Regular Article

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 IC₅₀ 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, 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, show greater advantages over anticancer agents targeting early or intermediate positions in the apoptotic cascade, such as p53 disruptors (CBL-0137 and COTI-2), XIAP inhibitors (AZD5582 and GDC-0152), MDM2 inhibitors (HDM201 and RG7112), and Bcl-2 inhibitors (GDC-0199 and ABT-737), 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 relationship (SAR) 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, a series of semicarbazone derivatives containing benzothiazole had been independently reported as procaspase-3 activators, 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 (e.g., substituted benzyloxy or heteroaryloxy groups) in the 2-hydroxy phenyl ring could enhance the anticancer activity.

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 T1/2 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 FeCl3·6H2O 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. 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 H1 (-NH-N=, 10.99 ppm) and H2 (-N=CH-, 8.24 ppm), which existed only in the E isomer due to the appropriate intramolecular H1–H2 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.

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 IC50 values ranging from 0.32 to 1.57 µM.

Further investigations were carried out to study the effect of different substituents of benzyloxy group on the activity (compounds 11a-p). The results revealed that incorporation of bulk substituent at the 4-position of the benzyloxy group could enhance anticancer activity (11b vs. 11c, 11h vs. 11i), indicating that increasing steric hindrance of the group at this region of benzyloxy group exhibited a positive effect on the activity. Moreover, chlorine (11d, 11j, R3=4-Cl) at the 4-position of benzyloxy group was more preferable than the fluorine (11e, 11m, R3=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, R3=4-Cl) or weak mono-electron-donating group (11b, 11h, R=4-CH3) 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.

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 IC50 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(T1/2) 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 T1/2 values of 72.19 min, 57.17 min and 57.01 min, 47.69 min(T1/2 = 30-120 min), respectively. However, compounds 11q and 11s were susceptible to metabolism in mouse microsomes, with T1/2 values of 16.72 and 4.04 min(T1/2 < 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. Procaspe-3 activation assay suggested that 11q and 11s could activate procaspe-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 ^1H and ^13C 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 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:
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 intermediates 7a-j, 8, 9 and 10 were prepared according to a known procedure21, 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]+; \(\text{^1}H\) NMR (400 MHz, DMSO-d6) \(\delta\) 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 C24H27ClN4O3 (%): C, 63.36; H, 5.98; N, 12.32. Found (%): C, 63.44; H, 5.92; N, 12.44.

\((E)-2-(2-hydroxy-4-((4-methylbenzyl)oxy)benzylidene)-N-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11b)\) Yellow solid; yield: 52%; MS (ESI) m/z: 432.5 [M+H]+; \(\text{^1}H\) NMR (400 MHz, DMSO-d6) \(\delta\) 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); Anal. Calcd. for C25H29ClN4O3 (%): 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]+; \(\text{^1}H\) NMR (400 MHz, DMSO-d6) \(\delta\) 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 (d, J = 4.8 Hz, 2H), 2.68 (d, J = 4.8 Hz, 6H); 13C NMR (101 MHz, DMSO) \(\delta\) 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 C28H35ClN4O3 (%): C, 65.81; H, 6.90; N, 10.96. Found (%): C, 65.88; H, 6.92; N, 10.86.

\((E)-2-(4-((4-(chlorobenzyl)oxy)-2-hydroxybenzylidene)-N-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11d)\) Yellow solid; yield: 54%; MS (ESI) m/z: 452.1 [M+H]+; \(\text{^1}H\) NMR (400 MHz, DMSO-d6) \(\delta\) 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); 13C NMR (101 MHz, DMSO) \(\delta\) 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 C24H22Cl2N4O3 (%): C, 58.90; H, 5.36; N, 11.45. Found (%): C, 58.81; H, 5.42; N, 11.52.

\((E)-2-(4-((4-fluorobenzyl)oxy)-2-hydroxybenzylidene)-N-(4-((dimethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11e)\) Yellow solid; yield: 54%; MS
(ESI) m/z: 436.1 [M+H]+; 1H NMR (400 MHz, DMSO-d6) 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 C24H26ClFN4O3 (%): 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-d6) 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, 2H), 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 C24H25Cl3N4O3 (%): 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-d6) 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), 7.71 (d, J = 2.0 Hz, 1H), 7.66 (d, J = 8.4 Hz, 2H), 7.54 – 7.48 (m, 2H), 5.08 (s, 2H), 4.19 (d, J = 4.8 Hz, 2H), 2.67 (d, J = 4.8 Hz, 6H). 13C 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 C24H25Cl3N4O3 (%): 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-d6) 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 = 12.4, 7.2 Hz, 4H), 2.31 (s, 3H), 1.24 (t, J = 7.2 Hz, 6H); Anal. Calcd. for C26H31ClN4O3 (%): 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-d6) δ 10.68 (s, 1H), 10.42 (s, 1H), 10.26 (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 C30H39ClN4O3 (%): 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-d6) 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 C26H30Cl2N4O3 (%): C, 60.35;
(E)-2-((3-chlorobenzyl)oxy)-2-hydroxybenzylidene)-N-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11k) Yellow solid; yield: 58%; MS (ESI) m/z: 480.5 [M+H]+; 1H NMR (400 MHz, DMSO-d6) 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 C26H30Cl2N4O3 (%): C, 60.35; H, 5.84; N, 10.83. Found (%): C, 60.45; H, 5.83; N, 10.79.

(E)-2-((2-chlorobenzyl)oxy)-2-hydroxybenzylidene)-N-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11l) Yellow solid; yield: 65%; MS (ESI) m/z: 480.2 [M+H]+; 1H NMR (400 MHz, DMSO-d6) 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), 3.57 (s, 4H), 3.03 (dd, J = 12.4, 7.2 Hz, 4H), 1.25 (t, J = 7.2 Hz, 6H); Anal. Calcd. for C26H30Cl2N4O3 (%): C, 60.35; H, 5.84; N, 10.83. Found (%): C, 60.30; H, 5.81; N, 10.88.

(E)-2-((4-fluorobenzyl)oxy)-2-hydroxybenzylidene)-N-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11m) Yellow solid; yield: 48%; MS (ESI) m/z: 464.5 [M+H]+; 1H NMR (400 MHz, DMSO-d6) 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 = 5.3 Hz, 2H), 3.02 (dd, J = 12.4, 7.2 Hz, 4H), 1.24 (t, J = 7.2 Hz, 6H); Anal. Calcd. for C26H30ClFN4O3 (%): C, 62.33; H, 6.04; N, 11.18. Found (%): C, 62.38; H, 6.14; N, 11.07.

(E)-2-((2,4-dichlorobenzyl)oxy)-2-hydroxybenzylidene)-N-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11n) Yellow solid; yield: 65%; MS (ESI) m/z: 514.4 [M+H]+; 1H NMR (400 MHz, DMSO-d6) 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 – 6.50 (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 C26H29Cl3N4O3 (%): C, 56.58; H, 5.30; N, 10.15. Found (%): C, 56.42; H, 5.39; N, 10.33.

(E)-2-((2,3-dichlorobenzyl)oxy)-2-hydroxybenzylidene)-N-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11o) Yellow solid; yield: 55%; MS (ESI) m/z: 514.3 [M+H]+; 1H NMR (400 MHz, DMSO-d6) δ 10.56 (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 – 6.50 (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 C26H29Cl3N4O3 (%): 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-((diethylamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride (11p) Yellow solid; yield: 52%; MS (ESI) m/z: 514.0 [M+H]+; 1H NMR (400 MHz, DMSO-d6) 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), 7.50 (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 C26H29Cl3N4O3 (%): C, 56.58; H, 5.30; N, 10.15. Found (%): C, 56.50; H, 5.41; N, 10.23.
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_{3}N_{4}O_{3} (%): C, 58.86; H, 5.49; N, 10.17. Found (%): C, 58.93; H, 5.41; N, 10.28.

\((E)-2-(3,5\text{-di-tert-butyl}-2\text{-hydroxybenzylidene})\text{-N-(4-((dimethylamino)methyl)phenyl)}hydrazine-1\text{-carboxamide hydrochloride (11q)}\) Yellow solid; yield: 61%; MS (ESI) m/z: 424.5 [M+H]^+; \(^1\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\) 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), 6.59 – 6.52 (m, 2H), 5.23 (s, 2H), 4.20 (s, 2H), 2.68 (s, 6H), 1.41 (s, 9H), 1.28 (s, 9H); 13C NMR (101 MHz, DMSO) \(\delta\) 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_{4}O_{2} (%): C, 65.13; H, 8.09; N, 12.15. Found (%): C, 65.28; H, 8.19; N, 12.10.

\((E)-2-(3\text{-allyl}-2\text{-hydroxybenzylidene})\text{-N-(4-((dimethylamino)methyl)phenyl)}hydrazine-1\text{-carboxamide hydrochloride (11r)}\) Yellow solid; yield: 44%; MS (ESI) m/z: 352.5 [M+H]^+; \(^1\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\) 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_{4}O_{2} (%): C, 61.77; H, 6.48; N, 14.41. Found (%): C, 61.89; H, 6.42; N, 14.55.

\((E)-2-(3,5\text{-di-tert-butyl}-2\text{-hydroxybenzylidene})\text{-N-(4-((diethylamino)methyl)phenyl)}hydrazine-1\text{-carboxamide hydrochloride (11s)}\) Yellow solid; yield: 57%; MS (ESI) m/z: 452.5 [M+H]^+; \(^1\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\) 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), 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_{27}H_{41}ClN_{4}O_{2} (%): C, 66.30; H, 8.45; N, 11.46. Found (%): C, 66.41; H, 8.40; N, 11.33.

\((E)-2-(3,5\text{-di-tert-butyl}-2\text{-hydroxybenzylidene})\text{-N-(4-((diethylamino)methyl)phenyl)}hydrazine-1\text{-carboxamide hydrochloride (11t)}\) Yellow solid; yield: 46%; MS (ESI) m/z: 380.5 [M+H]^+; \(^1\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\) 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_{4}O_{2} (%): C, 63.37; H, 7.01; N, 13.44. Found (%): C, 63.45; H, 7.09; N, 13.34.

\((E)-2-(4\text{-((2-(benzo[d][1,3]dioxol-5-ylmethyl)thiazol-4-yl)methoxy)-2\text{-hydroxybenzylidene})\text{-N-(4-((dimethylamino)methyl)phenyl)}hydrazine-1\text{-carboxamide hydrochloride (11u)}\) Yellow solid; yield: 67%; MS (ESI) m/z: 559.2 [M+H]^+; \(^1\)H NMR (400 MHz, DMSO-d_{6}) \(\delta\) 10.60 (s, 1H), 10.75 (s, 1H), 9.70 (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_{28}H_{28}ClN_{4}O_{5}S (%): C, 63.73; 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-diethy lamino)methyl)phenyl)hydrazine-1-carboxamide hydrochloride(11v) Yellow solid; yield: 71%; MS (ESI) m/z (%): 587.1 [M+H]+; $^1$H 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$_5$O$_5$S (%): 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 $4 \times 10^3$ 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 100 µl 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: $(F_{\text{compound}}-F_{\text{dmso}})/(F_{\text{PAC-1}}-F_{\text{dmso}})\times100$. 

**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 compounds (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.
References


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, FeCl₃·6H₂O, activated carbon, ethanol, 65 °C to 78 °C, 5 h; (iii) Phenyl chloroformate, pyridine, CH₂Cl₂, 0 °C to rt, 4-6 h; (iv) 80% hydrazine monohydrate, 1,4-dioxane, 80 °C, 6 h; (v) Na₂CO₃, KI, 65 °C, 30 h; (vi) NaHS, MgCl₂·6H₂O, DMF, H₂O, rt, 15 h; (vii) 1,3-dichloro-2-propanon, acetonitrile, 50 °C, 4 h; (viii) 2,4-dihydroxy benzaldehyde, NaHCO₃, 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.

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<th>Compd.</th>
<th>R1</th>
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<th>R3</th>
<th>IC50±SD(μM)</th>
<th>HT29</th>
<th>SK-N-SH</th>
<th>MDA-MB-231</th>
<th>MKN45</th>
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<tr>
<td>11q</td>
<td>N</td>
<td>N</td>
<td>3,5-di-tert-butyl</td>
<td>0.32±0.13</td>
<td>0.83±0.21</td>
<td>1.56±0.42</td>
<td>0.91±0.21</td>
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</tr>
<tr>
<td>11r</td>
<td>N</td>
<td>N</td>
<td>3-allyl</td>
<td>1.39±0.29</td>
<td>4.50±0.21</td>
<td>9.04±1.09</td>
<td>4.60±0.93</td>
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</tr>
<tr>
<td>11s</td>
<td>N</td>
<td>N</td>
<td>3,5-di-tert-butyl</td>
<td>0.37±0.21</td>
<td>0.87±0.15</td>
<td>1.57±0.37</td>
<td>1.50±0.39</td>
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</tr>
<tr>
<td>11t</td>
<td>N</td>
<td>N</td>
<td>3-allyl</td>
<td>2.21±0.29</td>
<td>6.21±0.47</td>
<td>13.70±1.03</td>
<td>1.80±0.20</td>
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</tr>
<tr>
<td>11u</td>
<td>N</td>
<td>N</td>
<td>-</td>
<td>2.44±0.53</td>
<td>2.07±0.61</td>
<td>1.96±0.48</td>
<td>2.32±0.21</td>
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</tr>
<tr>
<td>11v</td>
<td>N</td>
<td>N</td>
<td>-</td>
<td>2.11±0.73</td>
<td>1.64±0.18</td>
<td>1.92±0.36</td>
<td>2.06±0.31</td>
<td></td>
</tr>
<tr>
<td>PAC-1</td>
<td>N</td>
<td>N</td>
<td>-</td>
<td>1.16±0.51</td>
<td>3.91±0.61</td>
<td>1.52±0.13</td>
<td>1.16±0.38</td>
<td></td>
</tr>
</tbody>
</table>

*a* IC50: 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.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>IC$_{50}$±SD(μM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U937</td>
</tr>
<tr>
<td>11q</td>
<td>2.57±0.51</td>
</tr>
<tr>
<td>11s</td>
<td>2.68±0.71</td>
</tr>
<tr>
<td>PAC-1</td>
<td>6.01±0.58</td>
</tr>
</tbody>
</table>

$^a$ 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. Procapase-3 activity of selected compounds 11q, 11s and PAC-1 \textit{in vitro}.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Procapase-3 (% activity at 10μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11q</td>
<td>68.4±0.8</td>
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<tr>
<td>11s</td>
<td>76.3±0.6</td>
</tr>
<tr>
<td>PAC-1</td>
<td>100</td>
</tr>
</tbody>
</table>
### Table 4. *In vitro* metabolic half-life (T\(_{1/2}\)) [min] and intrinsic clearance (Cl\(_{\text{int}}\)) of compounds 11q and 11s.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Index</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>human</td>
</tr>
<tr>
<td>ketanserin</td>
<td>T(_{1/2}) (min)</td>
<td>36.07</td>
</tr>
<tr>
<td></td>
<td>Cl(_{\text{int}}) (mL/min/kg)</td>
<td>48.19</td>
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<tr>
<td>11q</td>
<td>T(_{1/2}) (min)</td>
<td>72.19</td>
</tr>
<tr>
<td></td>
<td>Cl(_{\text{int}}) (mL/min/kg)</td>
<td>24.08</td>
</tr>
<tr>
<td>11s</td>
<td>T(_{1/2}) (min)</td>
<td>51.01</td>
</tr>
<tr>
<td></td>
<td>Cl(_{\text{int}}) (mL/min/kg)</td>
<td>34.08</td>
</tr>
</tbody>
</table>