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Design, synthesis and apoptosis-related antiproliferative activities of chelidonine derivatives

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ABSTRACT

To get chelidonine derivatives with enhanced antiproliferative activity and selectivity, a series of nitric oxide donating derivatives (**10a-f** and **11a-j**) were designed, synthesized and biologically evaluated. Compared with chelidonine, these compounds exhibited lower IC_{50} values against human hepatoma cells HepG2, breast cancer cells MCF-7, colon cancer cells HCT-116, as well as leukemia cells K562. Compound **11j** displayed the strongest antiproliferative activity with IC_{50} values of 3.91, 6.90, 4.36 and 1.12 μM against the above four cells, respectively. Nevertheless, it showed an IC_{50} value > 40 μM against human peripheral blood mononuclear cells (PBMCs), which demonstrated high selectivity between normal and cancer blood cells. In further mechanism studies, **11j** showed the capability to induce K562 cells apoptosis, S phase cell cycle arrest and mitochondrial membrane potential disorder. Besides, **11j** was found to be effective in promoting the expression of proapoptotic protein Bad and suppressing the expression of anti-apoptotic proteins Bcl-xL, catalase, survivin, claspin and clusterin.

Chelidonium majus L. is known as a commonly-used Chinese medicinal herb for cough inhibition, analgesia and detoxification.[1] In recent studies, the crude extract of *C. majus* was observed to inhibit tumor cells from migration and induce cell cycle arrest and apoptosis.[2,3] Alkaloids are the active components of *C. majus*.[4] Chelidonine (**1**), a benzophenanthridine alkaloid, is the major alkaloid in *C. majus* with the capability of performing various biological activities,[3] including antitumor,[5–8] anti-inflammatory,[9–11] antibacterial,[12,13] analgesic,[14] insecticide[15] and spasmolytic[16,17] effects. Chelidonine inhibits various cancers, including leukemia,[18] liver cancer,[19] breast cancer,[20] lung cancer[18] and so on. Its antitumor mechanisms have been investigated extensively. Chelidonine induces human *T*-leukemia cells apoptosis via a mitochondrial death pathway by slightly releasing cytochrome *c* in 12 h and causes intensive DNA damage in 24 h.[21,22] It also prompts cells mitotic arrest and modulates tyrosine kinase activity.[23] In gastric cancer SGC-7901 cells, chelidonine signally down-regulates the expression of Cdk1 and cyclinB1, up-regulates the protein p-Cdk1 (Thr14) and induces the G_2/M phase cell cycle arrest.[6] Chelidonine not only inhibits the formation of integrin-linked kinase, PINCH and α-parvin complex, but also reduces migration and invasion by inhibiting tubulin polymerisation.[24,25] In addition, chelidonine down-regulates hTERT.[20] As confirmed by Mahmoud Zaki El-Readi et al., chelidonine overcomes multidrug resistance (MDR) in cancer cells through interaction with ABC-transporters, CYP3A4 and GST, induction of apoptosis and cytotoxic effects.[26] Apart from these, chelidonine attenuates eosinophilic airway inflammation by suppressing IL-4 and eotaxin-2 expression in asthmatic mice.[10] It also exerts inhibitory effect on human neutrophil elastase activity.[11]

Abbreviations: PBMC, peripheral blood mononuclear cell; MDR, multidrug resistance; NO, nitric oxide; Et_3N, triethylamine; DMAP, 4-dimethylaminopyridine; EDC-HCl, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; DCM, dichloromethane; MeOH, methanol; THF, tetrahydrofuran; 5-Fu, 5-fluorouracil; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol caebocyanine; PBS, phosphate buffered solution; NMR, nuclear magnetic resonance; HR(ESI)MS, high resolution electrospray ionization mass spectrometry; *m/z*, mass-to-charge ratio; OR, optical rotation

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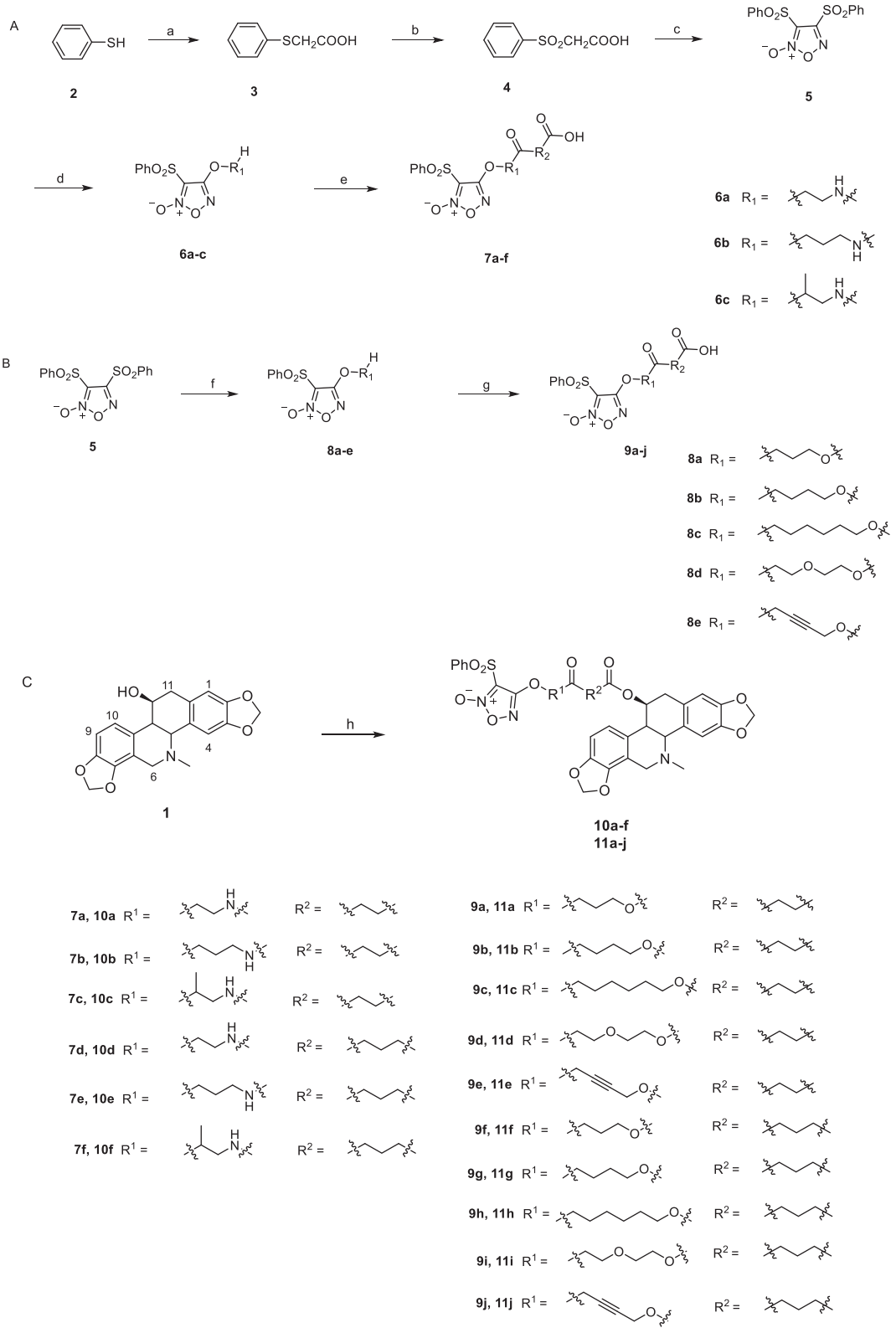

Scheme 1. (A) Synthesis of NO donors **7a-f**. Reagents and conditions: (a) $ClCH_2COOH$, NaOH (aq), 140 ℃, 2 h; (b) 30% H_2O_2, rt, 3 h; (c) fuming HNO_3, AcOH, 90 ℃, 4 h; (d) hydramine, NaH, 0 ℃, 5 h; (e) anhydride, anhydrous, pyridine, rt, 3 h. (B) Synthesis of NO donors **9a-j**. Reagents and conditions: (f) corresponding dihydric alcohol, THF, 30% NaOH, 0 ℃, 4–8 h; (g) corresponding anhydride, Et_3N, DMAP, DCM, rt, 3–4 h. (C) Synthesis of chelidonine derivatives **10a-f** and **11a-j**. Reagents and conditions: (h) **7a-f** or **9a-j**, EDC-HCl, DMAP, DCM, rt, 10–12 h.

Table 1
Antiproliferative activities of chelidonine and its 16 NO-releasing derivatives against four human cancer and two normal cell lines.

compound	IC_{50} (μM)[a]					
	HepG2	MCF-7	HCT-116	K562	L-02	PBMC
10a	11.36 ± 0.83	7.94 ± 0.55	11.54 ± 0.59	3.17 ± 0.14	27.68 ± 1.33	> 40
10b	16.83 ± 1.20	13.59 ± 1.04	17.26 ± 1.38	9.80 ± 0.46	36.48 ± 1.74	> 40
10c	19.43 ± 1.27	8.37 ± 0.51	16.45 ± 0.33	6.94 ± 0.31	> 40	> 40
10d	26.44 ± 1.73	14.58 ± 1.17	> 40	12.13 ± 0.34	> 40	> 40
10e	10.68 ± 0.62	5.29 ± 0.14	7.25 ± 0.48	3.18 ± 0.30	19.94 ± 0.97	> 40
10f	7.59 ± 0.45	4.92 ± 0.22	12.17 ± 0.46	2.68 ± 0.19	23.45 ± 1.38	> 40
11a	10.54 ± 0.72	3.68 ± 0.20	15.73 ± 0.94	3.08 ± 0.18	14.56 ± 1.05	> 40
11b	6.39 ± 0.24	4.78 ± 0.35	8.27 ± 0.43	2.91 ± 0.23	19.71 ± 0.80	> 40
11c	9.45 ± 0.59	7.94 ± 0.44	16.56 ± 1.87	7.61 ± 0.39	> 40	> 40
11d	8.65 ± 0.37	8.92 ± 0.30	8.68 ± 0.65	4.52 ± 0.23	23.93 ± 1.22	> 40
11e	14.39 ± 1.02	5.41 ± 0.31	23.54 ± 1.39	3.78 ± 0.22	28.19 ± 1.46	> 40
11f	13.27 ± 0.88	7.34 ± 0.52	12.88 ± 0.68	3.73 ± 0.26	20.32 ± 1.08	> 40
11g	19.58 ± 1.36	6.81 ± 0.40	19.14 ± 0.52	6.14 ± 0.52	> 40	> 40
11h	> 40	13.24 ± 0.83	> 40	9.59 ± 0.62	> 40	> 40
11i	6.55 ± 0.30	6.55 ± 0.28	8.47 ± 0.53	2.60 ± 0.17	16.52 ± 1.24	> 40
11j	3.91 ± 0.21	6.90 ± 0.35	4.36 ± 0.29	1.12 ± 0.11	6.04 ± 0.50	> 40
chelidonine	> 40	> 40	> 40	17.65 ± 1.56	> 40	> 40
5-Fu	32.57 ± 1.98	26.65 ± 1.92	6.86 ± 0.37	3.94 ± 0.17	> 40	> 40

[a] IC_{50}: concentration of the tested compound that inhibits 50% of cell growth by the MTT assay, the cells were incubated for 72 h. The values are expressed as averages ± standard deviations of three independent experiments.

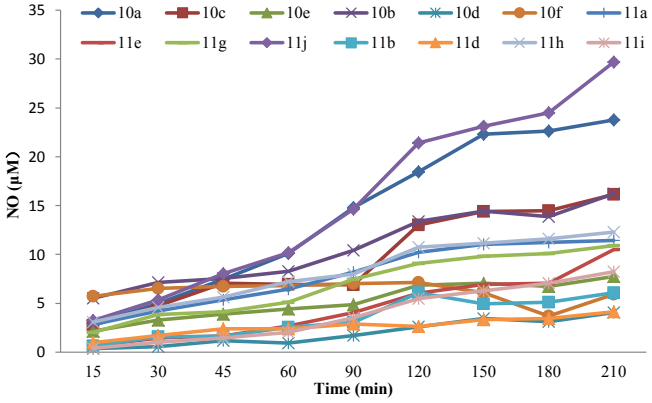

Fig. 1. NO-releasing ability of chelidonine derivatives. The values are expressed as averages of three independent experiments.

Structural modification of nature products is considered as a major approach of drug discovery. For example, the well-known anticancer drugs 10-hydroxycamptothecin, topotecan, belotecan and lurtotecan are all classed as derivatives of the alkaloid camptothecin. They are used to treat colon cancer, lung cancer and ovarian cancer in clinic.[27] Chelidonine possesses antitumor activities and is potential to be an antineoplastic precursor. It is reported in the literature, the alkaloids in *C. majus* L. show detectable hepatotoxicity.[28] In order to enhance the antiproliferative activity of chelidonine and improve the selectivity between cancer and normal cells, chelidonine is structurally modified in this study.

Nitric oxide (NO) represents a significant gaseous signaling molecule that is applied to the treatment of cancer by inducing apoptosis, increasing the sensitivity of tumor cells to drugs and attenuating cancer cell metastasis.[29–31] High levels of NO may exert anticancer activities through upregulating p53 to stimulate apoptosis, degradating the anti-apoptotic proteasomal molecule, increasing the mitochondrial permeability and releasing cytochrome *c*, cell cycle arrest, cell necrosis, cell necrosis and cytotoxicity.[32–34] In recent years, the research conducted into NO donating derivatives has been made a focus of attention. The antitumor effects dramatically attenuate by NO scavenger or elimination of the NO-releasing capability which indicates that the NO produced by NO-donating derivatives contributes to its cytotoxicity against cancer cells.[35,36] The combination of NO donors with natural products, such as terpenoids, alkaloids and flavonoids, is conducive to improving antitumor activity and reducing toxicity.[37–39] Among NO-donors, furazan has a relative stable structure and inhibits tumor proliferation by the release of NO.[40]

In this work, chelidonine was combined with a series of furazan-type NO donors to obtain 16 NO-donating derivatives, which were screened for their antiproliferative activities. Further mechanism study of **11j**, concerning apoptosis related properties, were also investigated.

Chelidonine was isolated from the aerial parts of *C. majus* L. and its structure was confirmed via a combination of ^{1}H NMR, ^{13}C NMR and optical rotation. The configuration of C-11 of chelidonine was confirmed by comparing the optical rotation with the literature.[41] The tested optical rotation of chelidonine was [α] + 135 (c 0.12, MeOH) which was fitted with [α] + 112 (c 0.65, MeOH) in the literature. Thiophenol (**2**) was reacted with chloroacetic acid in the presence of sodium hydroxide to generate phenylthio acetic acid **3**. In the presence of 30% H_2O_2, **4** was obtained from the oxidation of **3**. Further reaction with fuming nitric acid at 90 °C generated **5**. **5** was reacted with aminoalcohol, in THF to derive **6a-c**. **7a-f** were obtained by further reaction with anhydride and pyridine (Scheme 1A). In addition, **5** was reacted with propanediol, butanediol, hexanediol, diethylene glycol or 2-butyne-1,4-diol in THF containing 30% NaOH to generate **8a-e**, then treated with anhydride, triethylamine (Et_3N) and 4-dimethylaminopyridine (DMAP) to derive **9a-j** (Scheme 1B). In addition, **7a-f** or **9a-j** were reacted with chelidonine in the presence of DMAP and EDC-HCl in DCM, and then they were purified by silica gel column chromatography (DCM:MeOH = 200:1) to get the target compounds **10a-f** and **11a-j** (Scheme 1C).

The antiproliferative activities of chelidonine derivatives **10a-f** and **11a-j** were tested against four human cancer cells (hepatoma HepG2, breast cancer MCF-7, colon cancer HCT-116 and leukemia K562) and two normal cells (hepatocytes L-02 and peripheral blood mononuclear cells PBMC) in comparison with chelidonine (**1**). 5-Fu (5-fluorouracil) was used as positive control (Table 1).

As demonstrated by the results, a majority of the chelidonine derivatives exhibited antiproliferative activities against these four human tumor cells. All derivatives were indicated to perform significant cytotoxic activity with the IC_{50} values of 1.12–12.13 and 3.68–14.58 μM against K562 and MCF-7 cell lines, respectively. Most of the derivatives performed decent cytotoxic activity with the IC_{50} values of 4.36–23.54

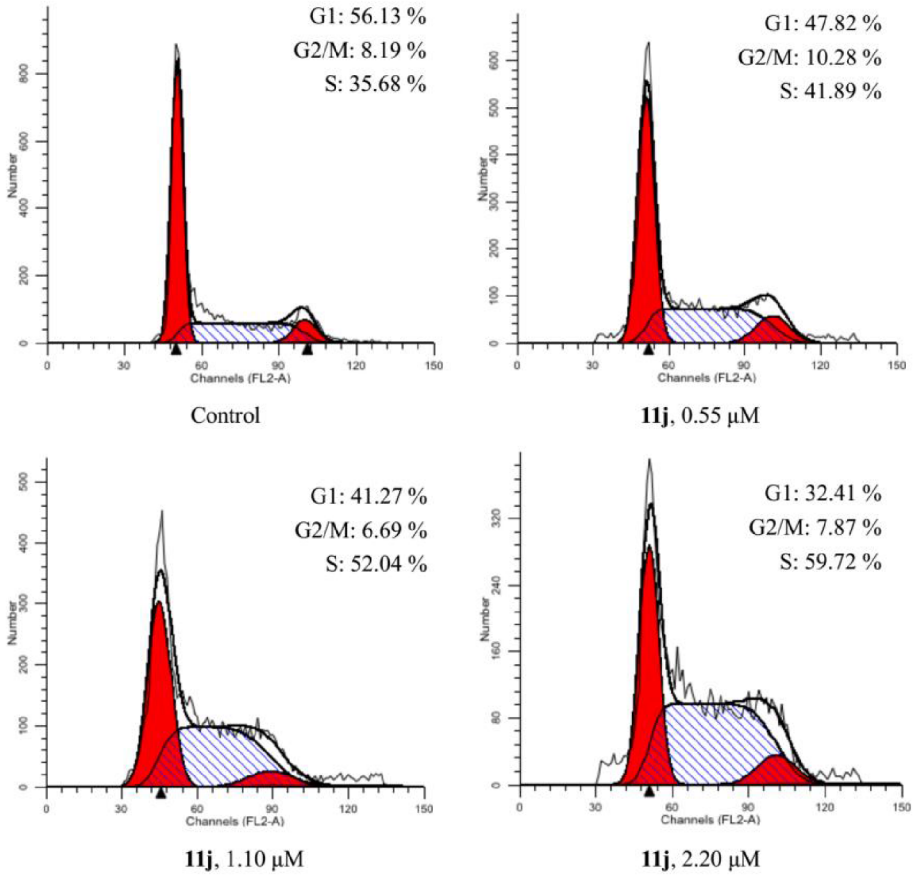

Fig. 2. Cell cycle analysis of different concentrations of **11j** in K562 cells. K562 cells were treated with **11j** (0, 0.55, 1.10 and 2.20 μM) for 72 h, then stained with PI. A flow cytometer was used to analyze.

and 3.91–26.44 μM against HCT-116 and HepG2 cell lines, respectively. For **10b**, **10d** and **10f** with the same R^2 of -$(CH_2)_3$-, generally in all four tested cancer cells, **10b** with R^1 of ethylamine showed the weakest antiproliferative potency while **10f** with R^1 of isopropyl moiety was the strongest. **11f** with R^1 of -$(CH_2)_6$- moiety and R^2 of -$(CH_2)_3$- showed the weakest antiproliferative activity. None of the derivatives performed antiproliferaitve activity against PBMCs with IC_{50}s > 40 μM, which showed selectivity between normal and cancer cells. Compounds **10b**, **10e** and **11d-f** were discovered to possess no cytotoxicity against L-02, which also showed selectivity. **11j**[42] with R^1 of butyne moiety and R^2 of -$(CH_2)_3$- was the strongest one against the above four tumor cells with IC_{50} values of 3.91, 6.90, 4.36 and 1.12 μM, respectively. Therefore, **11j** was selected for further mechanism study in K562 cell line.

The anti-neoplastic ability of NO was generally improved with the concentration of NO.[43] Therefore, to further investigate the association between the NO-releasing ability of NO-donating chelidonine derivatives and their antiproliferative activities, the target compounds were subjected to the test of NO release ability (Fig. 1). As indicated, the amounts of NO released from NO-donating derivatives increased markedly from 90 min and continued this increasing over time. Especially, **11j** exhibiting the strongest antiproliferative activity also showed the highest NO release ability, the amount of NO release was about 30 μM at 210 min, which suggested a certain association between the NO release ability and the antiproliferative effects.

To validate the effect of **11j** on K562 cell cycle, K562 cells were subjected to the treatment with varying concentrations of **11j** (0.55, 1.10 and 2.20 μM) for a 72 h spell. As indicated in Fig. 2, the G_1, S and G_2/M phases of solvent control were 56.13%, 35.68% and 8.19%, respectively. The proportion of cells in S phase increased from 35.68% to 41.89%, 52.04% and 59.72%, respectively. It suggested that **11j** exerted inhibitory effects on the proliferation of K562 cells by inducing cell cycle arrest in S phase in a concentration-dependent manner.

The morphological characteristics displayed by cells would manifest a string of changes like cell shrinkage, chromatin accumulation and nuclear membrane rupture when cell apoptosis occurs.[44] In order to test whether **11j** is capable to induce apoptosis, K562 cells were treated with varying concentrations of **11j** (0.55, 1.10 and 2.20 μM) for 48 h and subsequently stained with Hoechst 33258. The morphological changes were observed by fluorescence microscopy. The results clearly revealed (Fig. 3) that the morphology of cells in the control group underwent no noticeable change, and the cells showed round homogeneous nuclei. Whereas different concentrations of **11j** treated K562 cells showed significant morphological changes, including chromatin aggregation, nuclear rupture and other signs of apoptosis. The results showed that **11j** was effective in facilitating apoptosis in K562 cells.

To confirm the apoptosis-induced effects of **11j**, annexin-V FITC/PI binding tests were conducted. K562 cells were incubated with varying concentrations of **11j** (the same as cell cycle experiments) for 48 h, previous to being stained with annexin-V FITC and PI. Then, the percentages of apoptotic cells were determined by conducting flow cytometry analysis. As shown in Fig. 4, varying concentrations of **11j** caused the percentage of apoptosis to rise from 2.96% in the solvent group to 9.96%, 18.85% and 53.81%, respectively. Therefore, **11j** induced apoptosis in K562 cells in a dose-dependent manner.

As mitochondrial membrane potential is closely associated with apoptosis. The loss of mitochondrial membrane potential causes changes of the permeability of mitochondrial membrane, which induces cell apoptosis by releasing pro-apoptotic factors into cytosol.[45,46] The effects exerted by **11j** on mitochondrial membrane potentials in K562 cell line were detected. Cells were co-cultured with varying concentrations (the same as cell cycle experiment) of **11j** for a duration of 48 h prior to being stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-

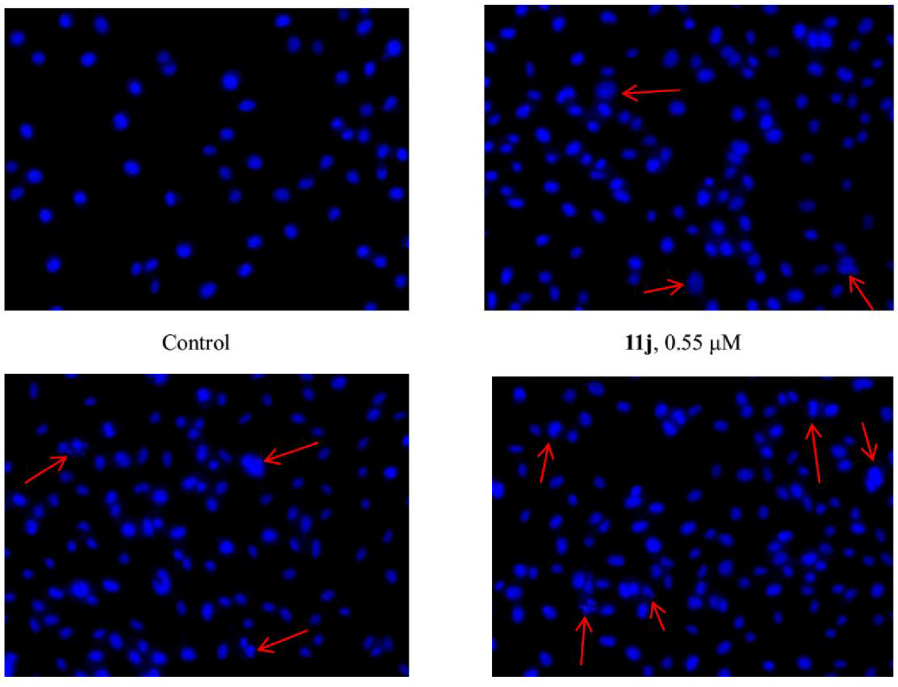

Fig. 3. Hoechst staining of **11j** in K562 cells. After the treatment with **11j** (0, 0.55, 1.10 and 2.20 μM) for 48 h, K562 cells were stained by Hoechst 33258 solution, then detected by fluorescent microscope.

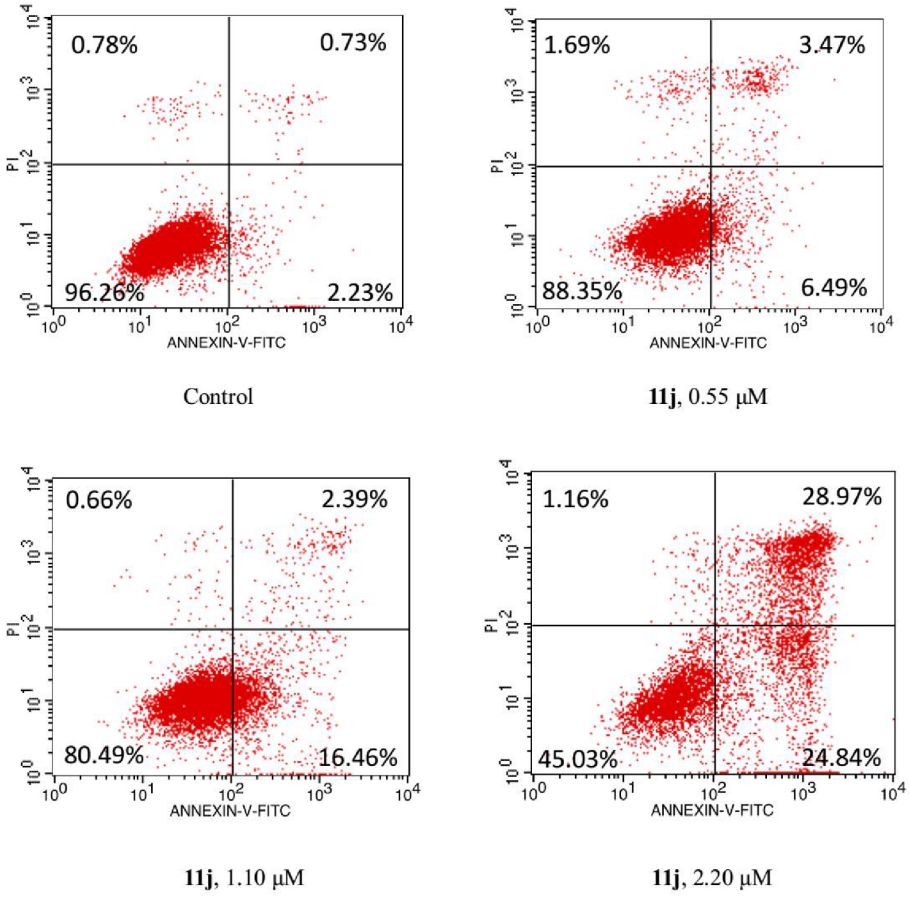

Fig. 4. Flow cytometry analysis of apoptosis induced by **11j** in K562 cells. K562 cells were treated with **11j** for 48 h and apoptosis was performed by flow cytometry via an annexin V-FITC/PI binding assay.

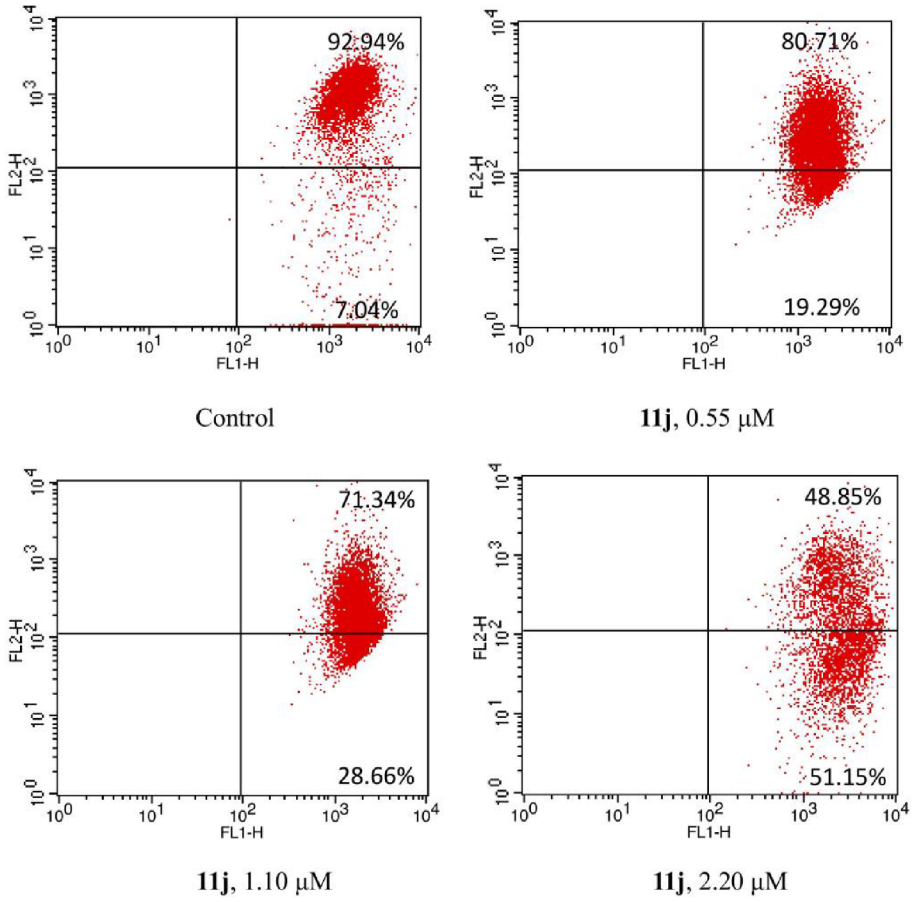

Fig. 5. Mitochondrial depolarization induced by **11j** in K562 cells. K562 cells were treated with **11j** and then stained with JC-1. The percentage of cells with collapsed mitochondrial membrane potentials was monitored by flow cytometry.

tetraethylbenzimidazol caebocyanine (JC-1). The results (Fig. 5) demonstrated that **11j** elevated the proportion of cells with mitochondrial depolarization in a concentration-dependent manner, raising the percentage from 7.04% in the control group to 19.29%, 28.66% and 51.15%, respectively. So, **11j** induced apoptosis by promoting the loss of mitochondrial membrane potential in K562 cells.

Pro- and anti-apoptotic factors affect apoptosis through cellular pathways. Mitochondria-dependent apoptotic pathways play important roles in cell apoptosis.[47,48] Bcl-2 family, including pro-apoptotic (Bax, Bak, Bid and Bad) and anti-apoptotic (Bcl-2, Bcl-xL and Bcl-w) proteins, induces apoptosis by influencing the permeability of mitochondria membrane, causing cytochrome *c* released into the cytosol and activating caspase-3 and -9.[49,50] Survivin is an anti-apoptotic factor, which controls the activities of caspase-3, −7 and −9 to inhibit apoptosis.[51–53] Claspin is a protein which is cleaved by caspase-7 during the initiation of apoptosis and causes the dysfunction of claspin to induce cell cycle arrest and repair subsequent damage. It regulates the activity of Chk1 and arrest cell cycle at S phase.[54–55] Therefore, down-regulated the expression of claspin inhibits cancer cells survival. The relations of these proteins and apoptosis are shown in Fig. 6. To figure out whether the expression of pro- and anti-apoptotic factors were influenced by **11j**, human apoptosis array was performed in K562 cells treated with **11j**. The relative levels of 35 apoptosis-related proteins in K562 cells were detected simultaneously (Fig. 7). The original image could be found in the supporting information (Fig. S49). A comparison was performed to determine the relative changes on apoptosis-related protein levels. As showed, **11j** up-regulated the expression of proapoptotic protein Bad while suppressing the expression of anti-apoptotic protein Bcl-xL, survivin and claspin. Moreover, the expression of anti-apoptotic factors catalase and clusterin were also reduced. Therefore, **11j** induced K562 cell apoptosis via up-regulating the pro-apoptotic proteins and down-regulating the anti-apoptotic proteins.

In summary, in order to improve its antiproliferative activity against

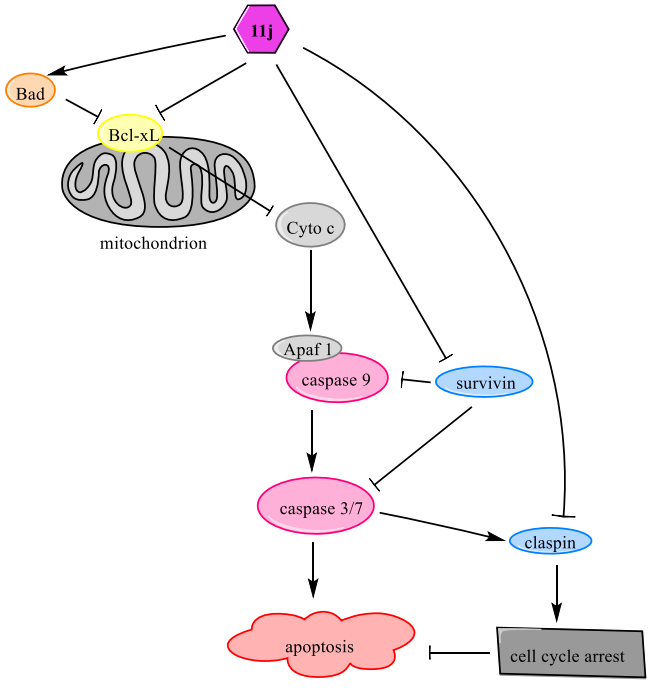

Fig. 6. The apoptosis pathways of apoptosis-related proteins influenced by **11j**.

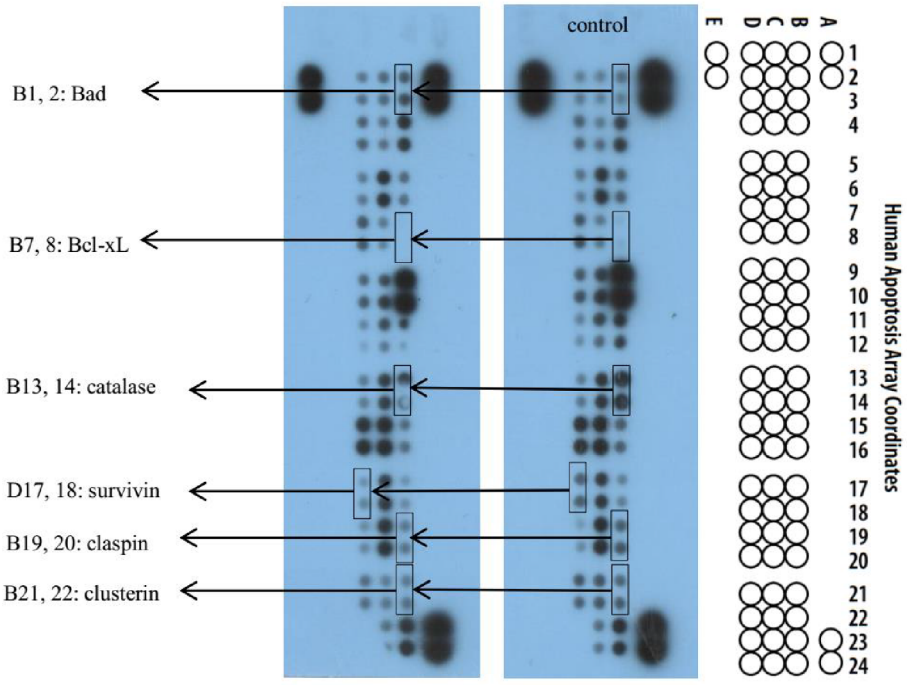

Fig. 7. The apoptosis protein array analysis of **11j**. Proteins were extracted from cells treated with **11j** for 24 h and detected the chemiluminescent signals by a digital imaging system.

tumor cells and reduce toxicity to normal cells, several furazan-type NO-donating derivatives of chelidonine were designed and synthesized. All the synthesized derivatives were tested for antiproliferative activities against four tumor cells HepG2, MCF-7, HCT-116 and K562, and two normal cells. Among them, **11j** exhibited good antiproliferative activity with IC_{50} values of 3.91, 6.90, 4.36 and 1.12 μM, respectively. NO-releasing ability results indicated that **11j** also released the highest amount of NO among the derivatives. In further mechanistic studies, **11j** was discovered to induce cell cycle arrest at S phase, cause disruption of mitochondrial membrane potential and enhance apoptosis in a dose-dependent manner. Subsequently, the results of human apoptosis array evidenced the effectiveness of **11j**-induced apoptosis through enhancing the expression of pro-apoptotic protein Bad and suppressing the expression of anti-apoptotic proteins Bcl-xL, catalase, survivin, claspin and clusterin in K562 cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126913.

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41. Mp: 51–53 °C, White solid, yield: 44%. 1H NMR (DMSO-d6, 600 MHz) δ (ppm): 8.01 (d, 2H, J = 7.8 Hz, Ar-H), 7.89 (t, 1H, J = 7.4 Hz, Ar-H), 7.74 (t, 2H, J = 7.6 Hz, Ar-H), 7.21 (d, 1H, J = 8.3 Hz, H-10), 7.10 (s, 1H, H-1), 6.75 (d, 1H, J = 8.3 Hz, H-9), 6.57 (s, 1H, H-4), 5.88-5.95 (m, 4H, 2 × -OCH2O-), 5.24 (s, 2H, -CH2-), 5.22 (m, 1H, H-11), 4.84 (s, 2H, -CH2-), 4.11 (d, 1H, J = 3.8 Hz, H-4b), 3.60 (d, 1H, J = 17.0 Hz, H-6), 3.53 (m, 1H, H-10b), 3.40 (d, 1H, J = 17.0 Hz, H-6), 2.79 (dd, 1H, J = 15.3, 4.7 Hz, H-12), 2.69 (m, 1H, H-12), 2.45 (s, 3H, N-CH3), 2.41 (m, 4H, 2 × -CH2-), 1.80 (m, 2H, -CH2-); 13C NMR (DMSO-d6, 150 MHz) δ (ppm): 171.82, 171.79, 158.0, 146.43, 146.35, 144.8, 143.5, 136.9, 136.2, 130.0, 130.0, 129.0, 128.4, 128.4, 127.0, 126.4, 121.2, 116.3, 110.7, 108.2, 107.4, 106.4, 100.9, 100.8, 84.1, 79.3, 71.7, 68.5, 60.6, 59.0, 54.9, 45.0, 41.0, 32.4, 30.8, 29.6, 19.7; HR(ESI)MS m/z calcd for C37H34N3O13S [M + H]+ 760.1786, found 760.1772. Spectrum data of compounds 10a-f, 11a-i, and experiment procedures of MTT assay, griess assay, cell cycle study, cell apoptosis assay, hoechst 333258 staining, mitochondrial membrane potential assay and Human Apoptosis Protein Array assay could be found in Supplementary Material.
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