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The synthesized novel fluorinated compound (LJJ-10) induces death receptor- and mitochondria-dependent apoptotic cell death in the human osteogenic sarcoma U-2 OS cells

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Abstract

We designed the 6-fluoro-2-(3-fluorophenyl)-4-substituted anilinoquinazoline derivatives as less toxic anti-cancer candidates. Our result demonstrated that LJJ-10 has greater cytotoxicity than that of the other compounds in human osteogenic sarcoma U-2 OS cells. LJJ-10-induced apoptosis was associated with enhancing ROS generation, DNA damage, and an increase of the protein levels of Fas, FasL, FADD, caspase-8, cytochrome *c*, Apaf-1, AIF, Endo G, caspase-9 and caspase-3 in U-2 OS cells. LJJ-10-triggered growth inhibition was significantly attenuated by *N*-acetylcysteine, cyclosporine A, anti-FasL monoclonal antibody, and caspase-8, -9 and -3 specific inhibitors in U-2 OS cells. We suggest that LJJ-10 induced apoptotic cell death in U-2 OS cells through death receptor and mitochondria-dependent apoptotic signaling pathways.

Keywords: 6-Fluoro-2-(3-fluorophenyl)-4-substituted anilinoquinazoline derivatives; LJJ-10; U-2 OS cells; death receptor; mitochondrial apoptotic signaling

1. Introduction

Osteosarcoma is the most common primary bone malignancy [1], especially in adolescents and young adults. Contemporary treatment of osteosarcoma requires therapy incorporating surgery and systemic chemotherapy [2]. With the advent of chemotherapy, the prognosis of osteosarcoma patients significantly improved. Several chemotherapeutic agents, doxorubicin, cisplatin, high-dose methotrexate with leucovorin rescue and ifosfamide, are considered the most active agents against osteosarcoma [3]. However, a plateau in the survival rate seems to have been reached, and high-grade osteosarcoma predicts a poor outcome with long-term survival rates between 10-40% [4]. In addition, most chemotherapeutic agents cause strong normal cells cytotoxicity and side effects [2]. Recently, studies in bone cancer or osteosarcoma treatment have been focused on novel target therapies including induction of apoptosis and reduction of cell growth in osteogenic sarcoma [5-7].

The importance of fluorine in medicinal chemistry is well recognized [8]. Indeed, an increasing number of drugs on the market contain fluorine, and the presence of which is often a major importance to activity. Many fluorinated analogues were recognized by macromolecular recognition sites as the natural substrate [9]. Fluorinated drugs are used in clinic for treatment of diseases of the central nervous system [10], various cardiovascular diseases [11], obesity [12], anti-bacterial agents [13], anti-fungal agents [14] and anticancer therapies [15]. Certain of these agents are well known, such as the fluoroquinolone, an anti-bacterial agent [16], fluoroazole, an anti-fungal agent [17] and 5-fluorouracil, an anticancer agent [18]. Herein, the fluorine substitution has played an important role in the development of more active and more selective agents. In addition, the advantages to drug design related to the special properties of fluorine including small size and high electronegativity, assured that the ability of fluorine-containing drugs in developing enzyme inhibitors or

rendering molecule resistant to chemical degradation. We designed the novel fluorinated compounds with a quinazoline core structure as anticancer candidates in order to increase their metabolic stability and be recognized by macromolecular recognition sites after entering human circulation.

The analogues of quinazoline nuclei were reported to have anti-malarial, anti-inflammatory, anti-bacterial and anticancer activities, etc [16-18]. In our study, we design the 6-fluoro-2-(3-fluorophenyl)-4-substituted anilinoquinazoline derivatives as less toxic anticancer candidates based on the characteristics of fluorine in medicinal chemistry in our previous study [19]. A series of 6-fluoro-2-(3-fluorophenyl)-4-substituted anilinoquinazoline derivatives were consequently synthesized and assayed for cytotoxicity *in vitro* against seven types of cancer cell lines. Our preliminary results also showed that these compounds (**11**, **12**, **13**, **16** and **18**) displayed anti-proliferate activities on human osteogenic sarcoma (U-2 OS), leukemia (HL-60), leukemic monocyte lymphoma (U937), colon adenocarcinoma (HT-29), colon cancer (colo 205), breast adenocarcinoma (MCF-7) and breast cancer (MDA-MB-231) cell lines. Compounds **26** and **29** indicated selective effects on HL-60, U937 and U-2 OS cells, and compounds **25**, **26** and **28** showed only cytotoxicity against the U-2 OS cell line. That is, **25**, **26** and **28** showed highly selectivity toward U-2 OS cells. The novel compound, 6-fluoro-(3-fluorophenyl)-4-(3-methoxyanilino) quinazoline (compound **13**, LJJ-10), has greater cytotoxicity than that of the other compounds in U-2 OS cells with the EC_{50} (half maximal effective concentration) about 30.26 μM. In this study, we also investigated the mechanism of apoptotic induction by LJJ-10 in U-2 OS cells. Also, the anticancer activity of LJJ-10 is not excellent but less cytotoxic effects in normal cells (human fetal normal osteoblastic cell line, hFOB). Our results suggest that LJJ-10 induced apoptosis in U-2 OS cells *via* death-receptor and

mitochondria-mediated apoptotic pathway. LJJ-10 might be selected as the lead compound of apoptotic anticancer agent for further structure modification in the future.

2. Chemistry

The 2-Aminobenzamide (**3a**) and 5-fluoro-2-aminobenzamide (**3b**) were prepared using standard methodology from 2-nitrobenzoic acid (**1a**) and 5-fluoro-2-nitrobenzoic acid (**1b**) respectively, by reaction with $SOCl_2$, NH_3, and H_2/Pd-C. The benzamide (**3a, 3b**) then were reacted with 3-substituted benzaldehydes in *N*,*N*-dimethylacetamide (DMAC) in the presence of $NaHSO_3$ at 150°C. Thermal cyclodehydration/dehydrogenation gave the 6-substituted-2-(3-substituted phenyl)-4-quinazolinones (**4**-**7**) as shown in scheme 1. 6-Substituted-2-(3-substituted phenyl)-4-quinazolinones (**4**-**7**) were chlorinated at the C-4 by reaction with $SOCl_2$, followed substitution by various substituted anilines to give the target 6-substituted-2-(3-substituted phenyl)-4-substituted anilinoquinazolines (**8**-**32**) as shown in scheme 2.

2.1. General Experimental Procedures

Reagents and solvents were obtained commercially and used without further purification. Reactions were monitored by thin-layer chromatography, using Merck plates with fluorescent indicator (TLC Silica Gel 60 F254). Melting points were determined on a Yanaco MP-500D melting point apparatus and were uncorrected. ^{1}H NMR spectra were obtained on a Bruker Advance DPX-200 FT-NMR spectrometer in DMSO-d_6. The following abbreviations are used: *s*, singlet; *d*, doublet; *m*, multiplet; *dd*, double doublet. EI-MS spectra were measured with an HP 5995 GC-MS instrument. ESI-MS spectra were measured with a Bruker HCT ultra PTM Discovery

system (Proteineer fc, UltiMate 3000). Elemental analyses (C, H, and N) were performed on a Perkin-Elmer 2400 Series II CHNS/O analyzer, and the results were within ±0.4% of the calculated values.

2.2. General procedure for synthesis of starting 2-aminobenzamides

(a) Thionyl chloride (1.5 g, 12.6 mmol) was added dropwise to a suspension of a 5-substituted-2-nitrobenzoic acid (**1a**: 1.1 g, 6.3 mmol; **1b**: 1.2 g, 6.3 mmol) in dichloroethane (30 mL) under reflux. The resulting mixture was stirred under reflux for 4 h and dried under vacuum. (b) The residue was dissolved in 200 mL of dichloroethane and treated with anhydrous ammonia gas at room temperature. (c) After removing solvent, the intermediate 5-substituted-2-nitrobenzamide (2**a**-**b**) was dissolved in MeOH and hydrogenated over 10% Pd/C for 1-2 h. The catalyst was removed by filtration, and the solution was dried under vacuum to afford the 5-substituted-2-aminobenzamides as pale yellow powders (**3a**: 0.7 g, 85%; **3b**: 0.8 g, 85%).

2.3. General procedure for synthesis of 6-substituted-2-(3-substituted phenyl)-4-quinazolinones (4-7)

Sodium hydrogen sulfite (0.8 g, 7.5 mmol) was added to a solution of 2-aminobenzamide (**3a**) (1.0 g, 7.3 mmol) and benzaldehyde (0.8 g, 7.3 mmol) in *N*,*N*-dimethylacetamide (DMAC) (20 mL). The mixture was heated with stirring at 150°C for 3 h and poured into ice water (200 mL). The precipitate was collected, washed with water, and dried in vacuo. After purification by column chromatography (silica gel; chloroform) and followed recrystallization from EtOH, 2-phenyl-4-quinazolinone (**4**) was obtained (1.1 g) as white needles. Yield, melting point, and spectral data of **4** and all subsequent compounds are summarized in Table 1.

The method used to prepare **4** was used with the indicated substituted benzaldehyde and benzamide to afford **5-7**.

2-(3-Fluorophenyl)-4-quinazolinone (5). 1.0 g from **3a** and 3-fluorobenzaldehyde (0.9 g, 7.3 mmol), white needles.

6-Fluoro-2-phenyl-4-quinazolinone (6). 1.1 g from **3b** and benzaldehyde (0.8 g, 7.3 mmol), brown powders.

6-Fluoro-2-(3-fluorophenyl)-4-quinazolinone (7). 1.1 g from **3b** and 3-fluorobenzaldehyde (0.9 g, 7.3 mmol), white needles.

2.4. General procedure for synthesis of 6-substituted-2-(3-substituted phenyl)-4-substituted anilinoquinazolines (8-32)

Thionyl chloride (1.5 g, 12.6 mmol) was added dropwise to a suspension of 2-phenyl-4-quinazolinone (**4**) (1.4 g, 6.3 mmol) in dichloroethane (30 mL) and *N*,*N*-dimethylformamide (DMF) (0.5 mL) at 50°C. The resulting mixture was stirred at 50°C for 30 min and dried under vacuum. The residue was dissolved in 20 mL of DMF and treated with aniline (0.6 g, 6.3 mmol) at 50°C for 2 h. The mixture was then poured into ice water (200 mL). The precipitate was collected, washed with water, and dried in vacuo. After purification by column chromatography (silica gel; chloroform) and followed recrystallization from the mixed solvent of $CHCl_3$ and EtOH, 4-anilino-2-phenylquinazoline (**8**) was obtained (1.3 g) as white needles. The method used to prepare **8** was used with the indicated substituted aniline and phenylquinazolinone to afford **9-32**.

4-Anilino-2(3-fluorophenyl) quinazoline (9). 1.5 g from **5** and aniline (0.6 g, 6.3 mmol), pale yellow powders.

4-Anilino-6-fluoro-2-phenylquiazoline (10). 1.5 g from **6** and aniline (0.6 g, 6.3 mmol), white needles.

4-Anilino-6-fluoro-2-(3-fluorophenyl)quinazoline (11). 1.6 g from **7** and aniline (0.6 g, 6.3 mmol), white needles.

6-Fluoro-2-(3-fluorophenyl)-4-(4-methoxyanilino)quinazoline (12). 1.6 g from **7** and 4-methoxyaniline (0.8 g, 6.3 mmol), brown powders.

6-Fluoro-2-(3-fluorophenyl)-4-(3-methoxyanilino)quinazoline (13). 1.6 g from **7** and 3-methoxyaniline (0.8 g, 6.3 mmol), yellow needles.

6-Fluoro-2-(2-fluorophenyl)-4-(3-methoxyanilino)quinazoline (14). 1.6 g from **7** and 2-methoxyaniline (0.8 g, 6.3 mmol), yellow needles.

6-Fluoro-2-(3-fluorophenyl)-4-(4-fluoroanilino)quinazoline (15). 1.6 g from **7** and 4-fluoroaniline (0.7 g, 6.3 mmol), white plate crystals.

6-Fluoro-2-(3-fluorophenyl)-4-(3-fluoroanilino)quinazoline (16). 1.6 g from **7** and 3-fluoroaniline (0.7 g, 6.3 mmol), white needles.

6-Fluoro-2-(3-fluorophenyl)-4-(2-fluoroanilino)quinazoline (17). 1.6 g from **7** and 2-fluoroaniline (0.7 g, 6.3 mmol), white needles.

6-Fluoro-2-(3-fluorophenyl)-4-(4-chloroanilino)quinazoline (18). 1.6 g from **7** and 4-chloroaniline (0.8 g, 6.3 mmol), white needles.

6-Fluoro-2-(3-fluorophenyl)-4-(3-chloroanilino)quinazoline (19). 1.6 g from **7** and 3-chloroaniline (0.8 g, 6.3 mmol), white needles.

6-Fluoro-2-(3-fluorophenyl)-4-(2-chloroanilino)quinazoline (20). 1.6 g from **7** and 2-chloroaniline (0.8 g, 6.3 mmol), pink needles.

6-Fluoro-2-(3-fluorophenyl)-4-(4-bromoanilino)quinazoline (21). 1.6 g from **7** and 4-bromoaniline (1.1 g, 6.3 mmol), yellow needles.

6-Fluoro-2-(3-fluorophenyl)-4-(3-bromoanilino)quinazoline (22). 1.6 g from **7** and 3-bromoaniline (1.1 g, 6.3 mmol), brown needles.

6-Fluoro-2-(3-fluorophenyl)-4-(2-bromoanilino)quinazoline (23). 1.6 g from **7** and 2-bromoaniline (1.1 g, 6.3 mmol), pink plate crystals.

6-Fluoro-2-(3-fluorophenyl)-4-(4-hydroxyanilino)quinazoline (24). 1.6 g from **7** and 4-aminophenol (0.7 g, 6.3 mmol), brown powders.

6-Fluoro-2-(3-fluorophenyl)-4-(3-hydroxyanilino)quinazoline (25). 1.6 g from **7** and 3-aminophenol (0.7 g, 6.3 mmol), yellow powders.

6-Fluoro-2-(3-fluorophenyl)-4-(2-hydroxyanilino)quinazoline (26). 1.6 g from **7** and 2-aminophenol (0.7 g, 6.3 mmol), yellow powders.

6-Fluoro-2-(3-fluorophenyl)-4-(4-aminoanilino)quinazoline (27). 1.6 g from **7** and *p*-phenylenediamine (0.7 g, 6.3 mmol), yellow needles.

6-Fluoro-2-(3-fluorophenyl)-4-(3-aminoanilino)quinazoline (28). 1.6 g from **7** and *m*-phenylenediamine (0.7 g, 6.3 mmol), yellow needles.

6-Fluoro-2-(3-fluorophenyl)-4-(2-aminoanilino)quinazoline (29). 1.6 g from **7** and *o*-phenylenediamine (0.7 g, 6.3 mmol), white needles.

6-Fluoro-2-(3-fluorophenyl)-4-(4-methylanilino)quinazoline (30). 1.6 g from **7** and *p*-toluidine (0.7 g, 6.3 mmol), yellow needles.

6-Fluoro-2-(3-fluorophenyl)-4-(3-methylanilino)quinazoline (31). 1.6 g from **7** and *m*-toluidine (0.7 g, 6.3 mmol), white needles.

6-Fluoro-2-(3-fluorophenyl)-4-(2-methylanilino)quinazoline (32). 1.6 g from **7** and *o*-toluidine (0.7 g, 6.3 mmol), pink plate crystals.

3. Pharmacology

3.1. Materials and reagents

MTT (3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide), *N*-acetylcysteine (NAC), cyclosporine A and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, McCoy’s 5a medium, RPMI 1640 medium, Dulbecco's modified eagle medium (DMEM), trypsin-EDTA,

2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and 3,3'-dihexyloxacarbocyanine iodide (DiOC6) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Caspase-3 inhibitor (Z-DEVE-FMK), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) (R&D systems, Minneapolis, MN, USA) were dissolved in DMSO and diluted in cell culture medium before use. Antibodies against caspase-8, caspase-9 and caspase-3 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for Fas/CD95, FasL, FADD, cytohrome *c*, Apaf-1, caspase-9, Endo G, AIF, Bcl-2 and Bax were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

3.2. Cell culture

The human leukemia cell line (HL-60), human monocyte lymphoma cell line (U937), human colon adenocarcinoma cell line (HT-29), human adenocarcinoma cell line (colo 205), human breast adenocarcinoma cell line (MCF-7), human breast cancer cell line (MDA-MB-231), human osteosarcoma cell line (U-2 OS) and human fetal normal osteoblastic cell line (hFOB) were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The HL-60, U937, HT-29 and colo 205 cells were cultured with RPMI 1640 medium. The MCF-7 and MDA-MB-231 cells were seeded in DMEM. The U-2 OS cells were cultured with 90% McCoy's 5a medium. The hFOB cells were cultured with DMEM/Ham's F12 medium (Invitrogen). All cells were plated onto 75 cm^2 tissue culture flasks with medium with 2 mM L-glutamine supplemented with 10% FBS, 100 Units/ml penicillin and 100 μg/ml streptomycin. All cells were grown at 37°C in a humidified atmosphere comprised of 95 % air and 5% CO_2 [20-22].

3.3. Determination of cell viability by MTT Assay

The cell viability was assessed using MTT assay. Cells (1×10^4 cells/well) onto 96-well plates were exposed test compounds, and 0.1% DMSO in media served as a vehicle control. After a 48 h-incubation, one hundred μl MTT (0.5 mg/ml) solution was added to each well, the plate was incubated at 37°C for 4 h. Then, one hundred μl 0.04 N HCl in isopropanol was added and the absorbance at 570 nm was measured for each well. The cell survival ratio was expressed as % of control. All results were performed from three independent experiments [21-22]. Cell viability was also used to examine in U-2 OS cells after pretreatment with 10 mM NAC, 1 μM cyclosporine A, FasL mAb, and caspase-3, -8 and -9 inhibitors. U-2 OS cells seeded at a density of 2.5×10^5 cells/well into 24-well plates were pretreated with NAC and cyclosporine A for 3 h or caspase-8, -9 and -3 inhibitors for 1 h, respectively, followed by treatment with 30 μM LJJ-10 and 0.1% DMSO as a control. Cells were then harvested after LJJ-10 for 48 h exposure to determine the percentage of viable cells as previously described [21-22].

3.4. Cell morphological examination

After U-2 OS cells (2.5×10^5 cells/well onto 24-well plate) were treated with 0, 15, 30 and 45 μM LJJ-10 for 48 h, the cell morphology was directly examined and photographed under a contrast-phase microscope [23].

3.5. Comet assay

After 48 h-treatment with 15, 30 and 45 μM LJJ-10, U-2 OS cells were harvested and mixed with low melting point (LMP, Sigma-Aldrich Corp.) agarose at 37°C. This mixture was placed on the top of previous layer of 0.5% agarose normal melting point (NMP) on the slide, and then covered with a covership at 41°C until solid. Subsequently, the covership was removed gently and some agarose was added onto

this slide, and then covered with the covership again. The slide was placed at 41°C until the mixture was solid, and put in chilled alkaline lysis buffer for electrophoresis. Afterwards, the slide was gently washed with neutralized buffer, and stained with DAPI (Invitrogen) [23]. All results were performed from three independent experiments. DNA damage was assessed by using free CometScore software (Tritek Corp., Sumerduck, VA, USA).

3.6. Assays for reactive oxygen species (ROS) and mitochondrial membrane potential *(ΔΨm)*

The levels of ROS and *ΔΨm* were determined by flow cytometry using H_2DCF-DA and DiOC6 as fluorescent probes, respectively. U-2 OS cells were seeded at a density of 2.5×10^5 cells/well into 24-well plates and treated with 30 μM LJJ-10 for 0, 2, 6, and 12 h. Then cells were incubated with 5 μM of H_2DCF-DA and 40 nM of DiOC6 at 37°C for 30 min and then analyzed by flow cytometry. The mean fluorescence intensity (MFI) was quantities by BD CellQuest Pro software [24-25]. All results were performed from three independent experiments.

3.7. Detection of Fas and Fas ligand (FasL) by flow cytometry

U-2 OS cells (2.5×10^5 cells/well) were seeded into 24-well plates and incubated with 30 μM LJJ-10 for 12 h. Cells were then harvested and washed twice by PBS. The cells were examined for Fas and Fas ligand (FasL) by being stained with anti-Fas-fluorescein isothiocyanate (FITC) and anti-FasL-FITC antibodies (BD Pharmingen Inc. San Diego, CA, USA) and then incubated 1 h and analyzed by flow cytometry [24].

3.8. Caspase-9, -8 and -3 activities assay

Approximately 1×10^7 cells of U-2 OS cells onto 75-T flask were pretreated with/without a pan-caspase inhibitor (Z-VAD-FMK) and selective inhibitors (Z-DEVE-FMK for caspase-3, Z-IETD-FMK for caspase-8 and Z-LEHD-FMK for caspase-9). After treatment with 30 μM LJJ-10 for 48 h, activities of caspase-3, -8 and -9 were determined according to the manufacturer's instructions (Caspase colorimetric kit, R&D systems). Briefly, cells were harvested and lysed in 50 μl lysis buffer containing 2 mM DTT for 10 min. After centrifugation, the supernatants containing 100 μg proteins were incubated with caspase-3, -8 and caspase-9 substrate in reaction buffer (R&D systems). Samples were incubated in a 96-well flat bottom microplate at 37°C for 1 h. The levels of released pNA (Z-DEVE-pNA, Z-IETD-pNA and Z-LEHD-pNA for caspase-3, -8 and -9, respectively) were measured with an ELISA reader (Anthos Labtec. Instruments) at a wavelength of 405 nm [26-27].

3.9. Western blotting analysis

Approximately 1×10^7 cells of U-2 OS cells onto 75-T flask were treated with 30 μM LJJ-10 for 0, 12, 24 and 48 h. Cells were harvested and the cytosolic proteins were collected as described elsewhere [28]. The cytosolic proteins (30 μg) from each sample were resolved on 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane by using the iBot Dry Blotting System (Invitrogen). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in Tris-buffered saline /Tween 20 (TBST) and incubated with primary antibodies (anti-Fas/CD95, -FasL, -FADD, -cytochrome *c*, -Apaf-1, -pro-caspase-3, -caspase-8, -casepase-9, -AIF, -Endo G, -Bcl-2, -Bax and -Bid (Santa Cruz Biotechnology, Inc.) at 4°C overnight. Membranes were washed three times with TBST for 10 min and incubated with secondary HRP-conjugated antibody. The blots were developed by using an ECL kit (Millipore Crop., Billerica,

MA, USA) and Kodak Bio-MAX MR film (Eastman Kodak). All results are from three independent experiments. Quantification of each band of protein levels were appeared by using the NIH ImageJ program [25, 29]

3.10. Statistical analysis

Data are presented as the mean ± S.E.M. for the indicated number of separate experiments. Statistical analyses of data were done by one-way ANOVA, and $P<0.05$ was considered significant.

4. Results

4.1. Chemistry

6-Substituted-2-(3-substituted phenyl)-4-quinazolinones (**4-7**) and 6-substituted-2-(3-substituted phenyl)-4-substituted anilinoquinazolines (**8-32**) were synthesized according to scheme 1-2. Structural elucidation of compounds **4-7** and **8-32** were characterized by correct elemental analysis and careful inspections of spectral data. ^{1}H NMR spectrum of **4-7** displayed signals at δ = 12.54-12.72 ppm characteristic for NH of quinazolinone ring. However, ^{1}H NMR spectrum of **8-32** displayed signals at δ = 9.27-9.98 ppm, except the compound 15 at 11.08 ppm, characteristic for NH of anilinoquinazoline. The physical and spectral data of compounds **4-32** were demonstrated in Table 1.

4.2. 6-Fluoro-2-(3-fluorophenyl)-4-substituted anilinoquinazolines induced growth inhibition in human cancer cells

The anti-proliferation effects of compounds **8-32** on U-2 OS, HL-60, U937, HT-29, Colo 205, MCF-7, MDA-MB-231 cells were evaluated by the MTT assay. The results, IC_{50} values of compounds **8-32** on cancer cell lines, are presented in Table

2. Our results demonstrated that the compounds **11**, **12**, **13**, **16** and **18** had cytotoxic effects toward a variety of cancer cells, but compound **16** was inactive in HT-29 and Colo 205 cells, and compound **18** was inactive in MCF-7 and MDA-MB-231 cells. Compounds **25**, **27** and **28** had highly selective effects on U-2 OS cells, and compounds **26** and **29** had selective effects on HL-60, U937 and U-2 OS cells. However, 6-fluoro-(3-fluorophenyl)-4-(3-methoxyanilino) quinazoline (compound **13**, LJJ-10; Figure 1A) was the most potent compound against human osteosarcoma U-2 OS cells.

4.3. LJJ-10 induced growth inhibition, apoptosis and DNA damage in human osteosarcoma U-2 OS cells

We investigated the growth inhibition effects of LJJ-10 on U-2 OS cells. As shown in Figure 1B, exposure to various concentrations of LJJ-10 (10, 20, 40 and 80 μM) for 48 h resulted in dose-dependent decreases on cell number of U-2 OS relative to control cultures. LJJ-10 caused little decrease on cell number of hFOB (Fig. 1B). The IC_{50} of LJJ-10-induced U-2 OS cell death was 30.26 ± 3.21 μM. LJJ-10-induced reduction of cell viability could occur due to DNA damage or apoptosis. It can be seen in Table 1 and Figure 1C and shown that LJJ-10 with 30 μM made the U-2 OS cells rounding and shrinking morphologically after 48 h-treatment. As shown in Figure 1D, LJJ-10 induced DNA damage of U-2 OS cells by the notice of extension of the tails by comet assay (Fig. 1D, left). Furthermore, analysis of the fluorescent intensity in the comet length of extending tails revealed that the LJJ-10 worked in a dose-dependent manner on U-2 OS cells (Fig. 1D, right). Our data suggest that LJJ-10 induced apoptotic death in U-2 OS cells. Moreover, LJJ-10 induced selective cytotoxicity in U-2 OS cells but had a less cytotoxic effect on human normal hFOB cells (Fig. 1B).

4.4. LJJ-10 induced the death receptor and mitochondria-dependent apoptotic signaling pathways in U-2 OS cells

Apoptosis is the process of programmed cell death which may originate in two major signaling pathways [30]. The death receptor pathway (the extrinsic pathway) activates the death receptor on cell surface (Fas/FasL) and then promotes caspase-8 activation.[31-32] In the mitochondrial pathway, some mitochondrial changes are occurred, including the decrease the $\Delta\Psi m$, elevated production of ROS, release of cytochrome *c*, Apaf-1 and pro-caspase-9 proteins from mitochondria to cytosol and then activation of caspases. To further evaluate the role of death receptor and mitochondrial pathways in LJJ-10-induced growth inhibition in U-2 OS cells, cells were pretreated with a ROS scavenger (NAC), mitochondrial membrane potential resting agent (cyclosporine A), and anti-FasL monoclonal antibody to block LJJ-10-induced growth inhibition and cell death. As shown in Figure 2A, LJJ-10-induced growth inhibition and cell death was significantly blocked by NAC, cyclosporine A and anti-FasL monoclonal antibody. As shown in Figure 2B, LJJ-10 increased the proportion of Fas and FasL when comparing with that of the control and the isotype control experiments. After exposure to 30 μM LJJ-10 for 2, 6 and 12 h, U-2 OS cells were harvested for determining the ROS and $\Delta\Psi m$. As shown in Figure 2C and 2D, LJJ-10 promoted ROS production and decreased the level of $\Delta\Psi m$ in U-2 OS cells in a time-dependent manner. Our results suggest that the death receptor and mitochondria-dependent apoptotic signaling pathways may be involved in LJJ-10-induced cell death in U-2 OS cells.

4.5. LJJ-10 stimulated caspase-8, caspase-9 and caspase-3 activity in U-2 OS cells

To investigate the mechanisms of LJJ-10-induced apoptosis, we determined the caspase-8, caspase-9 and caspase-3 activities in LJJ-10-treated U-2 OS cells. As

shown in Figure 3A, LJJ-10 caused an increase of caspase-8, caspase-9 and caspase-3 activities in 48-h treatment. LJJ-10-induced caspase-8, caspase-9 and caspase-3 activations were also significantly suppressed by a pan-caspase inhibitor (Z-VAD-FMK). Pre-incubation with caspase-8, caspase-9 and caspase-3 specific inhibitors significantly reduced LJJ-10-induced apoptotic cell death and growth inhibition in U-2 OS cells (Fig. 3B). Our results suggest that LJJ-10-induced apoptotic cell death is mediated through the caspase-8, caspase-9 and caspase-3 activations. In order to establish the all possible pathway that involved in LJJ-10-induced apoptosis in U-2 OS cells, a series of apoptosis-associated proteins were examined by Western blotting. As shown in left panel of Figure 3C, the protein levels of Fas/CD95, FasL, FADD, and caspase-8 in U-2 OS cells were increased after treatment with LJJ-10. Observing the protein level of pro-caspase-3 was decreased in LJJ-10-treated U-2 OS cells, we inferred that the protein level of caspase-3 was increased. Based on these results, LJJ-10 induced apoptosis in U-2 OS cells *via* the death receptor-dependent pathway. In the right panel of Figure 3C, we also showed that the protein levels of cytohrome *c*, Apaf-1, caspase-9, Endo G, AIF and Bax were increased. However, these of Bcl-2 and Bid were decreased in LJJ-10-treated U-2 OS cells. The results indicated that LJJ-10 induced apoptotic cell death in U-2 OS cells through the mitochondria-dependent pathway. As illustrated above, we suggest that LJJ-10 induced apoptosis in U-2 OS cells through death receptor- and mitochondria-dependent signaling pathways.

5. Discussion and Conclusions

In this study, the newly synthesized compounds **8**-**18** were assayed for anticancer activity against 7 types of human cancer cell lines. The IC_{50} values calculated from the dose-response data obtained from three independent experiments

were listed in Table 2. The results showed that compounds **11**, **12**, **13**, **16** and **18** inhibited cell proliferation in most of the cell lines, but compounds **8**-**10**, **14**-**15**, **17**, **19**-**24** and **30**-**32** were inactive. In terms of SAR information, those anilinoquinazoline derivatives without simultaneously 6- and 2-3-substitutions (**8**-**10**) were inactive and this finding is the same as that of our previous study [33]. Additionally, compounds bearing F (**16**), OH (**25**, **26**), OCH_3 (**12**, **13**) and NH_2 (**27**, **28**, **29**) substituents at 4-anilino moiety that may bind with some important proteins in cancer cells were active. However, compounds bearing CH_3 (**30**, **31, 32**), Cl (**19**, **20**) and Br (**21**, **22**, **23**) substituents at 4-anilino moiety that maybe have low potential for binding with some proteins in cancer cells were inactive. Among them, LJJ-10 induced significantly dose-dependent growth inhibition and apoptotic cell death, and the IC_{50} value of LJJ-10 in human osteogenic sarcoma U-2 OS cells was much lower than that in human fetal normal osteoblastic hFOB cells. Therefore, we suggest that LJJ-10 could be an efficacious and safer anticancer agent for treatment of human osteogenic sarcoma. LJJ-10 was supposed worthwhile to be the anticancer lead and investigate its mechanism. Moreover, further structural modification of compound **13** (LJJ-10) may lead to the discovery of more active anilinoquinazoline with good selectivity against various cancer types.

Recently, Jantova *et al.* demonstrated that the use of anilinoquinazoline derivatives to treat human leukemia cells, and had revealed some molecular mechanisms include the induction of caspase-3 activity and cell apoptosis [34]. In our study, we also demonstrated that 30 μM of LJJ-10 significantly inhibited the cell growth and cell death (Fig. 1B) and triggered apoptosis (Fig. 1C and D) through the activation of caspase-8, caspase-9 and caspase-3 cascades in U-2 OS cells (Fig. 3). Our results suggest that LJJ-10 induced anti-osteosarcoma effects through the caspase cascade-dependent apoptotic signaling pathways.

Two apoptotic pathways, the death receptor pathway and the death receptor pathway, were proved to be associated with the activation of the caspase family proteins when cells undergo apoptosis [35]. The death receptor pathway (extrinsic pathway) is triggered by binding the ligand to its surface receptors such as FasL and CD95/Fas, and then activating the caspase-8 and caspase-3 [36-37]. The mitochondrial dependent pathway (intrinsic pathway) that triggered by anticancer drugs, DNA damage and ROS involves the decrease of $\Delta\Psi m$, and the release of cytochrome *c*, Apaf-1, caspase-9, Endo G and AIF. The released cytochrome *c* and Apaf-1 form apoptosome and convert pro-caspase-9 into caspase-9. The pro-caspase-3 is then activated by caspase-9 and finally leads cells to apoptosis [38-40]. As shown in Figure 3A, LJJ-10 induced the cleavage and activation of the caspase-8, caspase-9, and caspase-3 after 12 h-treatment in U-2 OS cells. From the observations, we suggest that LJJ-10 induced cell apoptosis through both the death receptor and mitochondria-dependent signaling pathways. Our results can support the following evidences: (1) after pretreatment with NAC, cyclosporine A or FasL mAb, respectively, and then cells were treated with LJJ-10. There is a significant increase in cell viability (Fig. 2A). (2) LJJ-10 induced a time-dependent increase protein levels of Fas/CD95 and FasL (Fig. 3C) and then activation of both caspase-8 and caspase-3 (Fig. 3A). These results suggest that the involvement of LJJ-10 in the death receptor apoptotic pathway in U-2 OS cells. (3) the loss of $\Delta\Psi m$ and ROS production were mediated after LJJ-10 treatment for 2, 6 and 12 h (Fig. 2C and D) and led to the release of cytochrome *c*, Apaf-1, pro-caspase-9, Endo G and AIF from mitochondria into the cytosol (Fig. 3B) and then activation of caspase-9 cascade (Figs. 3A and C). Based on these results, we suggest that the mitochondria-dependent pathway is also involved in LJJ-10-induced apoptosis in U-2 OS cells. A signaling pathway that is involved in the cross-talk between both

mitochondria-dependent pathway and death receptor apoptotic pathway has been substantiated by the observations that the activation of caspase-8 causes the cleavage of Bid that in turn induces a subsequent activation of caspase-9 [41-43]. LJJ-10 exerts its effects through subsequent caspase-8 activation, Bid cleavage, caspase-9 activation, and finally caspase-3 activation, leading to apoptosis in U-2 OS cells.

In conclusion, LJJ-10 displays anti-osteosarcoma effects on U-2 OS cells by inducing cell apoptosis. Our studies have clearly pointed out that the death receptor protein Fas/CD95 and the mitochondrial environment are the targets of LJJ-10 and the effects of LJJ-10 on U-2 OS cells are through intrinsic and extrinsic apoptotic signaling pathways. LJJ-10 might be selected as the lead compound of anti-osteosarcoma agents for further structure modification in the future.

Acknowledgement

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References

[1] G. Ottaviani, N. Jaffe, The epidemiology of osteosarcoma, Cancer Treat Res, 152 (2010) 3-13.

[2] S. Ferrari, E. Palmerini, Adjuvant and neoadjuvant combination chemotherapy for osteogenic sarcoma, Curr Opin Oncol, 19 (2007) 341-346.

[3] S. Bielack, D. Carrle, P.G. Casali, Osteosarcoma: ESMO clinical recommendations for diagnosis, treatment and follow-up, Ann Oncol, 20 Suppl 4 (2009) 137-139.

[4] L. Kager, A. Zoubek, U. Kastner, B. Kempf-Bielack, J. Potratz, R. Kotz, G.U. Exner, C. Franzius, S. Lang, R. Maas, H. Jurgens, H. Gadner, S. Bielack, Skip metastases in osteosarcoma: experience of the Cooperative Osteosarcoma

Study Group, J Clin Oncol, 24 (2006) 1535-1541.

[5] T. Akiyama, C.R. Dass, P.F. Choong, Novel therapeutic strategy for osteosarcoma targeting osteoclast differentiation, bone-resorbing activity, and apoptosis pathway, Mol Cancer Ther, 7 (2008) 3461-3469.

[6] S.Y. Liu, S.X. Song, L. Lin, X. Liu, Molecular mechanism of cell apoptosis by paclitaxel and pirarubicin in a human osteosarcoma cell line, Chemotherapy, 56 (2010) 101-107.

[7] Y. Shapovalov, D. Benavidez, D. Zuch, R.A. Eliseev, Proteasome inhibition with bortezomib suppresses growth and induces apoptosis in osteosarcoma, Int J Cancer, 127 (2010) 67-76.

[8] H.J. Bohm, D. Banner, S. Bendels, M. Kansy, B. Kuhn, K. Muller, U. Obst-Sander, M. Stahl, Fluorine in medicinal chemistry, Chembiochem, 5 (2004) 637-643.

[9] S.G. Dimagno, H. Sun, The strength of weak interactions: aromatic fluorine in drug design, Curr Top Med Chem, 6 (2006) 1473-1482.

[10] S. Sun, A. Adejare, Fluorinated molecules as drugs and imaging agents in the CNS, Curr Top Med Chem, 6 (2006) 1457-1464.

[11] P. Jeschke, E. Baston, F.R. Leroux, alpha-fluorinated ethers as "exotic" entity in medicinal chemistry, Mini Rev Med Chem, 7 (2007) 1027-1034.

[12] S.A. Rice, K.J. Fish, J. Hoover-Plow, K. Jawaharlal, In vitro hepatic drug and anesthetic metabolism of rats with dietary-induced obesity, Arch Int Pharmacodyn Ther, 299 (1989) 286-293.

[13] F. Marco, D.J. Biedenbach, R.N. Jones, Anti-Legionella activity of trovafloxacin compared with seven other antimicrobial agents including an intermethod evaluation, Diagn Microbiol Infect Dis, 29 (1997) 55-57.

[14] W. Abuhammour, E. Habte-Gabr, Systemic antifungal agents, Indian J Pediatr, 68 (2001) 655-668.

[15] M.L. Miller, I. Ojima, Chemistry and chemical biology of taxane anticancer agents, Chem Rec, 1 (2001) 195-211.

[16] J. Guo, S.N. Han, J.X. Liu, X.M. Zhang, Z.S. Hu, J. Shi, L.R. Zhang, Z.Z. Zhao, Z. Zhang, The action of a novel fluoroquinolone antibiotic agent antofloxacin hydrochloride on human-ether-a-go-go-related gene potassium channel, Basic Clin Pharmacol Toxicol, 107 (2010) 643-649.

[17] S. Gognies, A. Belarbi, Use of a new gelling agent (Eladium(c)) as an alternative to agar-agar and its adaptation to screen biofilm-forming yeasts, Appl Microbiol Biotechnol, 88 (2010) 1095-1102.

[18] R.A. El-Awady, E.M. Saleh, J. Dahm-Daphi, Targeting DNA double-strand break repair: is it the right way for sensitizing cells to 5-fluorouracil?,

Anticancer Drugs, 21 (2010) 277-287.

[19] M.J. Hour, J.S. Yang, J.C. Lien, S.C. Kuo, L.J. Huang, Synthesis and cytotoxicity of 6-pyrrolidinyl-2-(2-substituted phenyl)-4-quinazolinones., J Chin Chem Soc, 54 (2007) 785-790.

[20] S.C. Hsu, J.S. Yang, C.L. Kuo, C. Lo, J.P. Lin, T.C. Hsia, J.J. Lin, K.C. Lai, H.M. Kuo, L.J. Huang, S.C. Kuo, W.G. Wood, J.G. Chung, Novel quinolone CHM-1 induces apoptosis and inhibits metastasis in a human osterogenic sarcoma cell line, J Orthop Res, 27 (2009) 1637-1644.

[21] S.W. Ip, H.C. Wei, J.P. Lin, H.M. Kuo, K.C. Liu, S.C. Hsu, J.S. Yang, D. Mei, T.H. Chiu, S.M. Han, J.G. Chung, Bee venom induced cell cycle arrest and apoptosis in human cervical epidermoid carcinoma Ca Ski cells, Anticancer Res, 28 (2008) 833-842.

[22] M.L. Lin, S.S. Chen, Y.C. Lu, R.Y. Liang, Y.T. Ho, C.Y. Yang, J.G. Chung, Rhein induces apoptosis through induction of endoplasmic reticulum stress and Ca2+-dependent mitochondrial death pathway in human nasopharyngeal carcinoma cells, Anticancer Res, 27 (2007) 3313-3322.

[23] J.S. Yang, G.W. Chen, T.C. Hsia, H.C. Ho, C.C. Ho, M.W. Lin, S.S. Lin, R.D. Yeh, S.W. Ip, H.F. Lu, J.G. Chung, Diallyl disulfide induces apoptosis in human colon cancer cell line (COLO 205) through the induction of reactive oxygen species, endoplasmic reticulum stress, caspases casade and mitochondrial-dependent pathways, Food Chem Toxicol, 47 (2009) 171-179.

[24] C.C. Lu, J.S. Yang, A.C. Huang, T.C. Hsia, S.T. Chou, C.L. Kuo, H.F. Lu, T.H. Lee, W.G. Wood, J.G. Chung, Chrysophanol induces necrosis through the production of ROS and alteration of ATP levels in J5 human liver cancer cells, Mol Nutr Food Res, 54 (2010) 967-976.

[25] J.H. Chiang, J.S. Yang, C.Y. Ma, M.D. Yang, H.Y. Huang, T.C. Hsia, H.M. Kuo, P.P. Wu, T.H. Lee, J.G. Chung, Danthron, an anthraquinone derivative, induces DNA damage and caspase cascades-mediated apoptosis in SNU-1 human gastric cancer cells through mitochondrial permeability transition pores and Bax-triggered pathways, Chem Res Toxicol, 24 (2011) 20-29.

[26] J.S. Yang, M.J. Hour, S.C. Kuo, L.J. Huang, M.R. Lee, Selective induction of G2/M arrest and apoptosis in HL-60 by a potent anticancer agent, HMJ-38, Anticancer Res, 24 (2004) 1769-1778.

[27] B.Z. Packard, D.D. Toptygin, A. Komoriya, L. Brand, Profluorescent protease substrates: intramolecular dimers described by the exciton model, Proc Natl Acad Sci U S A, 93 (1996) 11640-11645.

[28] J.G. Chung, J.S. Yang, L.J. Huang, F.Y. Lee, C.M. Teng, S.C. Tsai, K.L. Lin, S.F. Wang, S.C. Kuo, Proteomic approach to studying the cytotoxicity of

YC-1 on U937 leukemia cells and antileukemia activity in orthotopic model of leukemia mice, Proteomics, 7 (2007) 3305-3317.

[29] J.K. Shen, H.P. Du, M. Yang, Y.G. Wang, J. Jin, Casticin induces leukemic cell death through apoptosis and mitotic catastrophe, Ann Hematol, 88 (2009) 743-752.

[30] S.H. Wu, L.W. Hang, J.S. Yang, H.Y. Chen, H.Y. Lin, J.H. Chiang, C.C. Lu, J.L. Yang, T.Y. Lai, Y.C. Ko, J.G. Chung, Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways, Anticancer Res, 30 (2010) 2125-2133.

[31] K.M. Debatin, P.H. Krammer, Death receptors in chemotherapy and cancer, Oncogene, 23 (2004) 2950-2966.

[32] S. Fulda, K.M. Debatin, Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy, Oncogene, 25 (2006) 4798-4811.

[33] M.J. Hour, L.J. Huang, S.C. Kuo, Y. Xia, K. Bastow, Y. Nakanishi, E. Hamel, K.H. Lee, 6-Alkylamino- and 2,3-dihydro-3'-methoxy-2-phenyl-4-quinazolinones and related compounds: their synthesis, cytotoxicity, and inhibition of tubulin polymerization, J Med Chem, 43 (2000) 4479-4487.

[34] S. Jantova, A. Repicky, E. Paulovicova, S. Letasiova, L. Cipak, Antiproliferative activity and apoptosis induced by 6-bromo-2-(morpholin-1-yl)-4-anilinoquinazoline on cells of leukemia lines, Exp Oncol, 30 (2008) 139-142.

[35] N. Khan, H. Mukhtar, Multitargeted therapy of cancer by green tea polyphenols, Cancer Lett, 269 (2008) 269-280.

[36] E. Ma, X. Wang, Y. Li, X. Sun, W. Tai, T. Li, T. Guo, Induction of apoptosis by furanodiene in HL60 leukemia cells through activation of TNFR1 signaling pathway, Cancer Lett, 271 (2008) 158-166.

[37] M.L. Trincavelli, A. Falleni, B. Chelli, D. Tuscano, B. Costa, V. Gremigni, A. Lucacchini, C. Martini, A(2A) adenosine receptor ligands and proinflammatory cytokines induce PC 12 cell death through apoptosis, Biochem Pharmacol, 66 (2003) 1953-1962.

[38] P. Rustin, G. Kroemer, Mitochondria and cancer, Ernst Schering Found Symp Proc, (2007) 1-21.

[39] M. Saitoh, K. Nagai, K. Nakagawa, T. Yamamura, S. Yamamoto, T. Nishizaki, Adenosine induces apoptosis in the human gastric cancer cells via an intrinsic pathway relevant to activation of AMP-activated protein kinase, Biochem Pharmacol, 67 (2004) 2005-2011.

[40] A. Ashkenazi, Targeting the extrinsic apoptosis pathway in cancer, Cytokine Growth Factor Rev, 19 (2008) 325-331.

[41] R.K. Srivastava, TRAIL/Apo-2L: mechanisms and clinical applications in cancer, Neoplasia, 3 (2001) 535-546.

[42] B.C. Barnhart, E.C. Alappat, M.E. Peter, The CD95 type I/type II model, Semin Immunol, 15 (2003) 185-193.

[43] P. Clarke, K.L. Tyler, Reovirus-induced apoptosis: A minireview, Apoptosis, 8 (2003) 141-150.

Figure legends

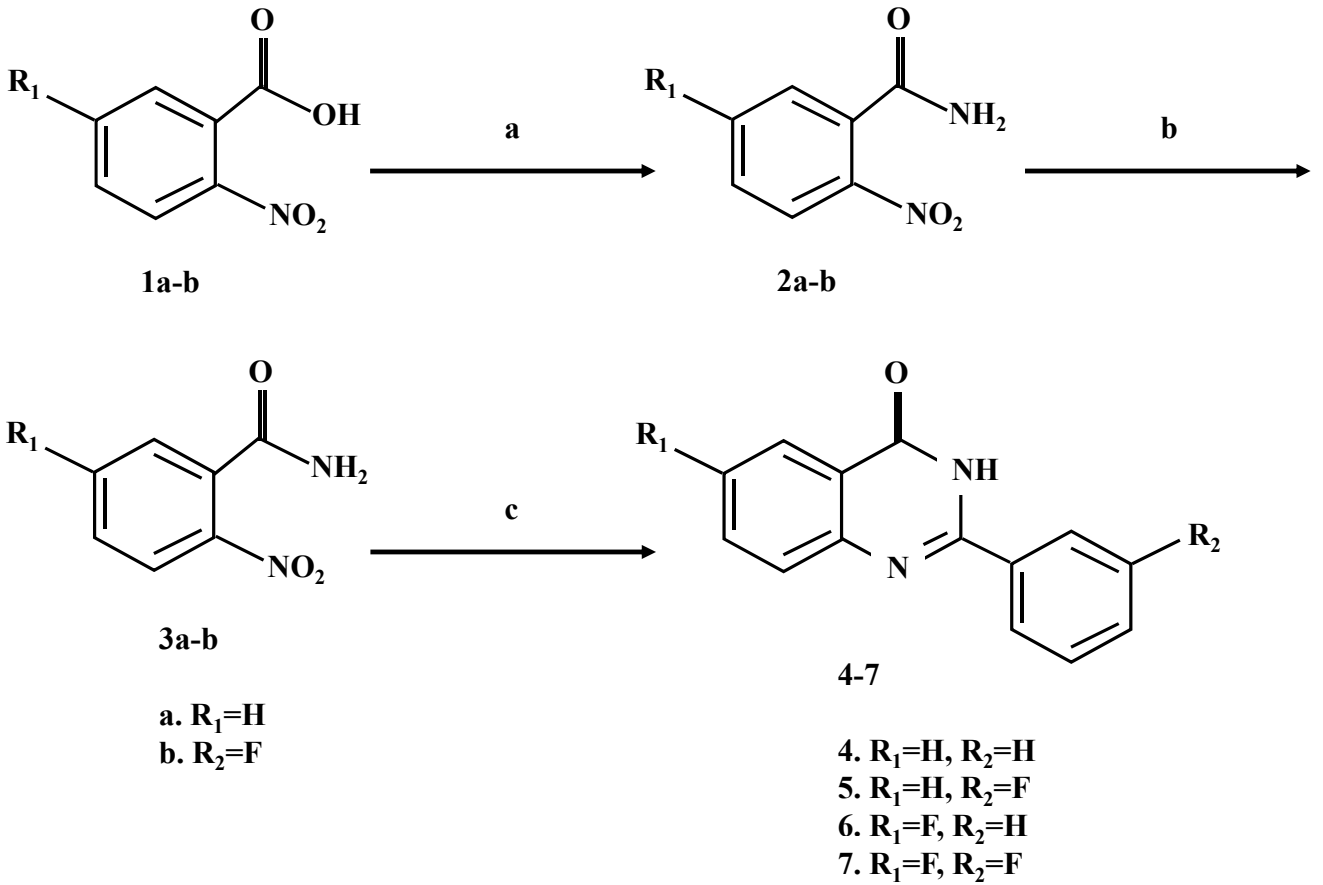
Fig. 1. Effects of LJJ-10 on cell viability, morphological changes, chromatin condensation and DNA damage in human osteosarcoma U-2 OS cells. The chemical structure of LJJ-10 (A). For cell viability assay, U-2 OS cells were cultured and treated with 0, 10, 20, 40 and 80 μM LJJ-10 for 48 h and the percentages of viable cells were detected by MTT assay described in Materials and Methods (B). After incubation with 0, 15, 30 and 45 μM LJJ-10 for 48 h, the cell morphological changes were examined and photographed under a phase-contrast microscope (C). DNA damage was determined by Comet assay as described in Materials and Methods (D). Cells were examined and photographed using the fluorescent microscopy (×200). Data from three independent experiments were presented (*p< 0.05 as compared with control treatments).

Fig. 2. Effects of LJJ-10 on the levels of Fas, FasL, reactive oxygen species (ROS) production and mitochondrial membrane potential *(ΔΨm)*. Cells were pretreated with *N*-acetylcysteine, cyclosporine A, and anti-FasL monoclonal antibody for 3 h after exposure to LJJ-10, and then incubated for 48 h. Cells were collected to determine the percentage of viable cells by MTT assay (A). U-2 OS cells were treated with 30 μM LJJ-10 for 12 h. Subsequently, cells were collected and incubated with Fas-FITC and FasL-FITC mAb for 1 h and then analyzed by flow cytometry (B). U-2 OS cells were treated with 30 μM LJJ-10 for 0, 2, 6 and 12 h, and the data from flow cytometric analysis was shown that ROS production increased (C) and loss of *ΔΨm* (D). Data from three independent experiments are presented (*p< 0.05 as compared with control treatments).

Fig. 3. Effects of LJJ-10 on caspase-8, caspase-9 and caspase-3 activity and

apoptosis-associated protein levels in U-2 OS cells. Cells were pre-incubated with or without a pan-caspase inhibitor (Z-VAD-FMK) for 1 h and then treated with 30 μM LJJ-10 for 48 h. After incubation, fifty μg proteins were incubated with caspase-3-, -8- and -9 specific substrates (Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA) with reaction buffer in a 96-well plate at 37°C for 1 h. The release of pNA was measured at 405 nm by a spectrophotometer (A). U-2 OS cells were pre-incubated with or without specific caspase inhibitors (Z-DEVE-FMK for caspase-3, Z-IETD-FMK for caspase-8 and Z-LEHD-FMK for caspase-9) for 1 h and then treated with 30 μM LJJ-10 for 48 h. Cells were collected to determine the percentage of viable cells by MTT assay. Data from three independent experiments are presented (*$p < 0.05$ as compared with control treatments) (B). Cells were treated with 30 μM LJJ-10 for 0, 12, 24 and 48 h, and cytosolic fractions were prepared and subjected to Western blotting. The resulting blots were probed for the protein levels of Fas, FasL, FADD, caspase-8, caspase-3, cytochrome *c*, Apaf-1, AIF, Endo G, caspase-9, Bcl-2, Bax, Bid and Actin (C).

Scheme 1



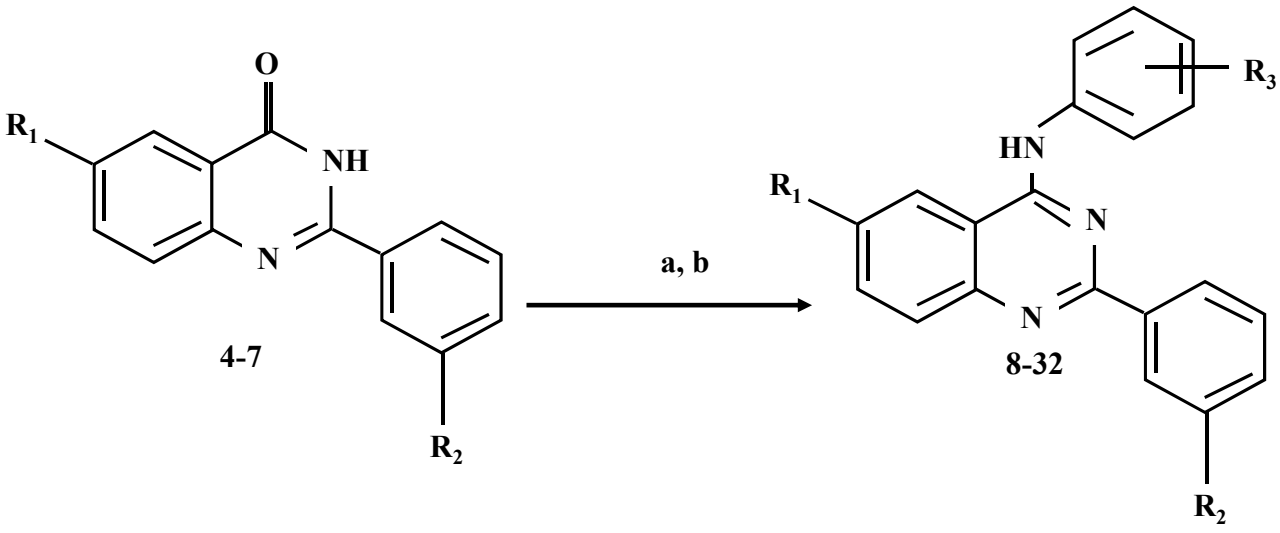
a. R_1=H
b. R_2=F

4. R_1=H, R_2=H
5. R_1=H, R_2=F
6. R_1=F, R_2=H
7. R_1=F, R_2=F

Reagents and conditions:

a. (i) $SOCl_2$ reflux; (ii) $NH_{3\,(g)}$, dichloroethane, TR.
b. H_2, Pd/C, methanol.
c. substituted benzaldehyde, DMAC, $NaHSO_3$, 150 ˚C

Scheme 2



Reagents and conditions:
a. $SOCl_2$, dichloroethane, DMF, 50-60 ˚C.
b. substituted anilines, DMF.

Compounds	R_1	R_2	R_3	Compounds	R_1	R_2	R_3
8	H	H	H	21	F	F	4-Br
9	H	F	H	22	F	F	3-Br
10	F	H	H	23	F	F	2-Br
11	F	F	H	24	F	F	4-OH
12	F	F	4-OCH_3	25	F	F	3-OH
13 (LJJ-10)	F	F	3-OCH_3	26	F	F	2-OH
14	F	F	2-OCH_3	27	F	F	4-NH_2
15	F	F	4-F	28	F	F	3-NH_2
16	F	F	3-F	29	F	F	2-NH_2
17	F	F	2-F	30	F	F	4-CH_3
18	F	F	4-Cl	31	F	F	3-CH_3
19	F	F	3-Cl	32	F	F	2-CH_3
20	F	F	2-Cl				

Figure 1

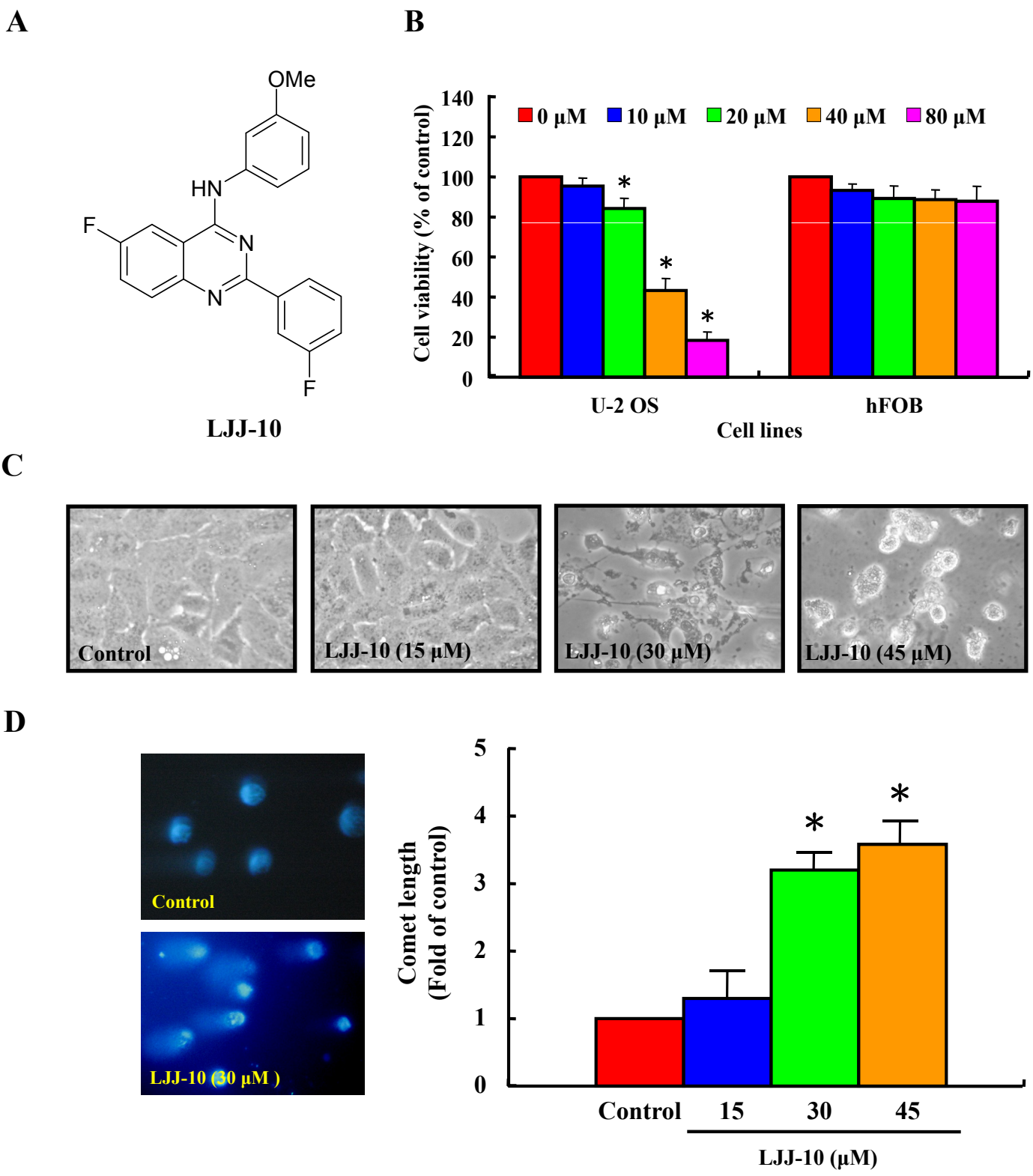

A
OMe
HN
F
N
N
F
LJJ-10
B
Cell viability (% of control)
140
120
100
80
60
40
20
0
0 μM
10 μM
20 μM
40 μM
80 μM
*
*
*
U-2 OS
hFOB
Cell lines
C
Control
LJJ-10 (15 μM)
LJJ-10 (30 μM)
LJJ-10 (45 μM)
D
Control
LJJ-10 (30 μM)
Comet length
(Fold of control)
5
4
3
2
1
0
*
*
Control
15
30
45
LJJ-10 (μM)

Figure 2

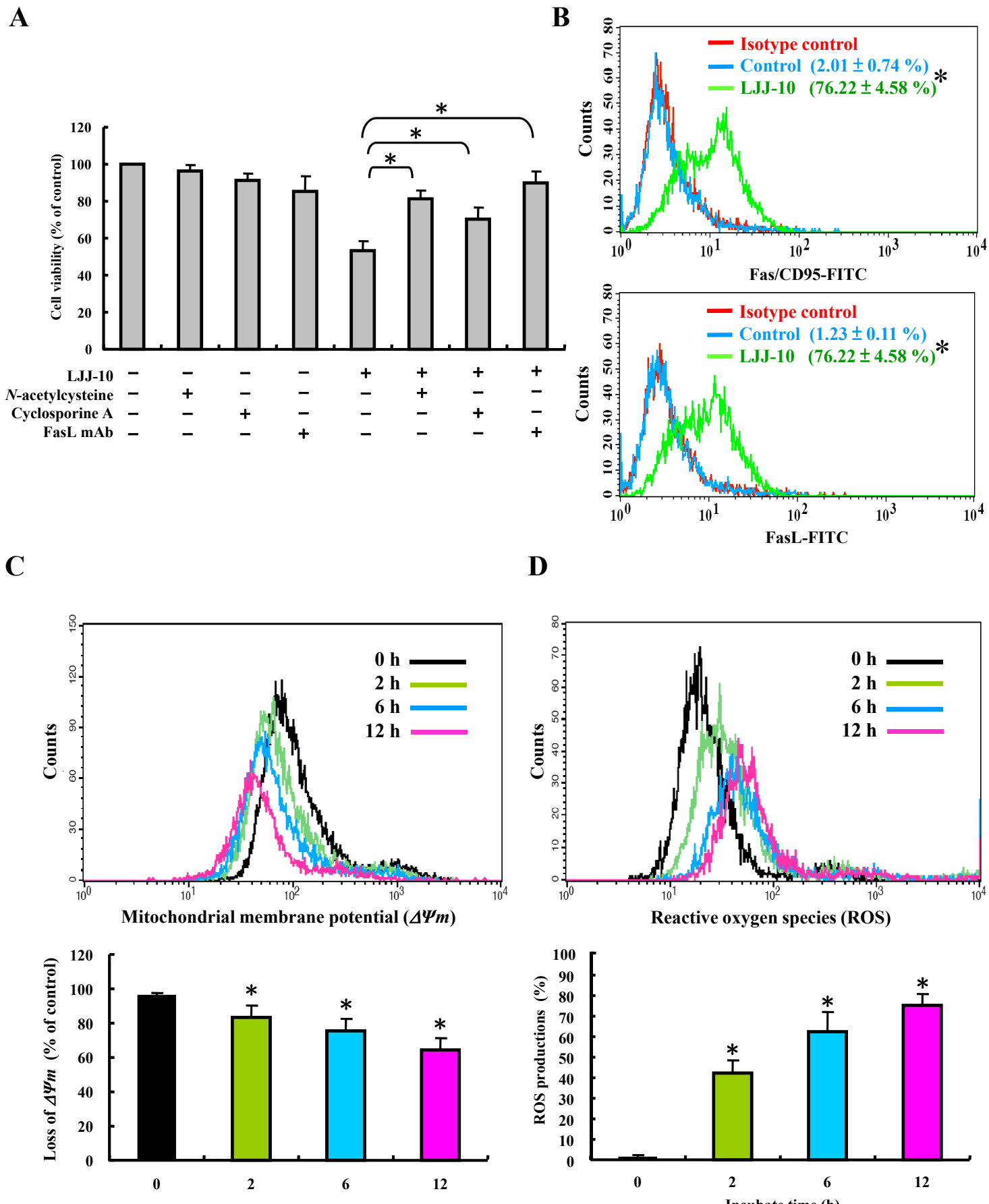
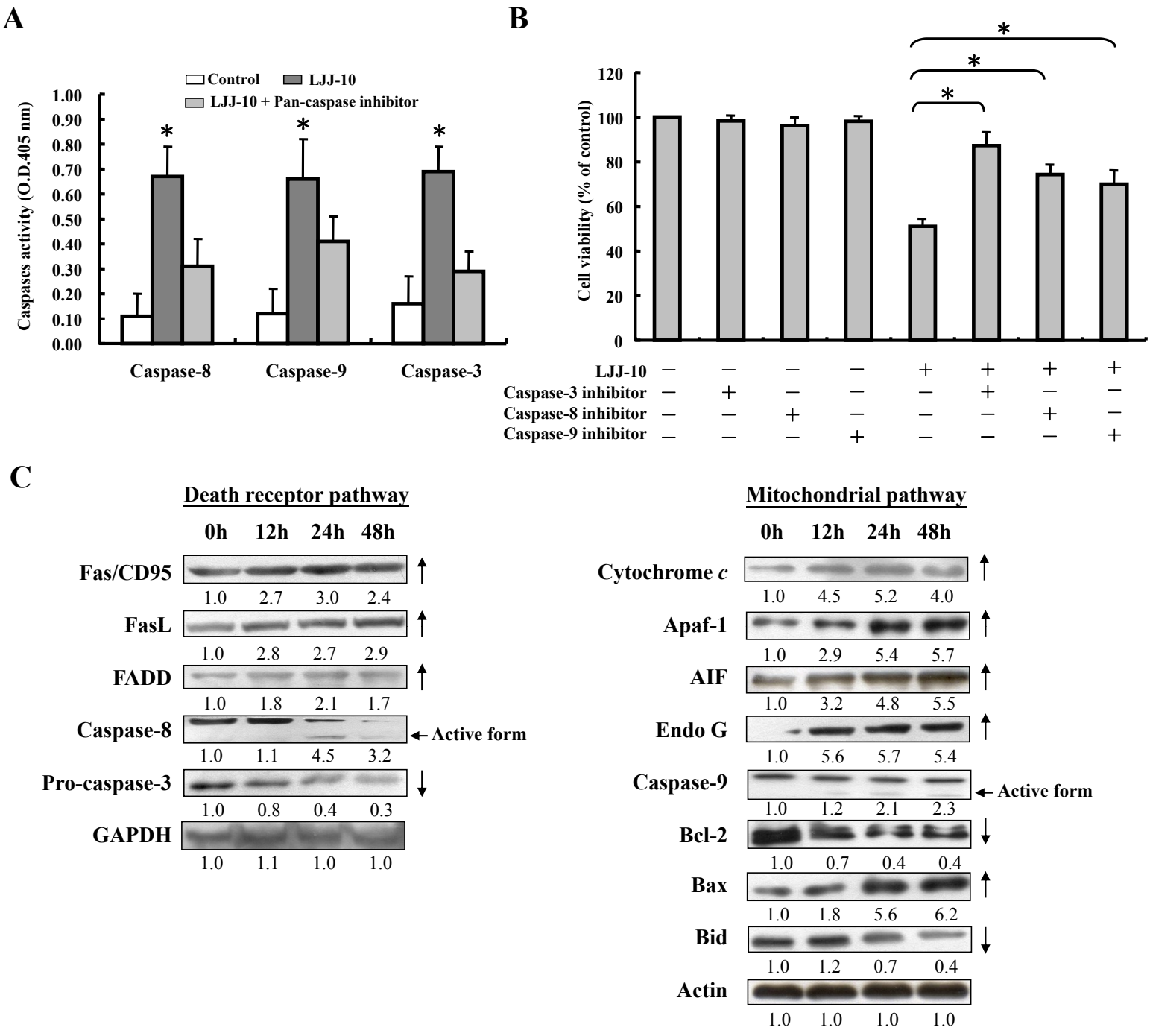

A
Cell viability (% of control)
120
100
80
60
40
20
0
*
*
*
LJJ-10 – – – – + + + +
N-acetylcysteine – + – – – + – –
Cyclosporine A – – + – – – + –
FasL mAb – – – + – – – +
B
Isotype control
Control (2.01 ± 0.74 %)
LJJ-10 (76.22 ± 4.58 %)*
Counts
Fas/CD95-FITC
Isotype control
Control (1.23 ± 0.11 %)
LJJ-10 (76.22 ± 4.58 %)*
Counts
FasL-FITC
C
0 h
2 h
6 h
12 h
Counts
Mitochondrial membrane potential (ΔΨm)
Loss of ΔΨm (% of control)
Incubate time (h)
D
0 h
2 h
6 h
12 h
Counts
Reactive oxygen species (ROS)
ROS productions (%)
Incubate time (h)

Figure 3


A
Control
LJJ-10
LJJ-10 + Pan-caspase inhibitor
Caspases activity (O.D.405 nm)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Caspase-8
Caspase-9
Caspase-3
B
Cell viability (% of control)
120
100
80
60
40
20
0
LJJ-10
Caspase-3 inhibitor
Caspase-8 inhibitor
Caspase-9 inhibitor
C
Death receptor pathway
0h 12h 24h 48h
Fas/CD95
1.0 2.7 3.0 2.4
FasL
1.0 2.8 2.7 2.9
FADD
1.0 1.8 2.1 1.7
Caspase-8
Active form
1.0 1.1 4.5 3.2
Pro-caspase-3
1.0 0.8 0.4 0.3
GAPDH
1.0 1.1 1.0 1.0
Mitochondrial pathway
0h 12h 24h 48h
Cytochrome c
1.0 4.5 5.2 4.0
Apaf-1
1.0 2.9 5.4 5.7
AIF
1.0 3.2 4.8 5.5
Endo G
1.0 5.6 5.7 5.4
Caspase-9
Active form
1.0 1.2 2.1 2.3
Bcl-2
1.0 0.7 0.4 0.4
Bax
1.0 1.8 5.6 6.2
Bid
1.0 1.2 0.7 0.4
Actin
1.0 1.0 1.0 1.0

Table(s)

Table 1. Physical and spectral data of compounds 4-32

Compounds	Yield (%)	mp (°C)	MS (M+) *m/z*	^{1}H NMR (DMSO-d_6)δ	^{13}C NMR (DMSO-d_6)δ
4	66	249-250	222	7.47-7.62 (4H, *m*, H-6, H-2', H-3', H-5'), 7.70-7.87 (2H, *m*, H-7, H-8), 8.12-8.19 (3H, *m*, H-5, H-2', H-6'), 12.54 (1H, *s*, NH)	120.95, 125.84, 126.57, 127.46, 127.75, 128.59, 131.38, 132.70, 134.59, 148.69, 152.33, 162.26
5	65	279-280	240	7.37-7.63 (3H, *m*, H-6, H-4', H-5'), 7.70-7.86 (2H, *m*, H-7, H-8), 7.95-8.05 (2H, *m*, H-2', H-6'), 8.13 (1H, *dd*, *J* = 1.2, 8.0 Hz, H-5), 12.58 (1H, *s*, NH)	114.29, 114.77, 118.04, 118.46, 121.11, 123.93, 125.87, 126.90, 127.60, 130.65, 130.81, 134.67, 134.92, 135.08, 148.44, 151.01, 159.66, 162.13, 164.51
6	69	>280	240	7.48-7.56 (3H, *m*, H-3', H-4', H-5'), 7.61-7.83 (3H, *m*, H-2', H-6', H-8), 8.12-8.16 (2H, *m*, H-5, H-7), 12.64 (1H, *s*, NH)	110.31, 110.77, 114.17, 122.11, 122.28, 122.86, 123.34, 127.78, 128.65, 130.21, 130.37, 131.46, 132.58, 145.58, 151.92, 151.95, 157.57, 161.72, 161.79, 162.25, 162.44, 168.23

7	44	>300	258	7.37-7.46 (1H, m, H-4'), 7.52-7.60 (1H, m, H-5'), 7.69-7.84 (3H, m, H-8, H-2', H-6'), 7.93-8.03 (2H, m, H-5, H-7), 12.72 (1H, *s*, NH)	110.78, 111.24, 114.72, 115.19, 118.52, 118.94, 122.72, 122.88, 123.34, 123.82, 124.32, 130.75, 130.91, 131.12, 131.29, 135.20, 135.36, 145.73, 151.04, 158.16, 160.09, 162.02, 163.04, 164.93
8	72	163-164	296	7.12-7.19 (1H, *m*, H-4''), 7.42-7.61 (6H, *m*, H-6, H-3', H-4', H-5', H-3'', H-5''), 7.79-7.90 (2H, *m*, H-7, H-8), 7.97-8.01 (2H, *m*, H-2'', H-6''), 8.46-8.49 (2H, *m*, H-2', H-6'), 8.58 (1H, *dd*, *J* = 1.2, 8.0 Hz, H-5), 9.88 (1H, *s*, NH)	114.03, 122.23, 123.02, 123.62, 125.86, 127.91, 128.12, 128.37, 128.47, 130.23, 133.13, 138.36, 139.32, 150.51, 157.92, 159.04
9	79	152-153	314	7.09-7.29 (2H, *m*, H-5', H-4''), 7.37-7.58 (4H, *m*, H-6, H-4', H-3'', H-5''), 7.74-7.81 (2H, *m*, H-7, H-8), 7.86-7.90 (2H, *m*, H-2'', H-6''), 8.05 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.21 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.50 (1H, *dd*, *J* = 1.2, 8.0 Hz, H-5),	114.01, 114.17, 114.47, 116.81, 117.23, 122.53, 123.08, 123.91, 126.26, 128.20, 128.52, 130.33, 130.49, 133.31, 139.16, 140.99, 141.14, 150.35, 157.85, 158.03, 160.03, 164.85

				9.87 (1H, *s*, NH)	
10	54	177-178	314	7.11-7.18 (1H, *m*, H-4"), 7.41-7.48 (5H, *m*, H-6, H-3', H-5', H-3", H-5"), 7.62-7.71 (1H, *m*, H-7), 7.83-7.98 (3H, *m*, H-8, H-2", H-6"), 8.37-8.42 (3H, *m*, H-5, H-2', H-6'), 9.73 (1H, *s*, NH)	107.25, 107.72, 114.34, 114.52, 122.11, 122.60, 123.67, 127.86, 128.34, 128.48, 130.19, 130.69, 130.85, 138.17, 139.18, 147.60, 156.91, 157.54, 157.62, 158.71, 161.77
11	45	169-170	332	7.13-7.33 (2H, *m*, H-4', H-4"), 7.41-7.56 (3H, *m*, H-5', H-3", H-5"), 7.68-7.78 (1H, *m*, H-7), 7.85-7.91 (3H, *m*, H-8, H-2", H-6"), 8.03 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.20 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.39 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.80 (1H, *s*, NH)	107.74, 108.21, 114.30, 114.75, 114.91, 115.10, 117.21, 117.64, 122.78, 123.27, 124.15, 124.20, 124.41, 128.94, 130.73, 130.89, 131.22, 131.40, 134.83, 139.35, 141.11, 141.27, 147.81, 147.83, 157.53, 157.81, 157.85, 157.92, 158.05, 158.13, 160.40, 162.40, 165.22
12	18	>270	363	3.79 (3H, *s*, OCH_3), 7.02 (2H, *d*, *J* = 9.0 Hz, H-3", H-5"), 7.27-7.33 (1H, *m*, H-4"), 7.45-7.56	55.63, 107.60, 108.08, 114.04, 114.26, 114.72, 114.81, 114.99, 117.05, 117.47,

				(1H, *m*, H-5'), 7.65-7.71 (1H, *m*, H-7), 7.73 -7.79 (1H, *m*, H-2", H-6"), 7.86-7.90 (1H, *m*, H-8), 8.02 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.18 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.34 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.72 (1H, *s*, NH)	122.45, 122.94, 124.14, 124.42, 130.58, 130.73, 131.06, 131.23, 132.21, 141.21, 141.36, 147.65, 156.28, 157.43, 157.96, 158.04, 160.34, 162.28, 165.16
13	12	144-145	362	3.82 (3H, *s*, OCH_3), 6.76 (1H, *dd*, *J* = 2.4, 8.0 Hz, H-4"), 7.28-7.39 (2H, *m*, H-4',H-5"), 7.47-7.60 (2H, *m*, H-2", H-5'), 7.70-7.82 (2H, *m*, H-7, H-6"), 7.89-7.97 (1H, *m*, H-8), 8.11 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.25 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.42 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.80 (1H, *s*, NH)	55.33, 70.96, 107.53, 107.70, 108.00, 110.12, 114.12, 114.54, 114.75, 114.93, 117.18, 117.60, 122.78, 123.27, 123.97, 129.56, 130.68, 130.83, 131.10, 131.27, 140.39, 140.85, 141.01, 147.58, 157.40, 157.79, 157.87, 159.63, 160.22, 162.27, 165.04
14	17	166-167	332	3.80 (3H, *s*, OCH_3), 6.99-7.32 (4H, *m*, H-4', H-3", H-4", H-5"), 7.39-7.50 (1H, *m*, H-5'), 7.65-7.76 (2H, *m*, H-7, H-6"), 7.84-7.96 (2H, *m*,	55.99, 107.59, 108.06, 112.11, 114.24, 114.69, 114.75, 114.92, 117.03, 117.46, 120.51, 122.63, 123.13, 124.03, 124.07,

				H-8, H-2'), 8.10 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.34 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.43 (1H, *s*, NH)	126.46, 127.00, 127.08, 127.25, 130.53, 130.69, 131.13, 131.30, 141.19, 141.35, 147.73, 153.55, 157.46, 157.90, 157.95, 158.01, 158.74, 158.82, 162.33, 165.14
15	22	232-233	351	7.30-7.50 (3H, *m*, H-4', H-3", H-5"), 7.56-7.67 (1H, *m*, H-5'), 7.81-7.97 (3H, *m*, H-7, H-2", H-6"), 8.08-8.28 (3H, *m*, H-8, H-2', H-6'), 8.71 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 11.08 (1H, *s*, NH)	109.27, 109.76, 114.58, 114.77, 115.36, 115.64, 115.83, 116.09, 119.33, 119.75, 124.39, 124.89, 125.26, 126.20, 126.36, 126.65, 131.37, 131.53, 134.19, 136.74, 141.56, 156.77, 157.61, 158.06, 158.83, 160.17, 162.45, 162.96, 165.02
16	16	107-108	351	6.89-6.96 (1H, *m*, H-4"), 7.20-7.27 (1H, *m*, H-4'), 7.33-7.49 (2H, *m*, H-5', H-5"), 7.61-7.64 (2H, *m*, H-2", H-6"), 7.73-7.94 (3H, *m*, H-7, H-8, H-2'), 8.08 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.22 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.70 (1H, s, NH)	107.54, 108.02, 109.25, 110.15, 110.57, 114.23, 114.92, 117.15, 117.57, 117.81, 122.72, 123.22, 124.01, 130.16, 130.35, 130.56, 130.72, 131.17, 131.34, 140.87, 141.03, 141.13, 141.36, 147.67, 157.62,

					160.01, 162.35, 162.37, 164.81, 165.17
17	14	200-201	351	7.22-7.69 (5H, *m*, H-4', H-5', H-3", H-4", H-5"), 7.74-7.98 (4H, *m*, H-2', H-7, H-8, H-6"), 8.08 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.35 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.98 (1H, *s*, NH)	107.37, 107.83, 113.74, 114.19, 115.77, 116.16, 116.84, 117.25, 122.68, 123.18, 123.63, 124.41, 125.91, 126.15, 127.58, 127.99, 130.47, 130.86, 131.02, 136.39, 137.62, 140.53, 147.43, 152.49, 154.26, 158.47, 159.17, 161.27, 161.96, 164.70
18	25	>300	367.5	7.21-7.28 (1H, *m*, H-4'), 7.40-7.51 (3H, *m*, H-5', H-3", H-5"), 7.62-7.97 (5H, *m*, H-7, H-8, H-2', H-2", H-6"), 8.11 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.26 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.75 (1H, *s*, NH)	107.22, 107.70, 113.80, 114.27, 114.38, 114.56, 115.28, 116.78, 117.20, 117.62, 122.39, 122.88, 123.62, 127.41, 128.31, 130.26, 130.42, 130.77, 130.94, 137.90, 140.47, 140.62, 147.30, 157.07, 157.33, 157.41, 159.92, 161.94, 164.74
19	21	223-224	367.5	7.12-7.28 (2H, *m*, H-4', H-4"), 7.34-7.49 (2H, *m*, H-5', H-5"), 7.58-7.96 (4H, *m*, H-7, H-8,	170.10, 107.58, 113.84, 114.30, 114.48, 116.72, 117.14, 119.90, 121.37, 122.29,

				H-2", H-6"), 8.08-8.14 (2H, *m*, H-2', H-6'), 8.22 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.68 (1H, *s*, NH)	122.78, 123.05, 123.60, 129.88, 130.08, 130.23, 130.75, 130.91, 132.76, 140.42, 147.53, 147.26, 157.12, 157.21, 159.93, 161.92, 164.76
20	20	185-186	367.5	7.19-7.28 (1H, *m*, H-4'), 7.35-7.51 (3H, *m*, H-3", H-4", H-5"), 7.67-7.71 (2H, *m*, H-5', H-6"), 7.75-7.96 (3H, *m*, H-2', H-7, H-8), 8.03 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.35 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.96 (1H, *s*, NH)	107.71, 108.18, 114.22, 114.51, 114.68, 117.19, 117.61, 123.05, 123.55, 124.06, 128.01, 128.33, 129.81, 130.23, 130.64, 130.79, 131.11, 131.30, 131.48, 136.06, 140.99, 141.14, 147.90, 157.52, 157.86, 159.03, 159.11, 160.30, 162.39, 165.12
21	22	231	412	7.22-7.31 (1H, *m*, H-4'), 7.41-7.70 (4H, *m*, H-7, H-5', H-3", H-5"), 7.79-7.83 (3H, *m*, H-8, H-2",H-6"), 7.92 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.09 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.25 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.78 (1H, *s*, NH)	107.30, 107.78, 113.88, 114.36, 114.57, 115.58, 116.90, 117.32, 122.46, 122.96, 123.81, 124.01, 130.31, 130.47, 130.60, 130.77, 131.25, 138.32, 140.29, 140.43, 147.06, 157.28, 159.94, 161.99, 164.76

22	13	224-225	412	7.23-7.51 (4H, *m*, H-4', H-5', H-4", H-5"), 7.61-.70 (2H, *m*, H-2", H-6"), 7.76-7.81 (2H, *m*, H-7, H-8), 7.96 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.13 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.25 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.74 (1H, *s*, NH)	107.21, 107.69, 113.90, 114.37, 114.56, 116.88, 117.30, 120.38, 121.24, 122.47, 122.96, 123.68, 124.35, 126.04, 130.34, 130.86, 131.03, 140.45, 140.69, 147.35, 157.20, 160.01, 162.01, 164.83
23	23	199-200	412	7.15-7.52 (4H, *m*, H-4', H-5', H-4", H-5"), 7.61-7.65 (1H, *m*, H-3"), 7.75-7.92 (4H, *m*, H-7, H-8, H-2', H-6"), 7.98 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.24 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.92 (1H, *s*, NH)	107.38, 107.86, 108.14, 110.21, 110.41, 113.99, 114.45, 114.55, 114.73, 116.73, 117.15, 122.21, 122.70, 123.86, 128.74, 130.30, 130.46, 130.73, 130.90, 139.43, 140.80, 140.95, 147.37, 148.98, 157.05, 157.57, 157.71, 157.79, 159.97, 161.91, 164.79
24	15	272	349	6.85 (2H, *d*, J = 12 Hz, H-3", H-5"), 7.24-7.34 (1H, *m*, H-4'), 7.46-7.53 (1H, *m*, H-5'), 7.58-7.76 (3H, *m*, H-7, H-2", H-6"), 7.83-7.90	107.39, 107.86, 114.09, 114.61, 114.79, 115.25, 116.98, 117.40, 122.39, 122.89, 123.95, 124.83, 130.34, 130.55, 130.70,

				(1H, *m*, H-8), 8.02 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.19 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.34 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.43 (1H, *s*, OH), 9.71 (1H, *s*, NH)	130.89, 131.06, 141.01, 141.16, 147.45, 154.38, 157.26, 157.82, 157.99, 158.06, 160.17, 162.12, 164.98
25	17	251-252	349	6.76 (1H, *dd*, *J* = 1.2, 7.8 Hz, H-4''), 7.19-7.42 (4H, *m*, H-4', H-5', H-2'', H-5''), 7.49-7.60 (1H, *m*, H-6''), 7.70-7.80 (1H, *m*, H-7), 7.88-7.95 (1H, *m*, H-8), 8.10 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.26 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.43 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.59 (1H, *s*, OH), 9.75 (1H, *s*, NH)	107.44, 107.91, 109.39, 111.14, 113.04, 114.00, 114.46, 114.58, 114.76, 116.88, 117.30, 122.43, 122.92, 123.88, 129.17, 130.39, 130.55, 130.84, 131.01, 139.96, 140.71, 140.87, 147.41, 157.13, 157.56, 157.72, 160.00, 161.99, 164.82
26	20	264	349	6.96-7.30 (4H, *m*, H-4', H-3'', H-4'', H-5''), 7.42-7.59 (2H, *m*, H-5', H-6''), 7.70-7.80 (1H, *m*, H-7), 7.87-7.96 (2H, *m*, H-8, H-2'), 8.11 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.36 (1H, *dd*, *J* = 2.5,	107.55, 108.03, 113.90, 114.36, 114.55, 114.73, 116.32, 116.78, 117.20, 118.90, 122.35, 122.84, 123.73, 125.54, 126.89, 127.31, 130.30, 130.46, 130.73, 130.90,

				9.8 Hz, H-5), 9.52 (2H, *s*, OH, NH)	140.81, 140.96, 147.33, 151.74, 157.03, 157.59, 158.69, 159.94, 161.89, 164.75
27	20	235-236	348	5.07 (2H, *s*, NH_2), 6.67 (2H, *d*, *J* = 8.2 Hz, H-3", H-5"), 7.26-7.30 (1H, *m*, H-4"), 7.42-.7.54 (3H, *m*, H-5', H-2", H-6"), 7.63-7.86 (2H, *m*, H-7, H-8), 8.02 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.17 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.30 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.59 (1H, *s*, NH)	107.29, 107.77, 113.77, 113.97, 114.43, 114.59, 114.77, 116.78, 117.20, 122.06, 122.55, 123.80, 124.50, 127.50, 130.35, 130.51, 130.71, 130.88, 141.06, 141.21, 145.83, 147.32, 157.08, 157.80, 157.88, 160.05, 161.94, 164.87
28	22	183-184	348	5.18 (2H, *s*, NH_2), 6.41-7.44 (1H, *m*, H-2"), 7.09-7.11 (3H, *m*, H-4", H-5", H-6"), 7.26-7.30 (1H, *m*, H-4'), 7.49-7.58 (1H, *m*, H-5'), 7.68-7.78 (1H, *m*, H-7), 7.85-7.93 (1H, *m*, H-8), 8.10 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.25 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.42 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.60 (1H, *s*, NH)	107.37, 107.84, 114.04, 114.22, 114.40, 114.49, 116.98, 117.39, 121.81, 122.82, 123.31, 123.86, 126.89, 130.42, 130.57, 131.04, 131.21, 133.16, 137.30, 140.72, 140.87, 147.62, 151.98, 157.30, 157.68, 158.78, 158.86, 160.06

29	33	213-215	348	5.05 (2H, *s*, NH_2), 6.65-6.71 (1H, *m*, H-3"), 6.87-7.28 (4H, *m*, H-4', H-4", H-5", H-6"), 7.40-4.51 (1H, *m*, H-5'), 7.68-7.77 (1H, *m*, H-7), 7.85-7.96 (2H, *m*, H-8, H-2'), 8.11 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.34 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.48 (1H, *s*, NH)	107.84, 108.30, 113.96, 114.41, 114.67, 114.84, 115.86, 115.97, 116.67, 117.09, 122.08, 122.57, 123.14, 123.75, 127.19, 127.84, 130.18, 130.34, 130.57, 130.74, 140.95, 141.10, 144.46, 147.33, 156.90, 157.70, 158.73, 158.80, 159.92, 161.75
30	25	197-198	347	2.31 (3H, *s*, CH_3), 7.19-7.29 (2H, *m*, H-3", H-5"), 7.41-7.85 (6H, *m*, H-7, H-8, H-4', H-5', H-2", H-6"), 8.01 (1H, *d*, *J* = 10.6 Hz, H-2'), 8.16 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.31 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.67 (1H, *s*, NH)	107.24, 107.71, 113.85, 114.31, 114.45, 114.63, 116.64, 117.07, 122.10, 122.25, 122.58, 123.70, 128.86, 130.15, 130.30, 130.68, 130.85, 132.90, 136.35, 140.76, 140.91, 147.29, 157.03, 157.46, 157.54, 159.94, 161.89, 164.76
31	22	173	347	2.49 (3H, *s*, CH_3), 7.07-7.10 (1H, *m*, H-2"), 7.38-7.61 (3H, *m*, H-4', H-4", H-5"), 7.76-7.90 (4H, *m*, H-7, H-8, H-5', H-6"), 8.13 (1H, *d*, *J* =	107.26, 107.73, 113.95, 114.48, 114.67, 116.70, 117.13, 119.26, 122.15, 122.65, 122.83, 123.70, 124.47, 128.28, 130.18,

				10.6 Hz, H-2'), 8.29 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.43 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.77 (1H, *s*, NH)	130.33, 130.75, 130.91, 137.59, 138.96, 140.77, 140.91, 147.36, 157.09, 157.43, 160.01, 161.96, 164.83
32	29	194-195	347	2.24 (3H, *s*, CH_3), 7.21-7.48 (6H, *m*, H-4', H-5', H-3", H-4", H-5", H-6"), 7.67-7.75 (1H, *m*, H-7), 7.85-7.89 (2H, *m*, H-8, H-2'), 8.03 (1H, *d*, *J* = 7.6 Hz, H-6'), 8.34 (1H, *dd*, *J* = 2.5, 9.8 Hz, H-5), 9.71 (1H, *s*, NH)	107.31, 107.78, 113.70, 114.17, 114.36, 116.61, 117.04, 122.24, 122.72, 123.54, 126.10, 126.39, 127.25, 130.11, 130.26, 130.45, 130.73, 130.90, 134.55, 136.79, 140.73, 140.88, 147.36, 157.01, 157.50, 158.60, 159.86, 161.87, 164.68

Table 2. ***In vitro*** **cytotoxicity of 6-substituted-2-(3-substituted phenyl)-4-substituted anilinoquinazolines (8-32)**

Compounds	EC_{50}[a](μM)						
	HL-60[b]	U937[b]	HT-29[b]	Colo 205[b]	U-2 OS[b]	MCF-7[b]	MDA-MB-231[b]
8	>50	>50	>50	>50	>50	>50	>50
9	>50	>50	>50	>50	>50	>50	>50
10	>50	>50	>50	>50	>50	>50	>50
11	48.26 ± 2.01	45.45 ± 1.06	48.32 ± 2.07	49.66 ± 3.04	47.62 ± 1.09	49.66 ± 3.47	47.82 ± 3.25
12	38.62 ± 4.89	41.09 ± 3.57	42.33 ± 3.29	46.82 ± 3.47	32.32 ± 3.98	48.36 ± 4.17	47.26 ± 4.18
13 (LJJ-10)	35.49 ± 3.69	39.62 ± 3.21	40.35 ± 4.58	44.68 ± 4.11	30.26 ± 3.21	46.33 ± 4.12	47.05 ± 6.32
14	>50	>50	>50	>50	>50	>50	>50
15	>50	>50	>50	>50	>50	>50	>50
16	43.11 ± 1.29	42.26 ± 3.26	>50	>50	49.62 ± 3.29	48.68 ± 4.47	47.63 ± 2.58
17	>50	>50	>50	>50	>50	>50	>50
18	45.69 ± 2.96	32.65 ± 6.24	40.32 ± 3.96	32.64 ± 4.17	38.36 ± 3.08	>50	>50

19	>50	>50	>50	>50	>50	>50	>50
20	>50	>50	>50	>50	>50	>50	>50
21	>50	>50	>50	>50	>50	>50	>50
22	>50	>50	>50	>50	>50	>50	>50
23	>50	>50	>50	>50	>50	>50	>50
24	>50	>50	>50	>50	>50	>50	>50
25	>50	>50	>50	>50	33.32 ± 2.36	>50	>50
26	44.36 ± 2.19	46.25 ± 3.26	>50	>50	41.36 ± 3.69	>50	>50
27	>50	>50	>50	>50	44.15 ± 6.98	>50	>50
28	>50	>50	>50	>50	49.65 ± 4.16	>50	>50
29	41.28 ±7.45	47.25 ± 5.01	>50	>50	37.85 ± 6.96	>50	>50
30	>50	>50	>50	>50	>50	>50	>50
31	>50	>50	>50	>50	>50	>50	>50
32	>50	>50	>50	>50	>50	>50	>50

[a] IC_{50} was the half maximal inhibitory concentration after 48 h of treatment. The unit is expressed as μM. [b] HL-60 (human leukemia cells), U937 (human leukemic monocyte lymphoma cells), HT-29 (human colon adenocarcinoma cells), Colo 205 (human colon cancer cells), U-2 OS (human osteosarcoma cells), MCF-7 (human breast adenocarcinoma cells) and MDA-MB-231 (human breast cancer cells).

Research Highlights

>We designed the 6-fluoro-(3-fluorophenyl)-4-(3-methoxyanilino)quinazoline (compound **13**, LJJ-10) and fount that it has greater cytotoxicity than that of the other compounds in human osteogenic sarcoma U-2 OS cells. >In this study, we investigated anticancer activity and apoptotic mechanisms of the LJJ-10 in U-2 OS cells *in vitro*. >Based on these observations, we suggest that LJJ-10 induced cell death in U-2 OS cells through death receptor and mitochondria-dependent apoptotic signaling pathways.