the averages of three determinations and calculated by using the Bacus Laboratories Incorporated Slide Scanner (Bliss) software.

Cell cycle HT29 cells were treated with different concentrations of **11q** and **11s** for 48 h and were harvested by digestion with trypsin and centrifugation (1500 rpm for 15 min). The cell pellets were suspended with 70 % ethanol at −20 °C overnight. Afterwards, cells were washed with PBS twice and incubated with RNase (180 μg/mL) for 30 min at 37 °C, and followed by incubation with PI solution (final concentration 50 μg/mL) for 30 min in the dark. Cells were analyzed by flow cytometry system (BD Biosciences) and results were analyzed by FlowJo V10 software.

Apoptosis study Apoptosis of HT-29 cells was detected using a flow cytometric assay. Briefly, cells were seeded in 6-well plates and incubated overnight. The following day, cells were treated with different concentrations of compounds **11q** and **11s** for 48 hours. The cells and supernatants were harvested and washed twice with cold PBS and then resuspended in 100μl 1× Binding Buffer. 5 μl of FITC Annexin V and 5 μl PI were added in each tube and the cells were then gently vortexed incubated for 15 min at RT (25°C) in the dark. 400 μl of 1× Binding Buffer then added to each tube. The stained cells were analyzed by a flow cytometer (FACS Calibur; BD).

Procaspase-3 activation assay Procaspase-3 was purchased from R&D Systems. Procaspase-3 was incubated at 100 nM, in the presence of selected compounds at 10 μM in a reaction buffer consisted 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. Fluorescence value was read at excitation and emission wavelengths of 400 nm and 505 nm. The activation rate (%) was calculated using the following equation: $(F_{compound}-F_{dmso})/(F_{PAC\text{-}1}-F_{dmso})\times 100$.

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 compouds(Ketanserin) and test compounds spiking solution: 500 μM spiking solution: add 10 μL of 10 mM DMSO stock solution into 190 μL ACN. 1.5 μM spiking solution in microsomes (0.75 mg/mL): add 1.5 μL of 500 μM spiking solution and 18.75 μL of 20 mg/mL liver microsomes into 479.75 μL of Buffer C on ice. (3) Prepare NADPH stock solution (6 mM) by dissolving NADPH into buffer C. (4) Dispense 30 μL of 1.5 μM spiking solution containing 0.75 mg/mL microsomes solution to the assay plates designated for different time points (0, 5, 15, 30, 45 min) on ice. (5) For 0-min, add 135 μL of ACN containing IS to the wells of 0-min plate and then add 15 μL of NADPH stock solution (6 mM). (6) Pre-incubate all other plates at 37 °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 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/min) and then centrifuge at 5594 g for 15 min (Thermo Multifuge × 3R). (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.

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Conflicts of interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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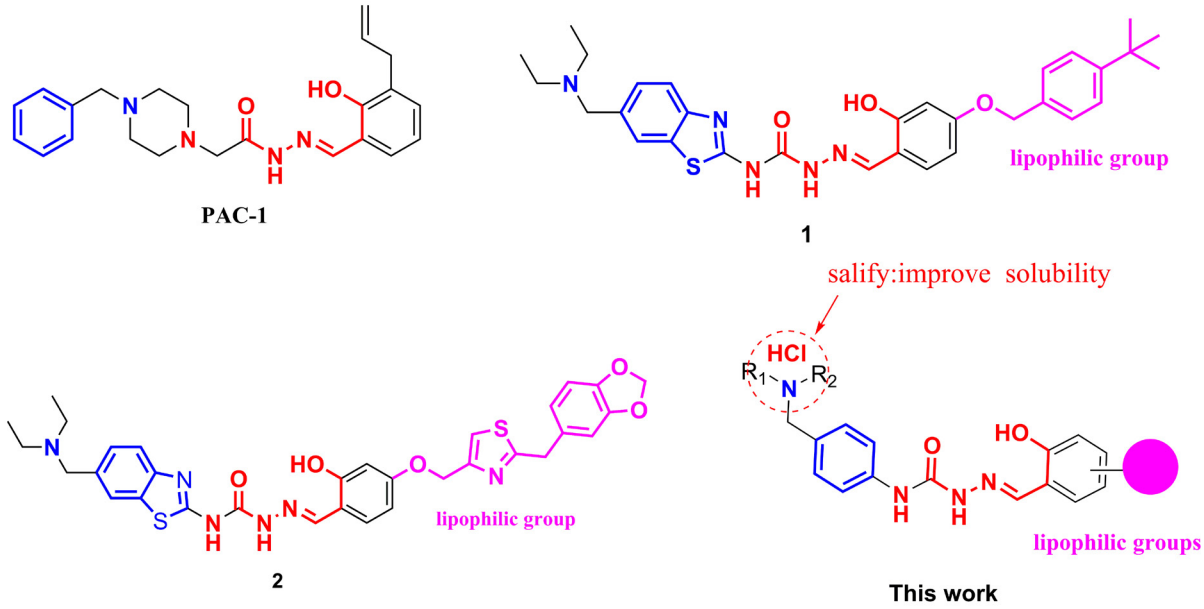


Fig. 1. Reported procaspase-3 activators and target compounds in this work

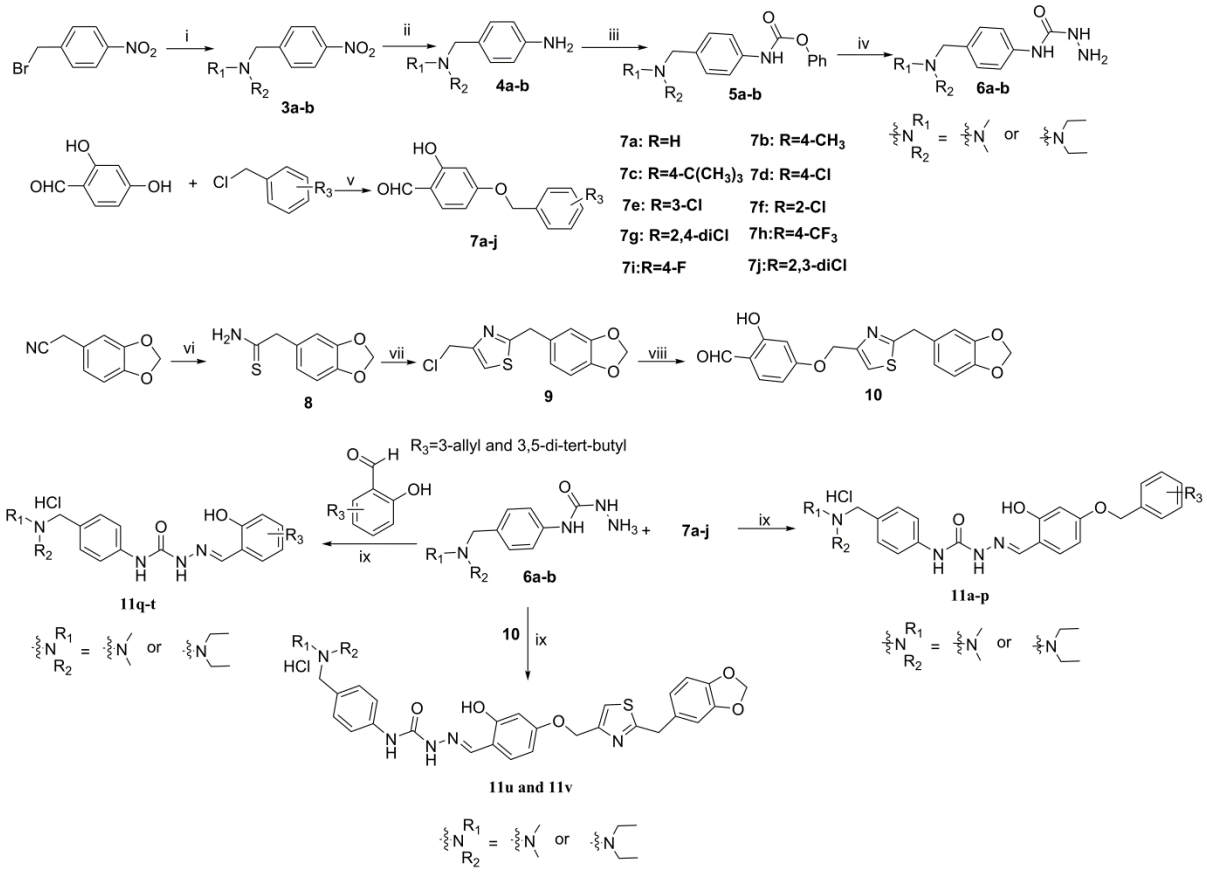


Chart 1. Reagents and conditions: (i) Acetonitrile, amine, rt, 3 h; (ii) 80% hydrazine monohydrate, $FeCl_3 \cdot 6H_2O$, activated carbon, ethanol, 65 °C to 78 °C, 5 h; (iii) Phenyl chloroformate, pyridine, CH_2Cl_2, 0 °C to rt, 4-6 h; (iv) 80% hydrazine monohydrate, 1,4-dioxane, 80 °C,6 h; (v) Na_2CO_3, KI, 65 °C, 30 h; (vi) NaHS, $MgCl_2 \cdot 6H_2O$, DMF, H_2O, rt, 15 h; (vii) 1,3-dichloro-2-propanon, acetonitrile, 50 °C, 4 h; (viii) 2,4-dihydroxy benzaldehyde, $NaHCO_3$, KI, acetonitrile, 80 °C, 2 h; (ix) Acetic acid, ethanol, reflux; HCl in ethanol, rt.

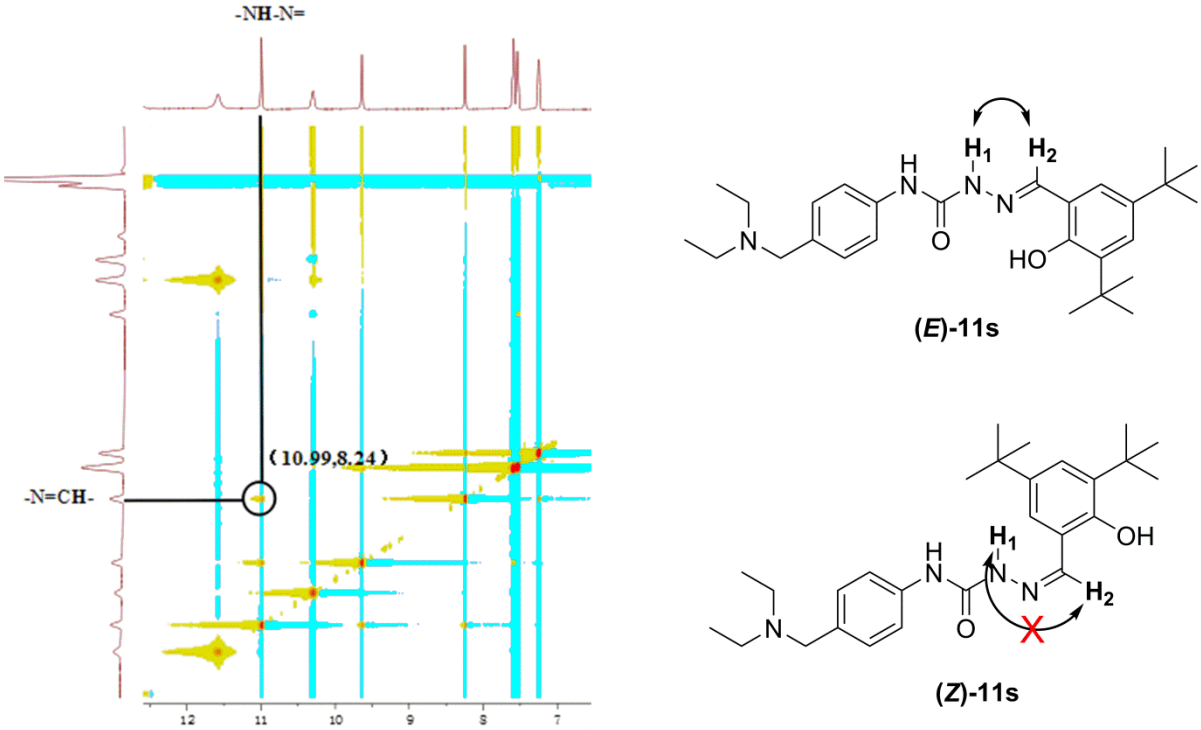
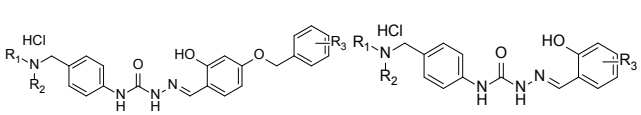


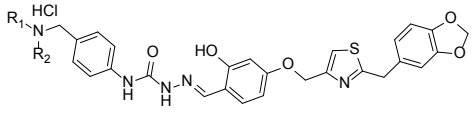
Fig. 2. NOE of the representative compound **11s**.

Table 1. Anticancer activities for synthesized compounds against tumor cell lines.



11a-p

11q-t



11u and 11v

Compd.	R_1 / R_2	R_3	IC_{50}±SD(μM)[a]			
			HT29	SK-N-SH	MDA-MB-231	MKN45
11a		H	9.06±0.57	8.43±0.39	>100	1.30±0.18
11b		4-CH_3	1.70±0.17	1.78±0.11	10.34±1.57	6.90±0.87
11c		4-$C(CH_3)_3$	1.40±0.23	1.44±0.18	3.32±0.62	2.60±0.49
11d		4-Cl	3.64±0.48	8.51±0.63	4.92±1.23	0.83±0.21
11e		4-F	4.45±0.24	8.56±0.53	8.88±0.86	1.40±0.23
11f		2,4-$(Cl)_2$	8.79±1.03	13.67±1.43	10.71±1.28	35.9±1.56
11g		2,3-$(Cl)_2$	3.94±0.51	4.74±1.21	9.4±1.01	4.20±1.03
11h		4-CH_3	1.92±0.18	2.70±0.33	12.36±1.73	1.11±0.83
11i		4-$C(CH_3)_3$	1.56±0.23	0.78±0.11	2.28±0.37	1.10±0.32
11j		4-Cl	1.37±0.54	1.01±0.38	4.40±0.93	0.51±0.21
11k		3-Cl	2.92±0.39	2.16±0.52	7.22±1.24	1.80±0.26
11l		2-Cl	2.35±0.41	4.02±0.91	8.94±0.72	2.71±0.58
11m		4-F	5.51±0.61	3.55±0.48	5.19±0.38	0.88±0.29
11n		2,4-$(Cl)_2$	6.09±1.46	5.65±1.11	12.77±1.88	4.01±0.45
11o		2,3-$(Cl)_2$	8.35±1.56	6.74±0.89	10.21±1.63	6.72±1.22
11p		4-CF_3	4.96±1.36	6.53±1.91	13.33±1.78	2.02±1.02
11q		3,5-di-tert-butyl	**0.32±0.13**	**0.83±0.21**	**1.56±0.42**	**0.91±0.21**
11r		3-allyl	1.39±0.29	4.50±0.21	9.04±1.09	4.60±0.93
11s		3,5-di-tert-butyl	**0.37±0.21**	**0.87±0.15**	**1.57±0.37**	**1.50±0.39**
11t		3-allyl	2.21±0.29	6.21±0.47	13.70±1.03	1.80±0.20
11u		-	2.44±0.53	2.07±0.61	1.96±0.48	2.32±0.21
11v		-	2.11±0.73	1.64±0.18	1.92±0.36	2.06±0.31
PAC-1	-	-	1.16±0.51	3.91±0.61	1.52±0.13	1.16±0.38

[a] IC_{50}: The biological data are generated from at least three independent experiments.

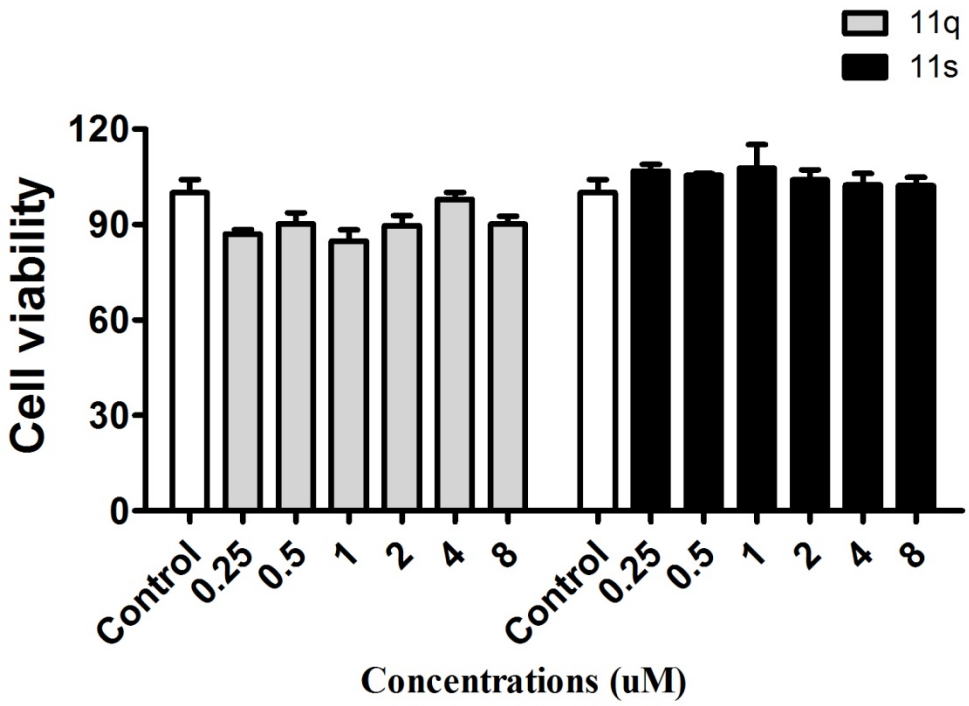


Fig. 3. Effect of **11q** and **11s** on viability of normal cells(HUVEC).

Table 2. Anticancer activities of **11q** and **11s** against three cancer cell lines.

Compd.	IC_{50}±SD(μM)[a]		
	U937	MCF-7	H226
11q	2.57±0.51	3.34±0.82	1.60±0.31
11s	2.68±0.71	3.85±0.67	1.62±0.38
PAC-1	6.01±0.58	>40	2.08±0.18

[a] IC_{50}: The biological data are generated from at least three independent experiments.

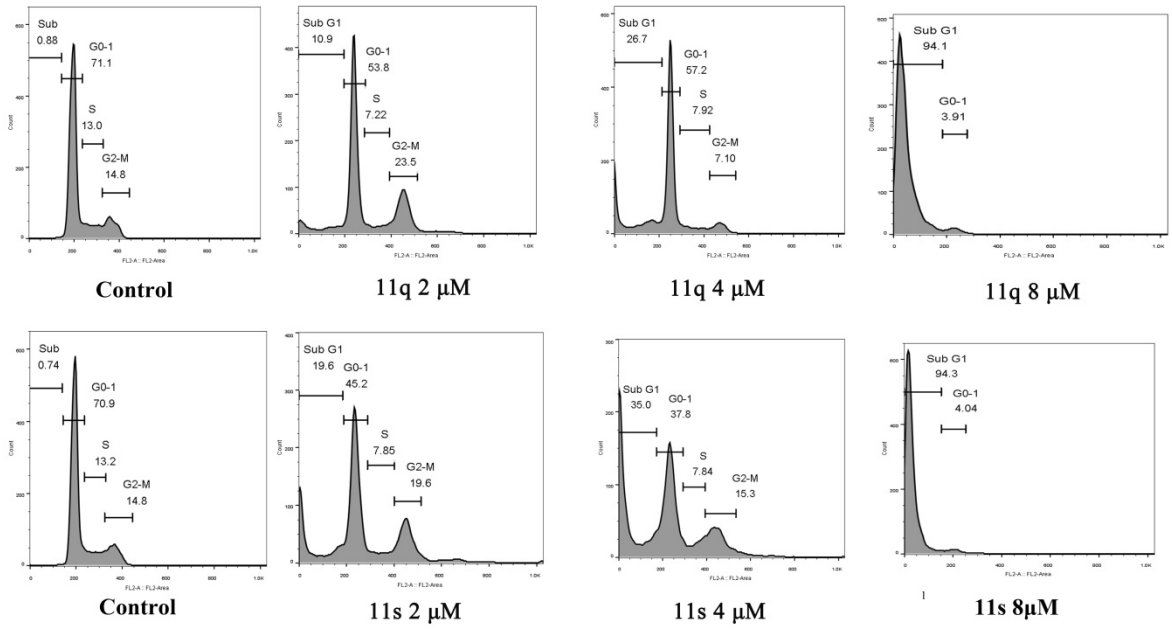


Fig. 4. Cell cycle arrest at Sub-G1 phase by **11q** and **11s** in HT-29 cells. HT-29 cells were incubated with the indicated concentrations of **11q** and **11s** for 48 h and the cells were stained with PI. Cellular DNA content, for cell cycle distribution analysis, was measured using a flow cytometry. The diagrams showed the distribution of the cells according to their DNA content. The inserts gave the percentages in different cell cycle phases.

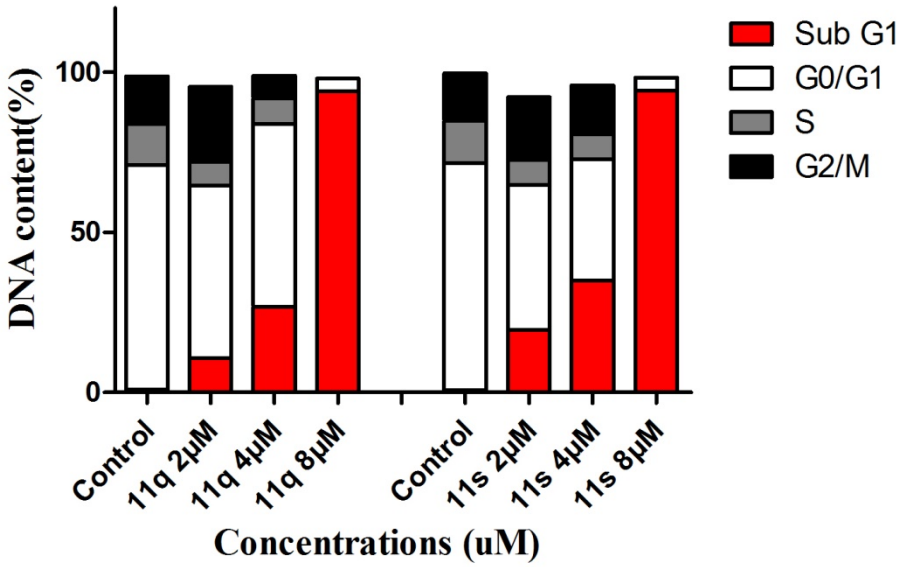


Fig.5. Column graph of DNA content in different cell cycle phases

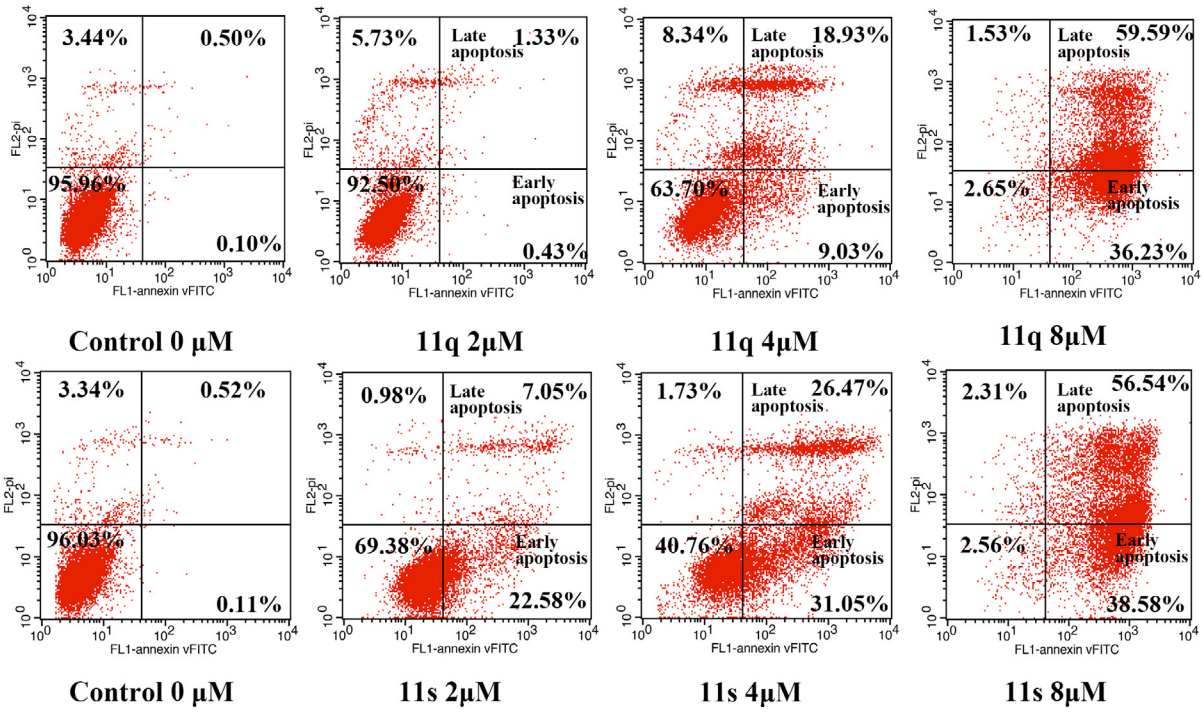


Fig.6. Apoptosis in HT-29 cells by the treatment with **11q** and **11s**. HT-29 cells were incubated with different concentrations of **11q** and **11s** for 48 h and the cells were stained with annexin V-FITC and PI, followed by flow cytometry analysis.

Table 3. Procaspase-3 activity of selected compounds 11q, 11s and PAC-1 *in vitro*.

Compd.	Procapase-3 (% activity at 10μM)
11q	68.4±0.8
11s	76.3±0.6
PAC-1	100

Table 4. *In vitro* metabolic half-life ($T_{1/2}$) [min] and intrinsic clearance (Cl_{int}) of compounds **11q** and **11s**.

Comp.	Index	Species		
		human	rat	mouse
ketanserin	$T_{1/2}$ (min)	36.07	14.40	20.46
	Cl_{int} (mL/min/kg)	48.19	172.52	266.76
11q	$T_{1/2}$ (min)	72.19	57.17	8.95
	Cl_{int} (mL/min/kg)	24.08	43.44	609.88
11s	$T_{1/2}$ (min)	51.01	47.69	4.04
	Cl_{int} (mL/min/kg)	34.08	52.08	1351.68