1,4-Diselenophene-1,4-diketone Triggers Caspase-Dependent Apoptosis in Human Melanoma A375 Cells through Induction of Mitochondrial Dysfunction

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Epidemiological, preclinical and clinical studies have supported the role of selenocompounds as potential cancer chemopreventive and chemotherapeutic agents. In this study, a novel selenophene-based compound, 1,4-diselenophene-1,4-diketone (DSeD), has been synthesized by Double Friedel–Crafts reaction and identified as a potent antiproliferative agent against a panel of six human caner cell lines. Despite this potency, DSeD was relatively nontoxic toward human normal cells, HS68 fibroblasts and HK-2 kidney cells. These results suggest that DSeD possesses great selectivity between cancer and normal cells. Induction of apoptosis in human melanoma A375 cells by DSeD was evidenced by accumulation of sub-G1 cell population, DNA fragmentation and nuclear condensation. Activation of caspase-9 and depletion of mitochondrial membrane potential indicated the initiation of the mitochondria-mediated apoptosis pathway. Pretreatment of cells with general caspase inhibitor z-VAD-fmk and caspase-9 inhibitor z-LEHD-fmk significantly suppressed the cell apoptosis, demonstrating the important roles of caspase and mitochondria in DSeD-induced apoptotic cell death. Furthermore, DSeD-induced apoptosis was found independent of reactive oxygen species generation. Taken together, our results suggest that DSeD induces caspase-dependent apoptosis in A375 cells through activation of mitochondria-mediated apoptosis pathway.

Key words selenium; apoptosis; caspase activation; mitochondria; reactive oxygen species

Selenium (Se) is an essential trace element with fundamental importance to humans and animals.[1)] Epidemiological, preclinical and clinical studies have supported the role of selenocompounds as potential cancer chemopreventive and chemotherapeutic agents.[2,3)] The chemical forms and metabolic activity are determinants of anticancer activities of selenocompounds. Comparing with inorganic Se, organoselenium compounds show several advantages, such as higher absorptivity, better anticancer activity and lower toxicity. Therefore, during the past decade, a number of potent organoselenium compounds have been designed to achieve greater chemopreventive efficacy and minimal side effects by structural modifications, such as ebselen, selenocyanate, selenobetaine and Se analogues of amino acids and other sulfur compounds with known antitumor activity.[3)] For instance, 1,2,5-selenadiazolo-[3,4-*d*]pyrimidine-5,7-(4*H*,6*H*)-dione has been identified as a potent antiproliferative agent against human breast carcinoma MCF-7 cells, human hepatoma HepG2 cells and human melanoma A375 cells.[4)] Dhimant Desai *et al.* found that Se analogs of suberoylanilide hydroxamic was potent histone deacetylase inhibitors.[5)] 1-Benzyl-3-(5-hydroxymethyl-2-furyl)selenolo[3,2-*c*]pyrazole derivatives have also been synthesized and found to display favorable anticancer activity.[6)] Nowadays, the application of synthetic organoselenium compounds in chemoprevention and chemotherapy is a fascinating field for cancer research.

Thiophenes are sulphur-containing compounds widely distributed in Asteraceae (Compositae), a plant with known medicinal use.[7)] Several naturally occurring and synthetic dithiophenes and terthiophenes have been proved to possess insecticidal, bactericidal, antifungal, antiviral and anticancer activities.[8)] Since sulfur and Se are in the same group (VIA) of periodic table of elements, it is feasible to substitute sulfur atom in thiophene-based compounds with Se. Recent studies have reported that substituting sulfur with Se in established chemopreventive agents may lead to more effective analogs.[8—10)] D-501036, a novel selenophene-based triheterocycle, has been identified as a promising anticancer compound with potential for therapy of human cancers.[9)] Das and co-workers[10)] synthesized a novel Se analog (Se-PBIT) of a chemopreventive agent *S*,*S*′-(1,4-phenylenebis[1,2-ethanediyl])bisisothiourea (PBIT) and found that Se-PBIT was superior to PBIT as remarkable inducer of apoptosis and inhibitor of cancer cell growth. Moreover, Desai and co-workers verified that substitution of sulfur with Se increased the compound's potency by several folds in different cancer cell lines by inhibition of inducible nitric oxide synthase (iNOS)/Akt.[11)]

Malignant melanoma is a rapidly spreading skin tumor with a high invasive capacity and growing incidence.[12)] Although great improvement in survival rate of disseminated melanoma by using the contemporary therapeutic strategies, severe side effects, such as thrombocytopenia, neutropenia and anemia are unavoidable. Therefore, searching for new agents capable of selectively killing melanoma cells constitutes an urgent priority in the field of cancer research. Apoptosis, an active mode of cell death, plays a vital role in the development, homeostasis, and prevention of cancer. The role of apoptosis in action of anticancer drugs is becoming more and more clear.[13)] Generally, apoptosis could occur *via* death receptor-dependent (extrinsic) or mitochondria-mediated (intrinsic) pathway. The extrinsic pathway is triggered through the formation of death inducing signaling complex, which subsequently activates initiator caspase-8 and then

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cleaves executioner caspases.[14] Intrinsic apoptotic cell death is initiated by the release of cytochrome *c* from mitochondria into cytosol and subsequent activation of caspase-9.[13] Mitochondria play a central role in cellular metabolism and control of cell apoptosis.[15] Disruption of mitochondrial membrane potential and release of apoptogenic factors are important for activation of both caspase-dependent and caspase-independent apoptosis pathways.[15] Apoptosis has been found as one of the most critical mechanisms for anticancer action of selenocompounds.[3] We have previously showed that, selenocystine, a nutritionally available selenoamino acid, exhibited potent antiproliferative effects on human melanoma cells through induction of apoptosis with the involvement of reactive oxygen species (ROS) generation and p53-mediated mitochondrial dysfunction.[16] Dietary supplementation of selenomethionine and high-Se soy protein was also found be able to reduce the metastasis of melanoma cells in mice.[17] Moreover, apoptosis was identified as the major mode of cell death induced in melanoma cells by synthetic selenadiazole derivatives.[18] However, so far very little information about the apoptosis-inducing activities of selenophene-based compounds and the underlying mechanisms are available.

In the present study, we have synthesized three heterocycles, 1,4-difuranyl-1,4-diketone (DOD), 1,4-dithienyl-1,4-diketone (DSD) and 1,4-diselenophene-1,4-diketone (DSeD), and compared their *in vitro* anticancer activities. The results showed that substitution of sulfur and oxygen with Se significantly enhanced the apoptosis-inducing activities of thiophenes and furans against human melanoma cells. Further investigation on the intracellular mechanisms showed that DSeD triggered caspase-dependent and ROS-independent apoptosis in A375 cells through induction of mitochondrial dysfunction.

Results and Disscussion

DSeD, DSD and DOD were synthesized by double Friedel–Crafts reaction,[19] and characterized by HNMR, electrospray ionization (ESI)-MS and IR. The *in vitro* anticancer activities of DSeD, DSD and DOD were firstly screened against a panel of six human cancer cell lines, including A375, MCF-7, HepG2, HeLa299, Neuro-2a and PC-3 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 72 h treatment. As shown in Table 1, DSeD exhibited broad inhibition on the tested cancer cells with IC_{50} values significantly lower than those of its analogs, DOD and DSD, indicating the higher cytotoxic effects of DSeD on cancer cells. Moreover, to investigate the kinetics of the antiproliferative effects of DSeD, we treated A375 cells with different doses of DSeD for various periods of time and analyzed the cell viability by MTT assay. Data in Fig. 1 showed that DSeD treatment resulted in time- and dose-dependent decrease in cell viability. Despite this potency, DSeD was relatively nontoxic toward human normal cells, including HS68 fibroblasts and HK-2 kidney cells (Table 1). Over 70% of HS68 and HK-2 cells remained viable after 72-h incubation with the presence of 400 μg/ml DSeD (data not shown). These results suggest that DSeD possesses great selectivity between cancer and normal cells.

We next investigated the underlying mechanism of DSeD-induced cell death. Inhibition of proliferation in cancer cells treated with anticancer drugs could be the result of induction

Table 1. Growth Inhibitory Effects of DOD, DSD and DSeD on Various Human Cancer and Normal Cells[a)]

Cell lines	IC_{50} (μg/ml)		
	DOD	DSD	DSeD
A375	>400	115.0±8.4	83.3±9.9
HepG2	371.0±40.3	236.6±17.4	109.8±10.7
MCF-7	>400	190.1±25.9	123.5±14.1
HeLa229	NE	>400	343.0±3.2
Neuro-2a	NE	343.0±3.2	103.5±4.4
PC-3NE	>400	174.3±23.6	
HK-2	>400	>400	353.7±1.6
HS68	>400	>400	>400

NE: no effect. *a*) Cells were treated with various concentrations of tested compounds for 72 h. Cell viability was determined by MTT assay and IC_{50} values were calculated as described in Experimental. Each value represents the mean±S.D. of three independent experiments.

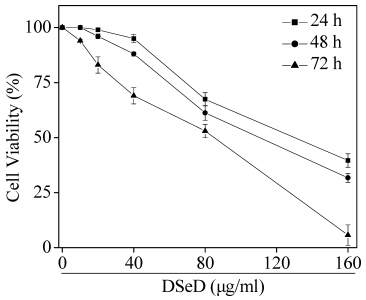


Fig. 1. Cell Growth Inhibition Induced by DSeD in A375 Cells

Cells were treated with different concentrations of DSeD for 24, 48, and 72 h. Cell viability was determined by MTT assay.

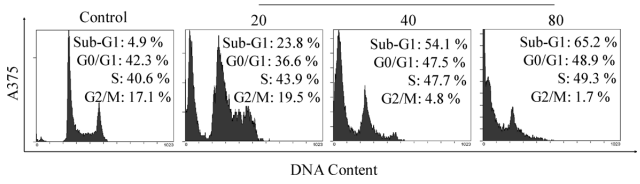


Fig. 2. Cell Cycle Analysis of A375 Cells Exposed to DSeD

The cells treated with DSeD for 72 h were collected and stained with PI after fixation. Each value represents the mean of three independent experiments.

of apoptosis or cell cycle arrest or a combination of these two modes. Therefore, we performed propidium iodide (PI)-flow cytometric analysis to determine whether apoptosis was involved in the cell death induced by DSeD in the most susceptible A375 human melanoma cells. Figure 2 showed the representative DNA histograms obtained after PI staining of permeabilized cells, which revealed that exposure of A375 cells to different concentrations of DSeD for 72 h leaded to dose-dependent increases in the proportion of apoptotic cells as reflected by the Sub-G1 cell population. Moreover, no significant change in cell cycle distribution was observed in DSeD-treated cells. Therefore, cell death induced by DSeD was primarily attributed to induction of apoptosis. This finding was further confirmed by DNA fragmentation and nuclear condensation as detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) enzymatic labeling and 4′,6-diamidino-2-phenyindole (DAPI) co-staining assay. DNA fragmentation is an important biochemical hallmark of cell apoptosis. TUNEL assay can be used to detect

early stage of DNA fragmentation in apoptotic cells prior to changes in morphology. As shown in Fig. 3, dose-dependent increase in DNA fragmentation and nuclear condensation were observed in A375 cells exposed to 40 and 80 μg/ml of DSeD.

Apoptosis may occur *via* two crucial pathways, including death receptor-mediated (extrinsic) and the mitochondria-mediated (intrinsic) pathways. The former is triggered by activation of death receptors, such as Fas and tumor nephrosis factor related apoptosis inducing ligand (TRAIL) receptors (DR4, DR5), and the subsequent cleavage of caspase-8/10.[20] The mitochondrial (intrinsic) pathway is mediated by Bcl-2 family proteins, a group of anti-apoptotic and pro-apoptotic proteins that regulate the release of apoptogenic factors from the mitochondria into cytosol, such as cytochrome *c*, Smac/Diablo and AIF, which then activate the caspase-dependent or -independent apoptotic pathways.[21] Activation of caspase-9 during this process will cleave and trigger the downstream apoptotic signal. To determine whether caspase activation was involved in the DSeD-induced apoptosis, the activity of an important effector caspase (caspase-3) and two initiator caspases (caspase-8 and caspase-9) were measured by fluorometric assays. As shown in Fig. 4A, treatments of A375 cells with DSeD activated caspase-3, -8 and -9 in a dose-dependent manner. Activities of caspase-9 and caspase-3 increased for 1.3—1.8 and 1.2—1.6 folds, respectively, in cells exposed to 20—80 μg/ml of DSeD by comparing with control. In contrast, only slight increase in activity of caspases-8 (1.1—1.2 fold) in response to DSeD treatment was observed. These results suggest that the contribution of extrinsic pathway to DSeD-induced apoptosis is likely to be insignificant.

In order to further evaluate the roles of caspases in DSeD-induced apoptosis, we examined the effects of various caspase inhibitors, including general caspase inhibitor z-VAD-fmk, caspase-8 inhibitor z-IETD-fmk and caspase-9 inhibitor z-LEHD-fmk, on cell apoptosis. As shown in Fig. 4B, DSeD-induced apoptotic cell death was remarkably suppressed by pretreatment of cells with 40 μM z-VAD-fmk as measured by flow cytometric analysis. These results indicate that DSeD-induced apoptosis mainly occurs in a caspase-dependent manner. Moreover, caspase-9 inhibitor z-LEHD-fmk significantly attenuated the DSeD-induced apoptotic cell death, but caspase-8 inhibitor z-IETD-fmk failed to do so. Based on these results, it can be concluded that mitochondria-mediated apoptotic pathway play the major role in DSeD-induced apoptosis in A375 cells. This conclusion was further verified by the observation of mitochondrial dysfunction in response to DSeD treatment.

Mitochondria play a critical role in the regulation of apoptosis. The intermembrane space contains several apoptogenic factors, such as cytochrome *c*, apoptosis-inducing factor, SMAC/Diablo and endonuclease G, which are liberated into cytosol during the disruption of $\Delta\Psi_m$.[22] The intrinsic and extrinsic apoptosis pathways can converge at the mitochondria level and trigger mitochondria membrane permeabilization.[23] Many studies have showed that permeabilization of the outer mitochondria membrane and the subsequent

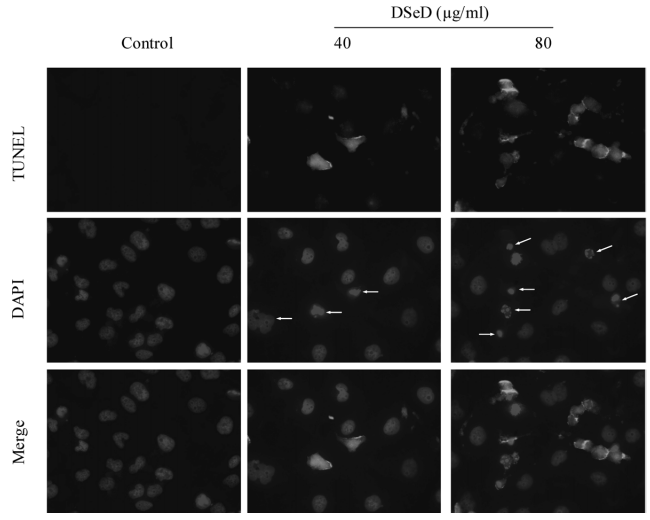


Fig. 3. Representative Photomicrographs of DNA Fragmentation and Nuclear Condensation in Response to DSeD Treatment as Detected by TUNEL Assay and DAPI Staining. A375 Cells Were Treated with 40 and 80 μg/ml DSeD for 24 h.

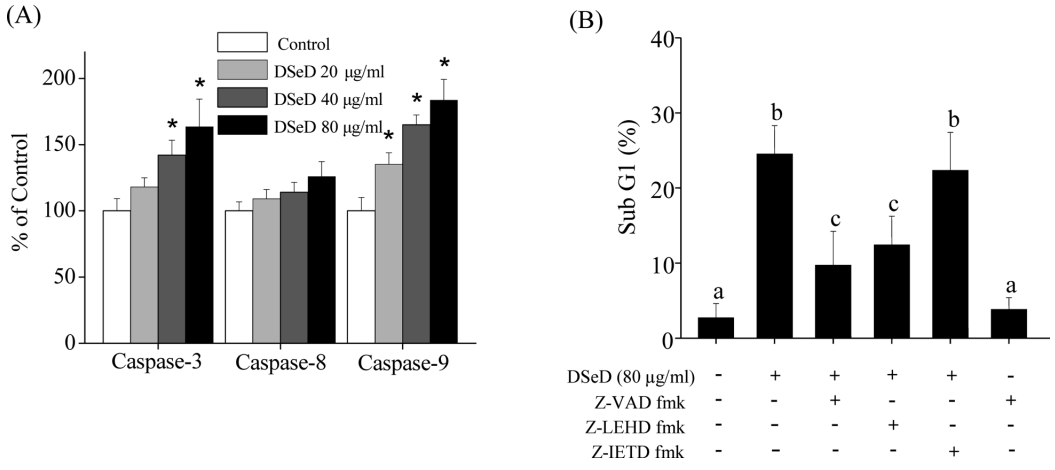


Fig. 4. DSeD Induces Caspase-Dependent Apoptosis in A375 Cells

(A) Analysis of caspase activation in DSeD-induced apoptosis in A375 cells. Caspase activities were measured using synthetic fluorescent substrates for caspase-3, caspase-8 and caspase-9 as described in Results and Discussion. (B) Effects of various caspase inhibitors (40 μM) on apoptosis induced by DSeD. Cells were pre-treated with caspase inhibitors for 2 h followed by co-incubation with DSeD for 24 h. Apoptotic cells were determined by flow cytometric analysis. All results were obtained from three independent experiments. Significant difference between treatment and control groups is indicated at $*\ p<0.05$ level.

release of pro-apoptotic proteins from the intermembrane space play an essential role in both caspase-dependent and caspase-independent apoptosis pathways.[24] JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria. During the loss of $\Delta\Psi_m$, the fluorescence of JC-1 dye shifts from red to green. The increase in green fluorescence indicates the loss of $\Delta\Psi_m$ in the treated cells. Therefore, we studied the status of $\Delta\Psi_m$ in DSeD-treated A375 cells by JC-1 flow cytometric analysis. As shown in Fig. 5, DSeD treatment induced a dose-dependent increase in depletion of $\Delta\Psi_m$. The percentage of depolarized mitochondria in cells exposed to 20, 40 and 80 μg/ml of DSeD increased from 1.2% (control) to 11.4%, 30.0% and 44.4%, respectively.

Mitochondria are the major intracellular source of ROS, especially superoxide and hydrogen peroxide.[25] ROS has been reported as an important regulator of cell apoptosis induced by various chemopreventive and chemotherapeutic agents.[26] Several studies have clarified that change in intracellular ROS level could induce cancer cell apoptosis, and inhibit the invasion and metastasis.[27] A mass of apoptotic stimuli cause cancer cell apoptosis through triggering cytochrome *c* release and overproduction of ROS.[28] Excess ROS could attack various components of DNA, leading to the generation of a variety of ROS-mediated modified products, including oxidized bases, DNA strand breaks, DNA intra-strand adducts, and DNA–protein crosslinks.[29] The detection of mitochondrial dysfunction by JC-1 probe led us to examine the role of ROS in DSeD-induced apoptosis. The intracellular ROS generation in A375 cells treated by DSeD was measured by DCF-flow cytometry. This assay is based on the cellular uptake of a non-fluorescent probe DCFH-DA,

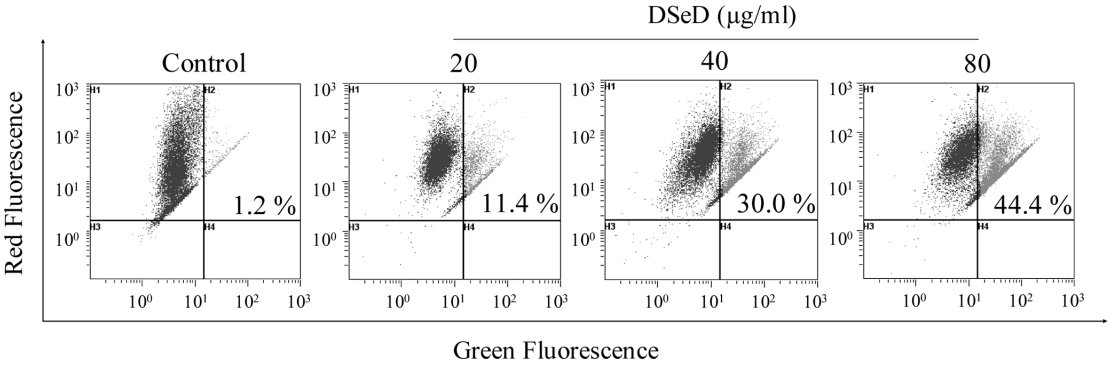


Fig. 5. DSeD Induces the Depletion of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Cells treated with DSeD were harvested and stained with the mitochondria-selective dye JC-1 and then analyzed by flow cytometry. The number in the right region of each dot plot represents the percentage of cells that emit green fluorescence due to the depletion of $\Delta\Psi_m$.

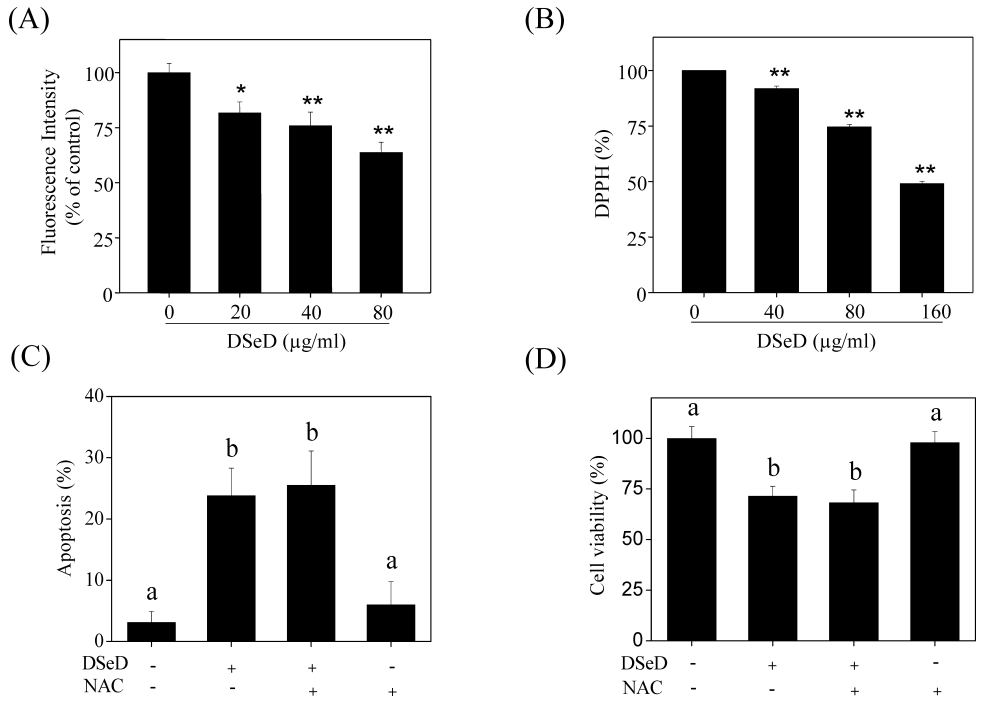


Fig. 6. ROS Production in A375 Cells Exposed to DSeD (A), *in Vitro* Antioxidant Activities of DSeD (B) and the Effects of NAC (2 mM) on DSeD-Induced Cell Apoptosis (C) and Cell Viability (D)

(A) Cells were exposed to the indicated concentrations of DSeD for 3 h and the levels of the intracellular ROS were analyzed by DCFH-DA fluorescence intensity. (B) *In vitro* antioxidant activity of DSeD as determined by DPPH free radical scavenging assays. (C, D) Cells were exposed to 80 μg/ml DSeD for 24 h with or without the pretreatment of NAC for 4 h. Apoptotic cell death was determined by flow cytometric analysis. Values expressed are means±S.D. of triplicates. Significant difference between treatment and control groups is indicated at $*p<0.05$ or $**p<0.01$ level.

which is subsequently hydrolyzed by intracellular esterase to form dichlorofluorescein, DCFH. The non-fluorescent substrate is oxidized by the intracellular free radicals, producing a fluorescent product DCF.[30] The treatment of DSeD led to a rapid disruption of $\Delta\Psi_m$ in A375 cells (Fig. 5). Therefore, we examined the intracellular ROS generation in A375 cells by measuring the DCF fluorescence intensity to test whether ROS was implicated in DSeD-induced apoptosis. As shown in Fig. 6A, DSeD treatments for 3 h resulted in dose-dependent decrease in DCF fluorescence intensity, indicating the down-regulation of intracellular ROS generation by DSeD. The total antioxidant activity of DSeD was thus evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. The results showed that DSeD dramatically inhibited the formation of DPPH free radicals in a dose-dependent manner (Fig. 6B), demonstrating the potent antioxidant activity of DSeD. Moreover, a thiol-reducing antioxidant *N*-acetylcysteine (NAC) failed to prevent the cells from DSeD-induced apoptosis (Fig. 6C) and growth inhibition (Fig. 6D). These results indicate that DSeD-induced apoptosis in A375 cells is independent of ROS generation.

Experimental

Materials Furan (AR, Aldrich), thiophene (AR, Aldrich), selenophene (AR, Sigma), $AlCl_3$ (AR, Aldrich) and succinyl chloride (AR, Sigma) were purchased commercially. DPPH, thiazolyl blue tetrazolium bromide (MTT), DAPI, propidium iodide (PI) and NAC were obtained from Sigma. Caspase-3 substrate (Ac-DEVD-AFC), caspase-8 substrate (Ac-IETD-AFC) and Caspase-9 substrate (Ac-LEHD-AFC) were purchased from Calbiochem. The general caspase inhibitor (z-VAD-fmk) was purchased from Calbiochem. The caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were obtained from Merck. Air-sensitive reagents were manipulated in an argon atmosphere. All solvents were dried and purified by standard procedures.

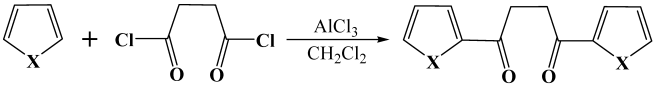
Synthesis and Characterization The schematic route for the synthesis of DOD, DSD and DSeD was showed in Chart 1.

Synthesis of DOD A CH_2Cl_2 solution containing furan (5 ml) and succinyl chloride (1 g) was added dropwise to an anhydrous CH_2Cl_2 solution (100 ml) containing $AlCl_3$ (5 g) under Ar_2 at 0 °C. The reaction mixture was stirred at 0 °C for 2 h, slowly warmed to room temperature, and stirred for 12 h. Then the mixture was poured into a beaker containing ice. Ethyl acetate was added and the organic layer was in a separatory funnel. After removing the solvent under vacuum, the residue crystallised with hexanes : ethyl acetate (7 : 3). Yields, 25%.[31] ^{1}H-NMR ($CDCl_3$) δ: 7.61 (2H, dd, J=1.7 Hz, 5-H), 7.25 (2H, dd, J=3.6 Hz, 3-H), 6.55 (2H, dd, J=3.6 Hz, 4-H) and 3.30 (4H, s, CH_2); IR (KBr) cm^{-1}: 1661 (C=O), 1651, 1574, 1496, 1324 and 1036; MS (EI) m/z: 218 $[M+H]^+$.

Synthesis of DSD DSD was prepared in a method similar to that of DOD, except, the residue crystallized from hexanes. Yields, 50%.[32] ^{1}H-NMR ($CDCl_3$) δ: 3.41 (4H, s, $COCH_2$), 7.16 (2H, dd, J=3.8, 1.1 Hz, H-4,4′), 7.66 (2H, dd, J=3.8, 5.0 Hz, H-3,3′), 7.83 (2H, dd, J=1.1, 5.0 Hz H-5,5′); IR (KBr) cm^{-1}: ν=3100, 2920, 1648 ; MS (EI) m/z: 250 $[M+H]^+$.

Synthesis of DSeD DSeD was synthesized in an approach similar to that of DOD, with the crude product was purified by crystallizing from petroleum. Yields, 25%.[33] ^{1}H-NMR ($CDCl_3$) δ: 8.36 (2H, dd, J=5.2, 0.8 Hz), 8.02 (H, dd, J=4, 1.2 Hz), 7.40 (2H, dd, J=5.6, 4.4 Hz), 3.39 (4H, s); IR (KBr) cm^{-1}: 1652 (C=O); MS (EI) m/z: 345.9 $[M+H]^+$.

Cell Culture The cell lines used in this study, including human melanoma A375 cells, human liver cancer HepG2 cells, human breast carcinoma MCF-7 cells, human cerical cancer HeLa299 cells, mouse neuroblastoma Neuro-2a cells, human Prostate cancer PC-3 cells, human proximal tubular epithelial HK-2 cells and human HS68 fibroblast were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). The cells were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) medium, which was supplemented with penicillin (100 units/ml), 10% fetal bovine serum and streptomycin (50 units/ml) at 37 °C in a humidified incubator with 5% CO_2 atmosphere.



DOD: X=O; DSD: X=S; DSeD: X=Se

Chart 1. Schematic Route for Synthesis of DOD, DSD and DSeD

MTT Assay Cell viability was examined by measuring the ability of cells to metabolize MTT to a purple formazan dye.[34] Cells were seeded in 96-well tissue culture plates for 24 h and then incubated with the tested compounds at different concentrations for different periods of time. After incubation, 20 μl/well of MTT solution (5 mg/ml phosphate buffered saline (PBS)) was added and incubated for 5 h. The medium was aspirated and replaced with 150 μl/well dimethyl sulfoxide (DMSO) to dissolve the formazan salt formed. The color intensity of the formazan solution, which reflects the cell growth condition, was measured at 570 nm using a microplate spectrophotometer (VSERSA Max).

Flow Cytometric Analysis The cell cycle distribution was examined by flow cytometry according to Li *et al.*[35] Briefly, the cells cultured with or without DSeD were trypsinized, washed with PBS and fixed with 70% ethanol overnight at −20 °C. The fixed cells were washed with PBS and stained with PI working solution (1.21 mg/ml Tris, 700 U/ml RNase, 50.1 μg/ml PI, pH 8.0) for 4 h in darkness. The stained cells were analyzed with Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL, U.S.A.). Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA, U.S.A.). The proportion of cells in G0/G1, S, G2/M phases was represented as DNA histograms. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment, 10000 events per sample were recorded.

TUNEL Assay and DAPI Staining Apoptotic DNA fragmentation induced by ASDO was examined by using an *in situ* cell death detection kit following the manufacturer's protocol. Briefly, cells cultured in chamber slides were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.1% triton X-100 in PBS. After then, the cells were incubated with 100 μl/well of TUNEL reaction mixture at 37 °C for 1 h. The nuclei of the cells were double stained with 1 μg/ml of DAPI for 15 min. Stained cells were examined on a fluorescence microscope (Nikon Eclipse 80i).

Caspase Activity Assay Harvested cell pellets were suspended in cell lysis buffer and incubated on ice for 1 h. After centrifugation at 11000×g for 30 min, supernatants were collected and immediately measured for protein concentration and caspase activities. The cell lysates were placed in 96-well plates and then specific caspase substrates (Ac-DEVD-AFC for caspase-3, IETD-AFC for caspase-8 and Ac-LEHD-AFC for caspase-9 substrate) were added. Plates were incubated at 37 °C for 1 h and caspase activity was determined by fluorescence intensity with the excitation and emission wavelengths set at 380 and 440 nm, respectively.

Evaluation of Mitochondrial Membrane Potential ($\Delta\Psi_m$) Cells in 6-well plates were trypsinized and resuspended in 0.5 ml of PBS buffer containing 10 μg/ml of JC-1. After incubation for 10 min at 37 °C in the incubator, cells were immediately centrifuged to remove the supernatant. Cell pellets were suspended in PBS and then analyzed by flow cytometry. The percentage of the green fluorescence from JC-1 monomers was used to represent the cells that lost $\Delta\Psi_m$.

Measurement of ROS Generation The effects of DSeD on ROS-initiated intracellular oxidation were evaluated by DCF fluorescence assay.[16] Briefly, collected cells were incubated with DCFH-DA at a final concentration of 10 μM at 37 °C for 30 min. The loaded cells were then washed twice with PBS and ROS level was determined by measuring the fluorescence intensity on a Tecan SAFIRE fluorescence reader.

DPPH Free Radical Scavenging Assays DPPH free radical scavenging activity of DSeD was measured according to the method as previously described.[36] Briefly, 20 μl of test samples at different concentrations was mixed with 180 μl of DPPH solution (60 μM in methanol) for 30 min in the dark, and then, the change in absorbance at 517 nm was measured. DMSO was used as a negative control.

Statistical Analysis Experiments were carried out at least in triplicate and results were expressed as mean±S.D. Statistical analysis was performed using SPSS statistical program version 13 (SPSS Inc., Chicago, IL, U.S.A.). Difference with $*p<0.05$ or $**p<0.01$ was considered statistically significant.

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