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Synthesis, Crystal Structure and Anticancer Activities of Tetrahydropyrido[4,3-*d*]dihydropyrimidine-2-thiones

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A new series of tetrahydropyrido[4,3-*d*]dihydropyrimidine-2-thiones (**3a—3x**) were designed and synthesized. Their structures were confirmed by ^{1}H NMR, IR, MS and elemental analysis, and the conformation of compound **3j** was confirmed by X-ray diffraction. Preliminary bioassays indicated that most of the target compounds presented good antiproliferative activities against leukemic K562 cells, ovarian cancer HO-8910 cells and liver cancer SMMC-7721 cells in vitro. Among them the compounds **3i** and **3m** afford the best activity, the IC_{50} of them were 3.22 and 3.65 μg/mL against leukemic K562 cells, respectively, which were lower than the anticancer drug of clinical practice 5-FU (IC_{50}=8.56 μg/mL). Preliminary mechanism of action studies revealed that compound **3i** caused DNA fragmentation and activated caspase-3/7 in leukemic K562 cells.

Keywords tetrahydropyrido[4,3-*d*]dihydropyrimidine, thione, synthesis, crystal structure, anticancer activities

Introduction

Cancer, the uncontrolled, rapid and pathological proliferation of abnormal cells, is the second leading cause of human death after cardiovascular diseases in developing as well as advanced countries.[1] Among them, coronic myelogenous leukemia (CML), ovarian cancer and liver cancer are main diseases to death, which are caused by clonal expansion of pluripotent stem cells retaining their differentiation potential.[2] At present, most of cancer are lack of special treatment methods, and cytotoxic drugs are still used in combination chemotherapy to treat these malignant diseases. Chemotherapy can only through a lot of damage to the proliferation of cancer cells and high patients get short-term relief, but can not cure this malignant disease.[3] And because the chemotherapy drugs currently used lack specificity, toxic effect is very large. The most difficulty is that most patients with recurrent use of the chemotherapy drug appear resistance to it.[4,5] Hence, the discovery of fast-acting new drugs to effectively cure cancer is imperative.

Pyrimidinone sub-structure is prevalent in many natural/synthetic biologically active compounds, which were found applications in pharmaceutical and biochemical areas.[6] Because dihydropyrimidinethiones (DHPMs)[7] can be converted into either guanidines or 1,3-diamines, which constitute the core structural elements commonly present in natural products, they were found applications as antihypertensive (SQ32926),[8] ala-adrenergic receptor antagonists,[9] antibacterial and antitumour agents.[10] On the other hand, pyridopyrimidine skeleton exists in many natural or synthetic biologically active materials, and its derivatives are applied in various pharmaceutical and biochemical fields.[11,12] Structure-activity studies disclose that anticancer activity of pyrimidinones and pyrimidinethiones is ascribable to the presence of (i) nitrogen heterocyclic ring and (ii) thione functionality, which generally enhance the activity.[13,14] This may provide some useful information for future design of new pyrimidinethiones.

Pursuing our searches on design, synthesis, and biological validation of small molecule modulators of pharmacologically targets, in 2011 we had reported the synthesis and antiproliferative activities against leukemic K562 cells of *N*-(substitutedbenzyl)-3,5-bis(benzylidene)-4-piperidone (**2**).[15] The data indicated that most of the compounds (**2**) have good antiproliferative activities against leukemic K562 cells, and some of them showed excllent antiproliferative activities, the IC_{50} values were lower than that of the anticancer drug of clinical practice 5-FU. While compared with the 5-FU, these results still could not do better, as well as their narrow anticancer spectrum. So many efforts should be made to develop **2** with the goal of increasing antiproliferative activities against cancer cells and ex-

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panding the anticancer spectra.

On the basis of above observations and obeying the principle of active-factor-addition,[16] in order to find some potent antiproliferative activity lead compounds, we have focused our attentions on derivatization of *N*-(substituted benzyl)-3,5-bis(benzylidene)-4-piperidones to get tetrahydropyrido[4,3-*d*]dihydropyrimidine-2-thiones (**3a—3x**), which contain not only DHPMs but also pyridopyrimidine skeleton.

Multi-component reactions (MCRs) constitute a highly valuable synthetic tool for the construction of polyfunctionalized heterocyclic compounds required for drug discovery programmes.[17] In the present investigation, a series of new tetrahydropyrido[4,3-*d*]dihydropyrimidine-2-thiones (**3a—3x**) were synthesized by a multicomponent reaction of *N*-(4-substituted benzyl)-4-piperidones (**1**), substituted aromatic aldehydes, thiourea and solid sodium hydroxide in ethanol. The structures of the title compounds were confirmed by ^{1}H NMR, IR, MS and elemental analysis, and the conformation of the compound **3j** was confirmed by X-ray diffraction. Preliminary bioassays indicated that most of the target compounds have good antiproliferative activities against leukemic K562 cells, ovarian cancer HO-8910 cells and liver cancer SMMC-7721 cells *in vitro*. The IC_{50} (μg/mL) of **3i** and **3m** against leukemic K562 cells were 3.22 and 3.65 respectively, which were lower than the standard 5-fluorouracil (IC_{50} = 8.56 μg/mL). Preliminary mechanism studies revealed that compound **3i** caused DNA fragmentation and activated caspase-3/7 in leukemic K562 cells.

Experimental

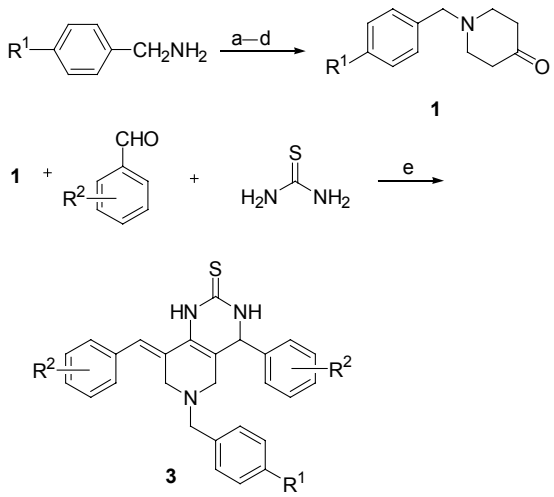
Materials and apparatus

All the chemical reagents purchased were of analytical grade and used without further purification, except for the toluene, which was dried by refluxing in the presence of sodium and distilled prior to use. RPMI 1640 was obtained from Gibco BRL (Grand Island, NY, USA) and bovine calf serum was supplied by Beijing Dingguo Biotechnology Co, China. Thin-layer chromatography (TLC) was carried out on silica gel 60 F254 plates (Merck KGaA). ^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz) spectrometer, using DMSO as solvents and tetramethylsilane (TMS) as internal standard. Melting points were determined by an RK1 micro scopic melting apparatus and uncorrected. Elemental analysis was performed with a Perkin-Elmer 2400 instrument. IR spectra were obtained on a Nicolet 5DX FT-IR spectrophotometer in the region 4000—400 cm^{-1} using KBr discs. MS spectra were recorded on a Trace DSQ mass spectrograph. X-ray diffraction data were recorded on a Bruker Smart CCD diffractometer.

Chemistry

Synthesis procedures for the title compounds are summarized in Scheme 1, *N*-(4-substituted benzyl)-4-piperidones (**1**) were prepared based on the procedures in the literatures.[15] In a microwave reactor, the title compounds (**3a — 3x**) were synthesized by a multicomponent reaction of *N*-(4-substituted benzyl)-4-piperidones (**1**), aromatic aldehydes, thiourea and solid sodium hydroxide in ethanol at 65 ℃ for 15—25 min. Compared with the conventional synthetic methods, controlled microwave heating had been shown to dramatically reduce reaction times, increase product yields, and enhance product purities by reducing side reactions.[18]

Scheme 1 Synthesis of the title compounds (**3a—3x**)



R^1 = Me, F, Br

R^2 = 2-F, 4-OMe, 4-CH(CH_3)$_2$, 4-Me, 4-F, 2-Cl, H, 4-Cl

Reagents and conditions: (a) methyl acrylate, absolute methanol, refluxing; (b) sodium/absolute methanol, toluene, refluxing; (c) 25% HCl, refluxing; (d) 35% sodium hydroxide; (e) solid sodium hydroxide, absolute ethanol, 65 ℃, microwave reaction, 15—25 min.

Possible mechanism of the multicomponent reaction for the synthesis of compounds **3** is described in Scheme 2. The intermediates **2** were formed by the aldol condensation of aromatic aldehyde with *N*-(4-substituted benzyl)-4-piperidone (**1**) , then an aza-Michael addition of thiourea to intermediate **2** leads to the formation of Michael adduct. Finally the tetrahydropyrido-[4,3-*d*]-dihydropyrimidine-2-thiones ring can be closed *via* nucleophilic attack of an NH_2 group on a carbonyl carbon, and the desired product **3** were obtained by eliminating a hydroxyl group. It is an efficient and promising method to construct the tetrahydropyrido-[4,3-*d*]dihydropyrimidine-2-thione skeleton.

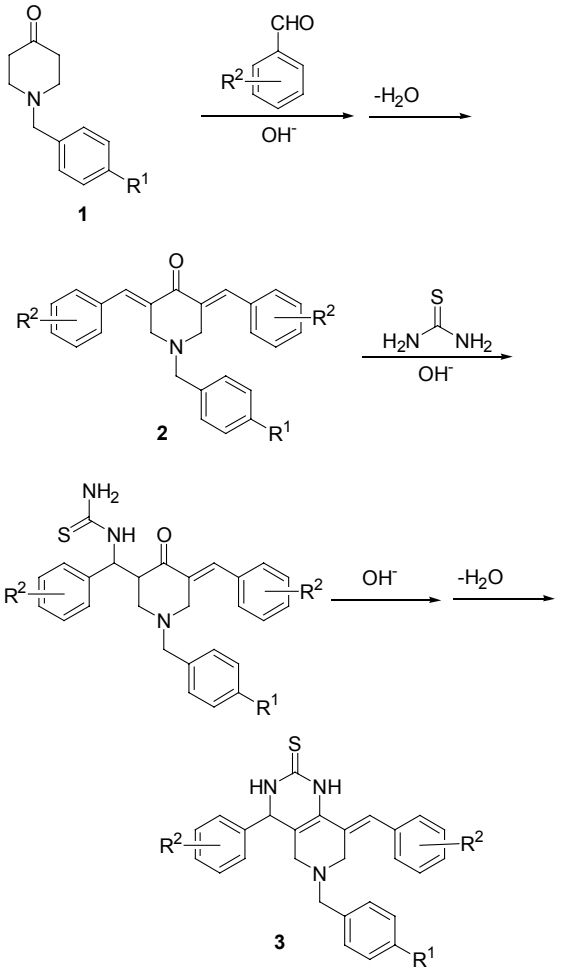
General synthetic procedures for the target compounds (3a—3x)

N-(4-Substituted benzyl)-4-piperidones (**1**) were synthesized according to the literature.[15]

In a microwave reactor, a mixture of *N*-(4-substituted benzyl))-4-piperidones **1a—1x** (1 mmol), substituted aromatic aldehydes (2 mmol), thiourea (1 mmol),

CHINESE JOURNAL OF CHEMISTRY

Scheme 2 Proposed reaction mechanism for the synthesis of the compounds **3**



and NaOH (2 mmol) in ethanol (50 mL), was heated to 65 ℃ for 5 min, and stirred at the same temperature for 10—20 min. After cooling to room temperature, 20 mL of water was added and the mixture was neutralized to pH 6 using 10% HCl solution. The separated solid was filtered, washed with water, dried, and recrystallized (ethanol) to afford the target compounds.

8-(2-Fluorobenzylidene)-4-(2-fluorophenyl)-6-(4-methylbezyl)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3a) Yield: 92%; white solid, m.p.: 132—134 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.75 (s, 1 H), 9.07 (s, 1H), 6.91—7.40 (m, 13H), 5.13 (s, 1H), 3.31—3.42 (m, 4H), 3.11 (d, J=18.0 Hz, 1H), 2.73 (d, J=16.6 Hz, 1H), 2.21 (s, 3H); IR (KBr) v: 3424, 1567, 1485, 1454, 1099, 988, 857 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 474.17. Anal. calcd for $C_{28}H_{25}F_2N_3S$: C 71.17, H 5.32, N 8.87; found C 71.20, H 5.30, N 8.88.

4-(4-Methoxylphenyl)-6-(4-methylbenzyl)-8-(4-methoxylbezylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3b) Yield: 78%; white solid, m.p.: 110—112 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.40 (s, 1H), 9.04 (s, 1H), 7.17—6.85 (m, 13H), 4.74 (s, 1H), 3.74 (s, 6H), 3.28—3.55 (m, 4H), 3.03 (d, J=16.45 Hz, 1H), 2.69 (d, J=16.3 Hz, 1H), 2.22 (s, 3H) ; IR (KBr) v: 3429, 1605, 1506, 1440, 1077, 988 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 498.21. Anal. calcd for $C_{30}H_{31}N_3O_2S$: C 72.40, H 6.28, N 8.44; found C 72.41, H 6.27, N 8.46.

8-(4-Isopropylbenzylidene)-4-(4-isopropylphenyl)-6-(4-methylbenzyl)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3c) Yield: 83%; yellow solid, m.p.: 179—181 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.48 (s, 1H), 9.07 (s, 1H), 7.25—7.07 (m, 13H), 4.77 (s, 1H), 3.59—3.37 (m, 4H), 3.08 (d, J=16.6 Hz, 1H), 2.87 (tt, J=13.5, 6.8 Hz, 2H), 2.75 (d, J=16.4 Hz, 1H), 1.19—1.17 (m, 12H); IR (KBr) v: 3434, 2390, 2340, 1718, 1637, 1421, 1363, 1120, 861 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 521.29. Anal. calcd for $C_{34}H_{39}N_3S$: C 78.27, H 7.53, N 8.05; found C 78.25, H 7.52, N 8.06.

4-(4-Methylphenyl)-6-(4-methylbenzyl)-8-(4-methylbenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3d) Yield: 87%; yellow solid, m.p.: 178—181 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.48 (s, 1H), 9.04 (s, 1H), 7.17—6.85 (m, 13H), 4.77 (s, 1H), 3.59—3.28 (m, 4H), 3.03 (d, J=16.45 Hz, 1H), 2.70 (d, J=16.4 Hz, 1H), 2.21 (s, 3H), 1.94 (s, 6H); IR (KBr) v: 3764, 2396, 1637, 1577, 1428, 1363, 1064, 861 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 465.65. Anal. calcd for $C_{30}H_{31}N_3S$ C: 77.38, H 6.71, N 9.02; found C 77.37, H 6.72, N 9.02.

4-(4-Fluorophenyl)-6-(4-methylbenzyl)-8-(4-fluorobenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3e) Yield: 78%; yellow solid, m.p.: 175—177 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.52 (s,1H) , 9.14 (s, 1H), 7.29—6.97 (m, 13H), 4.87 (s, 1H), 3.92—3.52 (m, 4H), 3.07 (d, J=16.7 Hz, 1H), 2.70 (d, J=16.5 Hz, 1H), 2.21 (s, 3H); IR (KBr) v: 3422, 2851, 2026, 1633, 1600, 1564, 1506, 1155, 1077, 854 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 473.71. Anal. calcd for $C_{28}H_{28}F_2N_3S$: C 71.01, H 5.32, N 8.87; found C 71.00, H 5.30, N 8.86.

8-(2-Chlorobenzylidene)-4-(2-chlorophenyl)-6-(4-methylbenzyl)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3f) Yield: 86%; white solid, m.p.: 102—104 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.59 (s, 1H), 9.20 (s, 1H), 7.47—6.96 (m, 13H), 4.92 (s, 1H), 3.53—3.34 (m, 4H), 3.12 (d, J=16.7 Hz, 1H), 2.75 (d, J=16.6 Hz, 1H), 2.22 (s, 3H); IR (KBr) v: 3422, 2342, 1560, 1506, 1451, 1075, 990, 856 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 507.11. Anal. calcd for $C_{28}H_{25}C_{l2}N_3S$: C 66.40, H 4.98, N 8.30; found C 66.41, H 4.98, N 8.31.

8-Benzylidene-4-phenyl-6-(4-methylbenzyl)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3g) Yield: 91%; white solid, m.p.: 174—176 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.53 (s, 1H), 9.08 (s, 1H), 7.53—6.96 (m, 15H), 4.85 (s, 1H), 3.61—3.36 (m, 4H), 3.12 (d, J=16.7 Hz, 1H), 2.74 (d, J=16.5 Hz, 1H), 2.22 (s, 3H); IR (KBr) v: 3425, 2347, 1561, 1546, 1456, 1175, 990, 856 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 437.20. Anal. calcd for $C_{28}H_{27}N_3S$: C 76.85, H 6.22, N 9.60; found C 76.85, H 6.20, N 9.61.

8-(4-Chlorobenzylidene)-4-(4-chlorophenyl)-6-(4-methylbenzyl)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3h) Yield: 76%; white solid, m.p.: 108—109 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.59 (s, 1H), 9.20 (s, 1H), 7.46—6.65 (m, 13H), 4.92 (s, 1H), 3.60—3.34 (m, 4H), 3.12 (d, J=16.7 Hz, 1H), 2.75 (d, J=16.7 Hz, 1H), 2.20 (s, 3H); IR (KBr) v: 3426, 1605, 1506, 1440, 1077, 988 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 486.17. Anal. calcd for $C_{28}H_{25}Cl_2N_3S$: C 66.40, H 6.98, N 8.30; found C 66.43, H 6.97, N 8.28.

4-(2-Fluorophenyl)-6-(4-fluorobenzyl)-8-(2-fluorobenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3i) Yield: 94%; white solid, m.p.: 106—108 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.53 (s, 1H), 9.14 (s, 1H), 7.40—6.90 (m, 13H), 5.14 (m, 4H), 3.13 (d, J=17.0 Hz, 1H), 2.74 (d, J=16.7 Hz, 1H); IR (KBr) v: 3426, 1567, 1465, 1453, 1077, 858; ESI-MS ([M + H]$^+$) m/z: 478.15. Anal. calcd for $C_{27}H_{22}F_3N_3S$: C 67.91, H 4.64, N 8.80; found C 67.90, H 4.65, N 8.81.

6-(4-Fluorobenzyl)-4-(4-methoxylphenyl)-8-(4-methoxylbenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3j) Yield: 75%; yellow solid, m.p.: 117—119 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.42 (s, 1H), 9.05 (s, 1H), 7.17—6.95 (m, 13H), 4.77 (s, 1H), 3.74 (s, 6H), 3.61—3.34 (m, 4H), 3.03 (d, J=15.7 Hz, 1H), 2.69 (d, J=17.6 Hz, 1H); IR (KBr) v: 3414, 1605, 1568, 1509, 1441, 1174, 1078, 858 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 501.19. Anal. calcd for $C_{29}H_{28}FN_3O_2S$: C 69.44, H 5.63, N 8.38; found C 59.45, H 5.61, N 8.40.

6-(4-Fluorobenzyl)-4-(4-isopropylphenyl)-8-(4-isopropylbenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3k) Yield: 85%; white solid, m.p.: 117—119 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.48 (s, 1H), 9.06 (s, 1H), 7.34—6.92 (m, 13H), 4.78 (s, 1H), 3.59—3.31 (m, 4H), 3.07 (d, J=16.7 Hz, 1H), 2.87 (d, J=12.1 Hz, 2H), 2.74 (d, J=16.4 Hz, 1H), 1.19—1.16 (m, 12H); IR (KBr) v: 3414, 1604, 1568, 1509, 1441, 1174, 988, 858 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 526.26. Anal. calcd for $C_{33}H_{36}FN_3S$: C 75.39, H 6.90, N 7.99; found C 75.41, H 6.88, N 8.00.

6-(4-Fluorobenzyl)-4-(4-methylphenyl)-8-(4-methylbenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3l) Yield: 80%; yellow solid, m.p.: 132—134 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.46 (s, 1H), 9.08 (s, 1H), 7.20—6.99 (m, 13H), 4.78 (s, 1H), 3.56—3.26 (m, 4H), 3.06 (d, J=16.6 Hz, 1H), 2.71 (d, J=16.3 Hz, 1H), 2.30 (s, 3H), 2.28 (s, 3H); IR (KBr) v: 3426, 1567, 1500, 1453, 1077, 989, 879, 858 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 469.70. Anal. calcd for $C_{29}H_{28}FN_3S$: C 74.17, H 6.01, N 8.95; found C 74.14, H 6.00, N 8.97.

4-(4-Fluorophenyl)-6-(4-fluorobenzyl)-8-(4-fluorobenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3m) Yield: 94%; white solid, m.p.: 106—108 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.53 (s, 1H), 9.14 (s, 1H), 7.30—6.98 (m, 13H), 4.88 (s, 1H), 3.61—3.36 (m, 4H), 3.08 (d, J=16.75 Hz, 1H), 2.70 (d, J=16.61 Hz, 1H); IR (KBr) v: 3416, 1570, 1500, 1473, 1077, 989, 879, 547 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 478.15. Anal. calcd for $C_{27}H_{22}F_3N_3S$: C 67.91, H 4.64, N 8.80; found C 67.90, H 4.65, N 8.81.

4-(2-Chlorophenyl)-8-(2-chlorobenzylidene)-6-(4-fluorobenzyl)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3n) Yield: 77%; yellow solid, m.p.: 110—112 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.81 (s, 1H) , 9.08 (s, 1H), 7.56—6.92 (m, 13H), 5.32 (s, 1H), 3.47—3.12 (m, 4H), 3.14 (d, J=16.7 Hz, 1H), 2.71 (d, J=16.8 Hz, 1H); IR (KBr) v: 3426, 1601, 1569, 1457, 1157, 1077, 858, 544 cm^{-1}; MS (ESI) ([M + H]$^+$) m/z: 509.21. Anal. calcd for $C_{27}H_{22}FCl_2N_3S$: C 63.53, H 4.34, N 8.23; found C 63.52, H 4.31, N 8.24.

6-(4-Fluorobenzyl)-8-benzylidene-4-phenyl-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3o) Yield: 91%; white solid, m.p.: 174—176 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.53 (s, 1H), 9.13 (s, 1H), 7.41—6.97 (m, 15H), 4.85 (s, 1H), 3.61—3.20 (m, 4H), 3.12 (d, J=16.5 Hz, 1H), 2.74 (d, J=16.4 Hz, 1H); IR (KBr) v: 3425, 1568, 1510, 1452, 1076, 985, 879, 858 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 441.19. Anal. calcd for $C_{27}H_{24}FN_3S$: C 73.44, H 5.48, N 9.52; found C 73.45, H 5.50, N 9.54.

4-(4-Chlorophenyl)-8-(4-chlorobenzylidene)-6-(4-fluorobenzyl)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3p) Yield: 75%; yellow solid, m.p.: 116—118 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.81 (s, 1H) , 9.05 (s, 1H), 7.55—6.92 (m, 13H), 5.32 (s, 1H), 3.42—3.12 (m, 4H), 3.14 (d, J=17.3 Hz, 1H), 2.71 (d, J=16.8 Hz, 1H); IR (KBr) v: 3427, 1601, 1569, 1457, 1157, 1077, 858 cm^{-1}; MS (ESI) ([M + H]$^+$) m/z: 510.09. Anal. calcd for $C_{27}H_{22}Cl_2N_3S$: C 63.53, H 4.34, N 8.23; found C 63.52, H 4.31, N 8.24.

6-(4-Bromobenzyl)-4-(2-fluorophenyl)-8-(2-fluorobenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3q) Yield: 80%; yellow solid, m.p.: 119—121 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.75 (s, 1H), 9.07 (s, 1H), 7.41—7.00 (m, 13H), 5.14 (s, 1H), 3.43—3.15 (m, 4H), 3.14 (d, J=16.7 Hz, 1H), 2.73 (d, J=16.8 Hz, 1H); IR (KBr) v: 3417, 1556, 1487, 1454, 1152, 1099, 1071, 856, 758 cm^{-1}; MS (ESI) ([M+H]$^+$) m/z: 537.06. Anal. calcd for $C_{28}H_{25}F_2BrN_3S$: C 60.23, H 4.12, N 7.80; found C 60.25, H 4.12, N 7.83.

6-(4-Bromobenzyl)-4-(4-methoxylphenyl)-8-(4-methoxylbenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3r) Yield: 86%; white solid, m.p.: 175—177 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.46 (s, 1H), 9.08 (s, 1H), 7.45—6.89 (m, 13H), 4.78 (s, 1H), 3.29—3.59 (m, 4H), 3.07 (d, J=16.6 Hz, 1H), 2.70 (d, J=16.4 Hz, 1H), 2.29 (s, 6H); IR (KBr) v: 3427, 1566, 1530, 1403, 1078, 988, 877, 856 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 562.12. Anal. calcd for $C_{29}H_{28}BrN_3O_2S$: C 65.65, H 5.32, N 7.92; found C

65.64, H 5.33, N 7.93.

6-(4-Bromobenzyl)-4-(4-isopropylphenyl)-8-(4-isopropylbenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3s) Yield: 89%; white solid, m.p.: 121—123 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.48 (s, 1H), 9.05 (s, 1H), 7.34—6.82 (m, 13H), 4.78 (s, 1H), 3.55—3.31 (m, 4H), 3.07 (d, J= 16.7 Hz, 1H), 2.87 (d, J=12.1 Hz, 2H), 2.74 (d, J= 16.4 Hz, 1H), 1.19—1.16 (m, 12H); IR (KBr) v: 3426, 1567, 1500, 1453, 1077, 989, 879, 858 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 587.26. Anal. calcd for $C_{33}H_{36}BrN_3S$: C 75.39, H 6.90, N 7.99; found C 75.41, H 6.88, N 8.00.

6-(4-Bromobenzyl)-4-(4-methylphenyl)-8-(4-methylbenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3t) Yield: 86%; white solid, m.p.: 175—177 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.48 (s, 1H), 9.07 (s, 1H), 7.37—7.04 (m, 13H), 4.78 (s, 1H), 3.59—3.29 (m, 4H), 3.07 (d, J=16.6 Hz, 1H), 2.70 (d, J=16.4 Hz, 1H), 2.31—2.29 (m, 6H); IR (KBr) v: 3427, 1567, 1500, 1453, 1077, 989, 879, 858 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 530.12. Anal. calcd for $C_{29}H_{28}BrN_3S$: C 65.65, H 5.32, N 7.92; found C 65.64, H 5.33, N 7.93.

6-(4-Bromobenzyl)-4-(4-fluorophenyl)-8-(4-fluorobenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3u) Yield: 81%; orange solid, m.p.: 134—136 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.53 (s, 1H), 9.15 (s, 1H), 7.47—7.02 (m, 13H), 4.88 (s, 1H), 3.53—3.29 (m, 4H), 3.09 (d, J=16.7 Hz, 1H), 2.70 (d, J=16.5 Hz, 1H); IR (KBr) v: 3416, 1601, 1569, 1506, 1484, 1429, 1157, 1073, 841, 647 cm^{-1}; MS (ESI) ([M+H]$^+$) m/z: 537.89. Anal. calcd for $C_{27}H_{22}BrF_2N_3S$: C 60.23, H 4.12, N 7.80; found C 60.20, H 4.10, N 7.81.

6-(4-Bromobenzyl)-4-(2-chlorophenyl)-8-(2-chlorobenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3v) Yield: 77%; yellow solid, m.p.: 110—112 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.58 (s, 1H) , 9.16 (s, 1H), 7.56—7.14 (m, 13H), 5.35 (s, 1H), 3.53—3.32 (m, 4H), 3.10 (d, J= 16.7 Hz, 1H), 2.71 (d, J=16.8 Hz, 1H); IR (KBr) v: 3427, 1601, 1569, 1457, 1157, 1077, 858 cm^{-1}; MS (ESI) ([M+H]$^+$) m/z: 571.31. Anal. calcd for $C_{27}H_{22}BrCl_2N_3S$: C 56.76, H 3.88, N 7.35; found C 56.77, H 3.89, N 7.34.

6-(4-Bromobenzyl)-8-benzylidene-4-phenyl-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3w) Yield: 91%; white solid, m.p.: 174—176 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.55 (s, 1H), 9.13 (s, 1H), 7.53—7.04 (m, 15H), 4.85 (s, 1H), 3.61—3.36 (m, 4H), 3.12 (d, J=16.7 Hz, 1H), 2.74 (d, J=16.5 Hz, 1H); IR (KBr) v: 3428, 1565, 1506, 1455, 1077, 989, 879, 858; ESI-MS([M+H]$^+$) m/z: 502.09. Anal. calcd for $C_{27}H_{24}BrN_3S$: C 64.54, H 4.81, N 8.36; found C 64.55, H 4.80, N 8.36.

6-(4-Bromobenzyl)-4-(4-chlorophenyl)-8-(4-chlorobenzylidene)-1,2,5,6-tetrahydropyrido[4,3-*d*]-3,4-dihydropyrimidine-2-thione (3x) Yield: 89%; white solid, m.p.: 179—181 ℃; ^{1}H NMR (400 MHz, DMSO) δ: 9.58 (s, 1H), 9.16 (s, 1H), 7.47—7.04 (m, 13H), 4.89 (s, 1H), 3.53—3.07 (m, 4H), 3.10 (d, J=16.8 Hz, 1H), 2.71 (d, J=16.6 Hz, 1H); IR (KBr) v: 3426, 1567, 1500, 1453, 1077, 989, 879, 858 cm^{-1}; ESI-MS ([M+H]$^+$) m/z: 570.01. Anal. calcd for $C_{27}H_{22}BrCl_2N_3S$: C 56.76, H 3.88, N 7.35; found C 56.75, H 3.88, N 7.36.

X-ray crystallography

A chartreuse crystal of compound **3j** recrystallized by slow evaporation from ethyl acetate at room temperature, with dimensions of 0.12 mm×0.10 mm×0.10 mm was mounted on a glass fiber for data collection which was made on α BRUKER SMART APEX 1000 CCD diffractometer equipped with a graphite-monochromatic MoKα radiation (λ=0.71073 Å) by using a φ-ω scan mode in the range of 0.85°≤θ≤22.87° (−36≤h≤36, 0≤k≤52, 0≤l≤28) at 173 K. A total of 21124 reflections were collected with 6366 unique ones (R_{int}=0.0545), of which 3804 with $I>2\sigma(I)$ were considered as observed and used in the succeeding refinements. The intensity data were corrected for Lp factors and empirical absorption. All of the non-hydrogen atoms were refined anisotropically, and hydrogen atoms were located at their idealized positions. The structure was solved by direct methods and refined by full-matrix least-squares techniques on F^2 using the SHELXTL program package. The final R=0.0601, wR=0.1505 ($w=1/[\sigma^2(F_o^2)+(0.0568P)^2+0.2960P]$, where $P=(F_o^2+2F_c^2)/3$), S=1.025, $(\Delta/\sigma)_{max}$=0.143, $(\Delta\rho)_{max}$= 0.519 and $(\Delta\rho)_{min}$=−0.315 e/Å^3. The structural graphics were drawn with SHELXTL-97 software package. Other details of the structure have been deposited with the Cambridge Crystallographic Data Centre, 120302F.

Treatment of tumor cell lines

The antiproliferative activities of the title compounds and 5-FU against leukemia K562 cells, HO-8910 cells and liver cancer SMMC-7721 cells were determined by the MTT assay according to the standard bioactivity test procedures.[19] Leukemic K562 cells, ovarian cancer HO-8910 cells and liver cancer SMMC-7721 cells were cultured in RPMI 1640 medium at 37 ℃ with 5% CO_2, and 95% air, supplemented with 10% (V/V) bovine calf serum and 80 U/mL gentamicin. The cells were seeded onto 96-well plates or other appropriate dishes containing the medium at the density of 6250/cm^2. About 10 mg of the title compounds were dissolved in 100 μL of dimethylsulfoxide (DMSO), then concentrations of 1.0, 10.0, 100.0 μg/mL were adjusted stepwise in each 96-well plates, and the concentration of DMSO was 1‰. One hundred thousand cells, 20 μL of FBS, RPMI-1640 and diversified concentration title compounds were seeded in each 100 μL well of a 96-well plate. Each group had eight wells. Set up the control well of remedy colour (5-FU) and incubate the well (containing cells). After 44 h in a humidified atmosphere with 5% CO_2 at 37 ℃, add 10 μL of MTT (5 mg/mL) into every well, then rejoin 100 μL

of 10% SDS to stop the reaction after 4 h. One night later at 37 ℃, the number of absorbency (*A*) of every well was measured by enzyme immunity at 570 nm. Equation of growth inhibition of cells was shown as follows.

$$\text{Inhibition} = [1 - A(\text{experiment})/A(\text{control})] \times 100\%$$

Apoptosis detection

Fluorescein isothiocyanate (FITC)-conjugated annexin V has been utilized to detect the externalization of phosphatidylserine that occurs at an early stage of apoptosis. Propidium iodide (PI) is used as a marker of necrosis due to cell membrane destruction.[21] To elucidate whether compound-induced cell death involved apoptosis or necrosis, we performed a biparametric cytofluorimetric analysis using annexin V and PI double-staining as shown in Figure 2. Treatment of leukemic K562 cells with 1, 10 and 100 μg/mL **3i** respectively, for 24 h induced apoptosis effects (annexin V^+/PI^-) in 2.70% (Control), 6.05% (1 μg/mL), 33.00% (10 μg/mL) and 81.26% (100 μg/mL) of annexin V-FITC cells.

Western blotting

After treatment, cell pellets were collected and lysed in a lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 8.0, 2 mmol/L ethylene glycol-bis(*β*-aminoethyl ether), 2 mmol/L EDTA, 25 mmol/L NaF, 25 mmol/L *β*-glycerophosphate, 0.2% Triton X-100, 0.3% Nonidet P-40, and 0.1 mmol/L phenylmethylsulfonyl fluoride]. Total protein concentrations of whole cell lysis were determined using BioRad BCA method (PIERCE, Rockford, IL). Equal amounts of protein sampled from whole cell lysis were subjected to electrophoresis on 10%—12% Tris-Glycine pre-cast gels (Novex, San Diego, CA) and electroblotted onto Immobilon-P Transfer Membrane (Millipore Corporation, Billerica, Massachusetts), and probed with primary antibodies and then incubated with a horseradish peroxidase (HRP) conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (ECL) Western Blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

Electrophoretic analysis of DNA fragmentation

Leukemia K562 cells were lysed in 200.0 mL lysis buffer (10.0 mmol/L EDTA; 50.0 mmol/L Tris-HCl, pH 8.0; 0.5% sodium lauryl sulfate; 100.0 mg/mL proteinase K) at 37 ℃ for 12 h, then incubated with RNase (50.0 mg/mL) at 37 ℃ for an additional 1 h. After incubation, DNA in the lysate was extracted with equal volume of phenol/chloroform/isoamyl alcohol (25∶24∶1), then with chloroform. DNA was precipitated with two volumes of ethanol in the presence of 0.3 mol/L sodium acetate. After centrifugation at 12000 g for 15 min, the DNA pellets were washed with 70% ethanol, air-dried, and resuspended in 20.0 mL TE (10.0 mmol/L Tris-HCl and 1.0 mmol/L EDTA, pH 8.0). DNA was separated on 1.5% agarose gels containing 0.5 mg/mL ethidium bromide and photographed by Bio-Rad GD2000 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Caspase-3/7 data are presented as means ±SE of at least three independent experiments. In addition, each caspase-3/7 experiment was performed using triplicate and duplicate wells, respectively. All fluorescence caspase-3/7 data were first blank corrected and then converted into rates of AFC produced per hour. These values were statistically compared to the controls using one-way ANOVA with a Dunnett's post test using InStat version 3.0a (GraphPad) where $p < 0.05$ was considered significant.

Result and Discussion

Evaluation of anticancer activities

The antiproliferative activities (IC_{50}) of compounds **3a**—**3x** and 5-FU were presented in Table 1, and the effects of **3i** on the morphology of leukemic K562 cells at 1, 10, 100 μg/mL cultured after 44 h are shown in Figre 1.

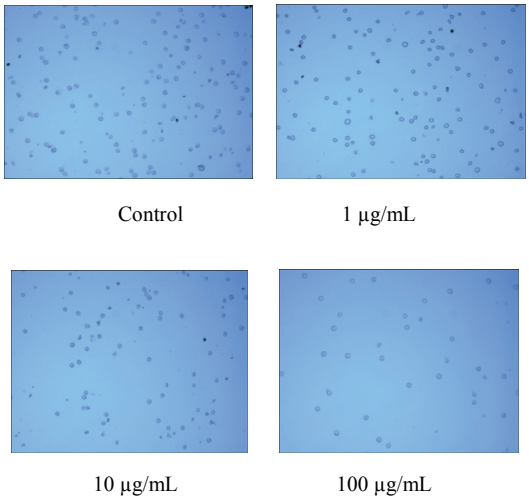


Figure 1 Leukemic K562 cells act on **3i** at 1, 10, 100 μg/mL cultured after 44 h.

According to the Table 1, most of the title compounds exhibited good antiproliferative activities against leukemic K562 cells, ovarian cancer HO-8910 cells and liver cancer SMMC-7721 cells *in vitro*. Among them **3i** and **3m** afforded the best activities *in vitro*. The IC_{50} of them were 3.22 and 3.65 μg/mL against leukemic K562 cells respectively, which were lower than the anticancer drug of clinical practice 5-FU (IC_{50} = 8.56 μg/mL).

As shown in Table 1, when the different substituents R^1 and R^2 were introduced to the title compounds, the antiproliferative activities varied greatly. Considering the influence of R^1 group, when the R^2 group is same, the compounds bearing F (**3i**—**3p**) had shown relatively

CHINESE JOURNAL OF CHEMISTRY

better antiproliferative activities than those bearing Br and Me, the activities increased in the order: **3e** (R^1=Me, R^2=4-F)<**3u** (R^1=Br, R^2=4-F)<**3m** (R^1=F, R^2=4-F). When the titie compounds bearing different R^2, such as **3i**—**3p**, their activities decreased in the orde: **3i** (R^2=2-F)>**3m** (R^2=4-F)>**3p** (R^2=4-Cl)>**3n** (R^2=2-Cl)>**3o** (R^2=H)>**3j** (R^2=4-OMe)>**3l** (R^2=4Me)>**3k** [R^2=4-CH(Me)$_2$]. These results clearly suggest that, when R were electron-withdrawing substituents, such as single F or Cl atom, the activities of the title compounds were higher than those bearing electron-donating substituents, such as OMe, Me, CH(Me)$_2$. Considering the discussion above, we found that the antiproliferative activities of our designed compounds could be increased by the introduction of fluoro, chloro on benzene rings, further study is underway. The results further suggested that small differences between the structures could lead to large differences in the overall activities, which implies further possibilities for lead compound development.

Table 1 Antiproliferative activities (IC_{50}) of the compounds **3a**—**3x** and 5-FU at 48 h

Compd.	R^1	R^2	IC_{50}/(μg•mL^{-1})		
			Leukemic K562	Ovarian cancer HO-8910	Liver cancer SMMC-7721
3a	Me	2-F	17.17	33.21	47.17
3b	Me	4-OMe	38.43	62.54	88.43
3c	Me	4-CH(CH$_3$)$_2$	55.67	87.65	145.67
3d	Me	4-Me	45.42	63.17	105.42
3e	Me	4-F	16.21	39.46	46.21
3f	Me	2-Cl	17.45	40.21	48.45
3g	Me	4-H	31.06	43.55	51.06
3h	Me	4-Cl	19.67	41.36	46.67
3i	F	2-F	3.22	20.17	21.22
3j	F	4-OMe	14.32	32.65	64.32
3k	F	4-CH(CH$_3$)$_2$	24.56	78.32	124.5
3l	F	4-Me	21.65	34.65	71.65
3m	F	4-F	3.65	19.65	23.65
3n	F	2-Cl	9.74	24.33	26.74
3o	F	4-H	11.21	34.72	31.21
3p	F	4-Cl	9.11	22.56	27.11
3q	Br	2-F	7.32	24.36	29.32
3r	Br	4-OMe	11.24	43.54	83.24
3s	Br	4-CH(CH$_3$)$_2$	27.82	78.45	127.82
3t	Br	4-Me	21.42	35.32	91.42
3u	Br	4-F	6.27	32.17	31.27
3v	Br	2-Cl	13.56	33.46	46.56
3w	Br	4-H	15.06	39.43	40.06
3x	Br	4-Cl	17.32	35.71	47.32
5-FU			8.56	14.46	13.7

The results of preliminary mechanism studaies are shown in Figure 1s—3s. The Figure 1s is the flow analysis figure of **3i** induced apoptosis of K562 cells, the effect of **3i** on the activation of caspase-3/7 is shown in Figure 2s and the DNA electrophoresis is shown in Figure 3s. Figures 1s—3s are in the Supporting Information.

As shown in Figure 1s, treatment of leukemic K562 cells with 1, 10 and 100 μg/mL **3i** respectively, for 24 h, induced apoptosis effects (annexin V^+/PI^-) in 2.70% (Control), 6.05% (1 μg/mL), 33.00% (10 μg/mL) and 81.26% (100 μg/mL) of annexin V-FITC cells. The results showed that **3i** induced more apoptotic cells than that of the untreated control. In addition, the apoptosis of leukemic K562 cells was determined by caspase-3/7 Kit and Electrophoretic Analysis of DNA Fragmentation (Figure 2s and Figure 3s). We found that compared with control cells, cells exposed to **3i** slightly increased caspase-3/7 activity, and resulted in a characteristic fragmentation of DNA, a common feature of apoptotic cell death. DNA fragmentation was later conducted in apoptotic cells, compared with increasing caspase-3/7 activity. These theoretical events may explain that treating with 10 μg/mL **3i**, a severely high dose, could induce leukemic K562 cells DNA fragmentation. Taken together, all results suggested that apoptosis pathway was involved in **3i** induced antiproliferative activity in Leukemic K562 cells.

To confirm the three-dimensional information of the title compounds, the single-crystal structure of compound **3j** was investigated and determined by X-ray diffraction analysis. Molecular structure of compound **3j** with atom-labeling is shown in Figure 2 and the packing diagram in Figure 4s. The crystallographic information files of **3j** and Figure 4s are in the Supporting Information.

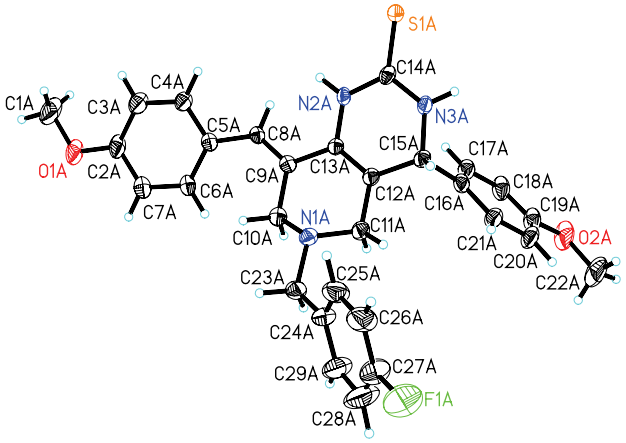


Figure 2 Molecular structure of compound **3j** (Number 120302F) with atom-labeling.

Conclusions

A new series of tetrahydropyrido[4,3-*d*]dihydropyrimidine-2-thiones (**3a**—**3x**) have been designed and synthesized. The conformation of the compound **3j** was confirmed by X-ray diffraction. Most of the title compounds showed good antiproliferative activity against human cancer cell lines, including leukemic K562, ovarian cancer HO-8910 and liver cancer SMMC-7721 *in vitro*. Among them, the **3i** and **3m** were the most

promising compounds against the leukemic K562 cells, the IC_{50} of them were 3.22 and 3.65 μg/mL, respectively, which were lower than the anticancer drug of clinical practice 5-FU (IC_{50} = 8.56 μg/mL). In addition, preliminary mechanism of action studies revealed that compound **3i** caused DNA fragmentation, and activated caspase-3/7 in leukemia K562 cells. The results provided valuable information for the design of anticancer agents. Future progress on related series will be reported in due course.

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