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ORIGINAL PAPER

Apoptotic effects of 1,5-bis-(5-nitro-2-furanyl)-1,4-pentadien-3-one on Drosophila SL2 cells

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Abstract In recent years, concerns over the potential impact of synthetic pesticides on the environment have promoted the research and development of environmentally friendly "green" pesticides. In the current study, we utilized a green biomimetic insecticide, 1,5-bis-(5-nitro-2-furanyl)-1,4-pentadien-3-one (compound C), to examine its cytotoxicity on an insect cell line, *Drosophila melanogaster* (SL2). Results from MTT assay showed cells treated with 100 μM of compound C for 48 h significantly inhibited the growth of SL2 cells by 87.71 ± 0.96%. We subsequently attempted to illustrate the act on mechanism of compound C at the cellular level and found that it initiated apoptosis through a mitochondrial-dependent mechanism that increased the activity of caspase-3 and altered the cell cycle. These results suggest that the green biomimetic insecticide, compound C, is a novel and potent inducer of insect cell apoptosis.

Keywords 1,5-Bis-(5-nitro-2-furanyl)-1,4-pentadien-3-one, Biomimetic insecticide, Apoptosis, Cytotoxicity, SL2

Agricultural pests are a serious threat to crop production around the world. For years, scientists have been dedicated to the search of novel insecticides in order to cope with the global food shortage. However, controlling insect pests with traditional pesticides have been shown to produce various side effects, such as pollution, residue with high toxicity for humans, etc.[1]. Thus, it is desirable to find new compounds that possess high bioactivity and low toxicity.

Compounds extracted from plants have often been regarded as bio-insecticides and have become valuable as new chemical candidates for the development of novel insecticide molecules[2,3]. As such, in 2001, our laboratory was the first to extract two compounds from *Stellera chamaejasme* L. (compounds A and B) (Figure 1A, B) with good insecticidal properties[4]. Bioassay results showed that the two compounds had strong bioactivity against *Aphis gossypii*[5,6]. In addition, compounds with furan rings have often been reported to display a variety of biological activities, such as insecticidal, fungicidal, antimicrobial and antitumor[7-9]. Therefore, to search for useful pesticide candidates, we previously designed and synthesized a series of furanyl compounds based on compounds A and B, replacing the benzene ring with a furan ring. The results showed that the newly synthesized compounds had excellent biological activity[10,11].

Of these synthesized compounds, we subsequently found that 1,5-bis-(5-nitro-2-furanyl)-1,4-pentadien-3-one (compound C) (Figure 1C) had the highest bioactivity against *Aphis gossypii*[12]. Moreover, compound C also showed better cytotoxic effects; following exposure to compound C at 100 μM for 48 h the rate of inhibition on SL2 cells was nearly 90%. However, to our knowledge, there have been few reports that further focused on the toxic effect and the mechanisms of action of compounds with furan rings in insect cells.

Therefore, in the current paper we used the *Drosophila melanogaster* cell line (SL2) to investigate the potential of compound C to induce apoptosis in cells. We also investigated cell inhibition, DNA fragmentation, caspase-3 activation, mitochondrial membrane potential (ΔΨm), and the cell cycle.

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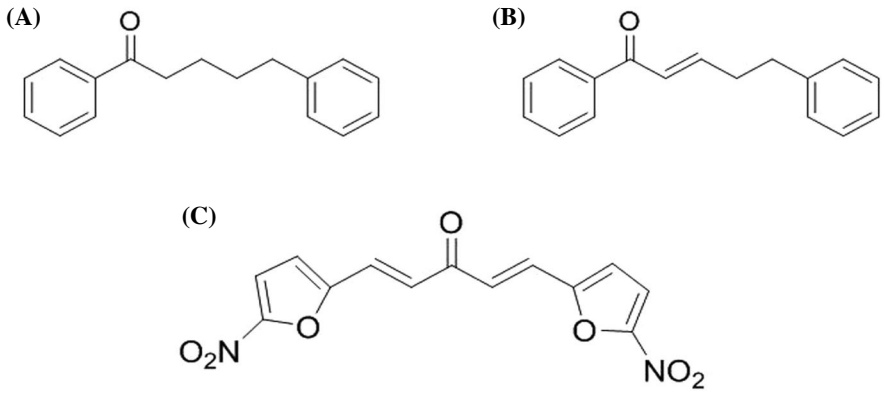

Figure 1. Structure of compound A, B and C. A: 1,5-Diphenyl-1-pentanone; B: 1,5-Diphenyl-2-penten-1-one; C: 1,5-Bis-(5-nitro-2-furanyl)-1,4-pentadien-3-one.

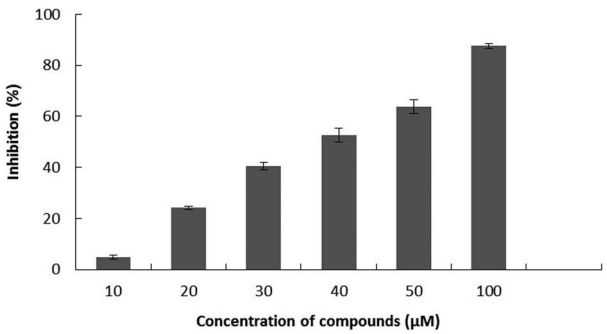

Figure 2. Compound C effects SL2 proliferation in a dose-dependent manner. Inhibition (%) = (OD490 of the 0.1% DMSO-treated cells-OD490 of compound C-treated cells)/OD of 0.1% DMSO-treated cells × 100%. Values are shown as mean ± SEM.

Effect of compound C on cell viability

The cytotoxicity of compound C on SL2 was assessed at different concentrations (10, 20, 30, 40, 50 and 100 μM). As showed in Figure 2, the inhibition of SL2 proliferation was 6.29 ± 0.75%, 25.50 ± 0.67%, 41.56 ± 1.52%, 53.48 ± 2.65%, 64.40 ± 2.60% and 87.71 ± 0.96 % (mean ± SD) respectively. These results indicate that compound C has excellent cytotoxic effects on SL2 cells.

Compound-induced apoptosis on SL2 cells

After exposure to the compound for 24 h, the cells tend to have only minor morphological changes, characteristic of early apoptosis, including cytoplasmic dehydration with a concomitant modification of the cell shape and size and/or condensation of the nuclear chromatin into a concave shape resembles half-moon (Figure 3B). Following a 48 h treatment, we found that the cells had morphological changes indicative of late apoptosis, including condensation of the cytoplasm with concomitant shrinking and alterations in the shapes of the cells, in addition to apoptotic bodies with membrane bubbles (Figure 3C).

DNA fragmentation assays also revealed characteristics of apoptosis. Following agarose gel electrophoresis, a typical ladder pattern of inter nucleosomal fragmentation was observed on SL2 cells after treatment for 24 and 48 h (Figure 4).

Caspase-3 activity

The activation of caspase is a key feature of apoptosis. As such, SL2 cells were exposed to 50 μM of compound C for 0, 4, 8, 12, and 24 h and the resultant activities of caspase-3 were determined. As shown in Figure 5, the activity of caspase-3 increased significantly following 4 h and 8 h ($P < 0.05$), reached its maximum at 12 h ($P < 0.01$). Then declined at 24 h ($P < 0.01$).

Mitochondrial membrane potential (ΔΨm) change

Mitochondrial membrane potential (ΔΨm) is a marker of mitochondrial function often associated with apoptosis[13,14]. Results indicated a remarkable decrease in ΔΨm (Figure 6). The peak values of Rh 123 fluorescence in the treated cells reduced by 37.41% ($P < 0.05$).

Cell cycle analysis

Incubation of compound C (50 μM) for 12 h produced a decrease in the percentage of cells in G1 phase as compared with the control (28.43% ($P < 0.01$)). A concomitant increase in cells during the S (23.92% ($P < 0.01$)) Phase was also observed. These results indicated that the inherent cells hyperplastic cycle was disrupted after treatment; some cells were blocked in S phase and some cells failed to go through mitosis.

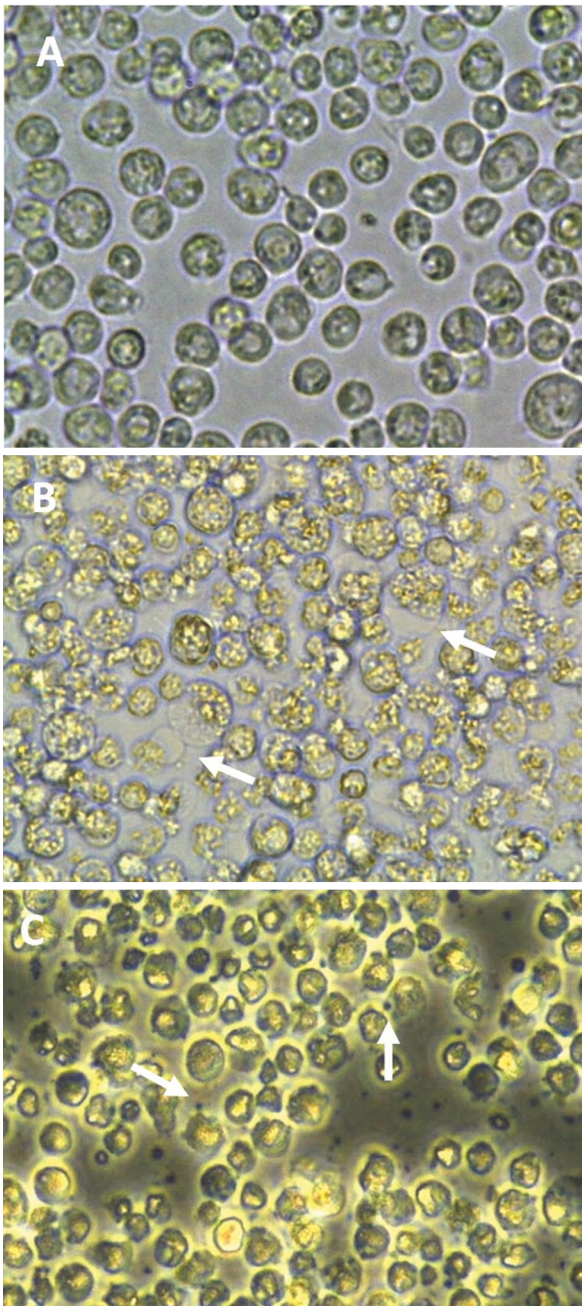

Figure 3. Morphological changes in the SL2 cell after treatment with compound C. A: control cells; B: 50 μM of compound C for 24 h; C: 50 μM of compound C for 48 h. Early apoptotic changes (half-moon structure) are seen in B and late apoptotic changes (apoptotic bodies) can be seen in C (demonstrated by arrows).

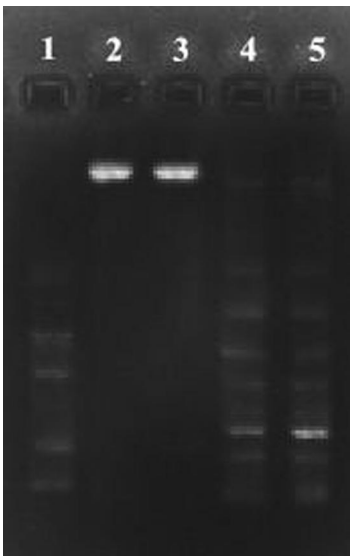

Figure 4. Electrophoresis analysis of the DNA. Lane 1: 100 bp DNA ladder; lane 2: untreated cells; lane 3: cells treated with 0.1% DMSO; lane 4: cells treated with compound C for 24 h; lane 5: cells treated with compound C for 48 h.

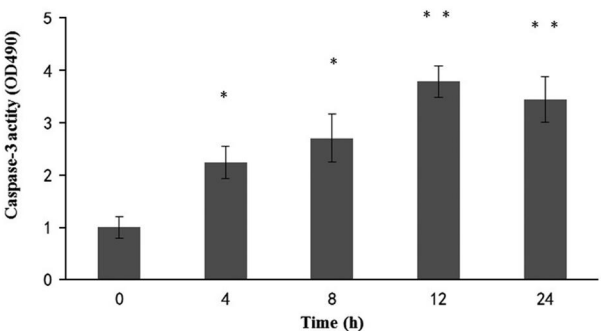

Figure 5. Caspase-3 activity of SL2 cells after treated with 50 μM compound C for 0, 4, 8, 12 and 24 h. Values are shown as mean ± SEM. Bars having asterisks are significantly different from each other (*$P < 0.05$ or **$P < 0.01$).

Discussion

The data revealed that phytochemicals can modify specific physiological processes of insects[15]. Meanwhile previous research also showed that a series of furan compounds possessed high insecticidal activity[7,16-18]. Then the new furan compound, 1,5-bis-(5-nitro-2-furanyl)-1,4-pentadien-3-one (compound C), was synthesized.

Studies at the molecular and cellular level can provide detailed information how chemicals interact with target sites[19]. Cell-based assays can quickly enable the discovery of new insecticide candidates and provide a new angle for investigating the toxicology and mechanism of action of biomimetic-synthesized insecticides at the molecular and cellular level[20]. Nonetheless, novel insecticide candidates are often missed using these techniques. However, in recent years, few studies have

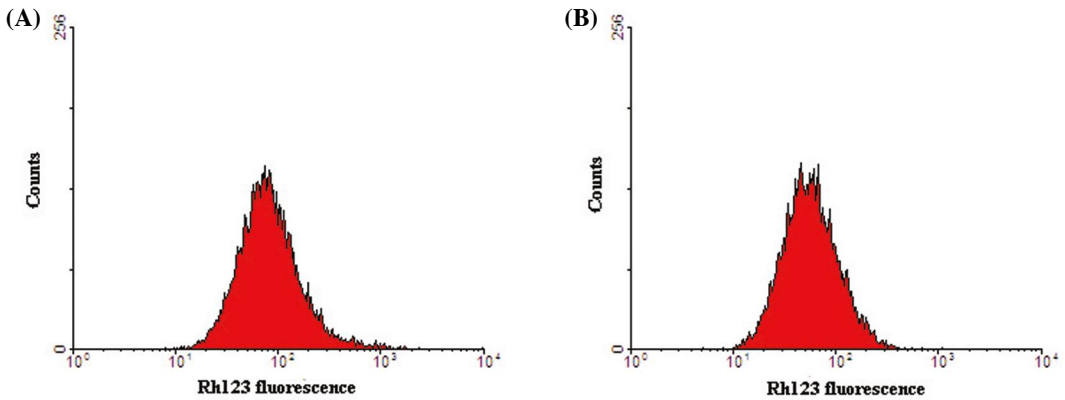

Figure 6. Effect of compound C on mitochondrial membrane potential. A: control cells; B: treated cells by 50 μM compound C for 12 h.

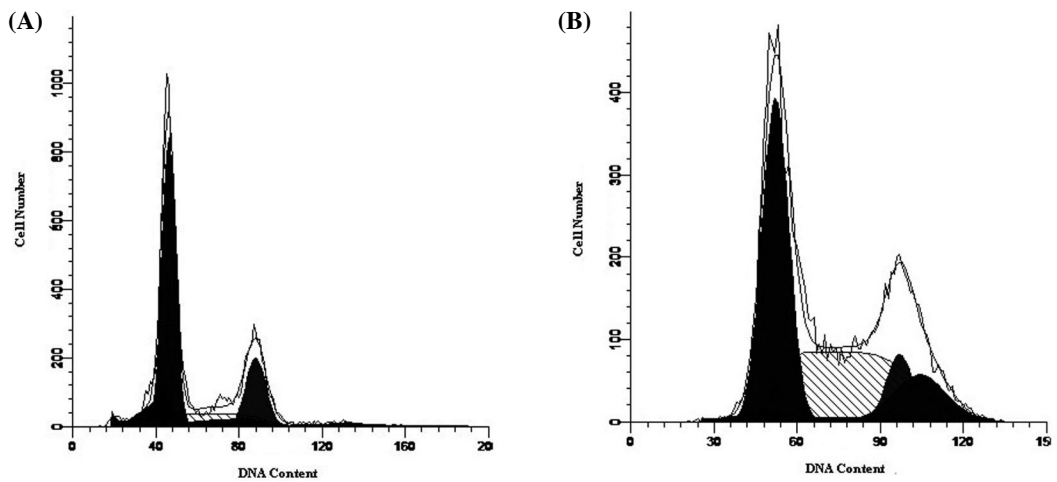

Figure 7. Cell cycle analysis. A: control cells; B: treated cells by 50 μM compound C for 12 h.

shown that compounds with insecticidal activity that have the addition of a furan ring can induce "typical" apoptosis on insect cell lines. Furthermore, furan compounds primarily appear to act through their antimicrobial activity instead of insecticidal activity[8].

In the present study, we used the SL2 insect cell line in order to investigate the effects of compound C at the cellular level and found it to be highly cytotoxic in a dose-dependent manner. Upon further evaluation, characteristics of apoptosis were detected.

Apoptosis is an active process of cell death, which includes morphogenesis, DNA fragmentation, cell cycle arrest, inhibition of caspase-3 activity, and reduction of the mitochondrial membrane potential.

Changes in morphogenesis have been defined as "proofs of physiological cell death", characterized by cell shrinkage, membrane blebbing, formation of apoptotic bodies, and fragmentation of nuclear DNA[21,22]. In the current study, we saw morphological changes typical of apoptosis in compound C-treated cells. Meanwhile, we also clearly detected a fragmentation in the DNA ladder. Taken together, these results suggested that the compound mediates apoptosis.

Recent studies have also confirmed that mitochondria play a decisive role in apoptosis by controlling the membrane potential ($\Delta\Psi m$)[13]. In fact, a loss of mitochondrial membrane potential ($\Delta\Psi m$) is considered the earliest events in process of apoptosis cascade and is one of specific signs of apoptosis[14]. Moreover, many apoptotic responses are initiated by the delivery of cytochrome-c from mitochondria, leading to the activation of caspase-3 and subsequent apoptosis[13]. Our results clearly show that mitochondrial membrane potential ($\Delta\Psi m$) reduced. Furthermore, we also detected a significant increase in the activity of caspase-3 during the early phase of apoptosis. These results fur-

ther implicate compound C in the induction of apoptosis.

The control of the cell cycle is the major regulatory mechanism involved in cell growth. Many cytotoxic agents arrest the cell cycle at G1, S, or G2/M phase causing apoptotic cell death[23]. In the present study we found that compound C induces an accumulation of SL2 cells in the S phase of the cell cycle thus leading to an overall inhibition in proliferation.

Taken together, the results from this study provide an essential foundation of knowledge necessary for elucidating the mechanism of action of 1,5-bis-(5-nitro-2-furanyl)-1,4-pentadien-3-one. This information is essential for developing novel series of compounds for use as insecticides.

Materials & Methods

Materials

Compound C was synthesized in our laboratory (Key Laboratory of Bio-Resources and Eco-Environment, Ministry of Education, China). Dimethyl sulfoxide (DMSO), Rhodamine 123 (Rh 123), phosphate buffer saline (PBS), thiazolyl blue tetrazolium bromide (MTT), agarose, propidium iodide (PI) and Schneider's insect medium were purchased from Sigma Chemical Co., Ltd. (St. Louis, USA). Fetal bovine serum (FBS) was the product of Thermo Fisher Scientific Co., Ltd. (Utah, USA) and the caspase-3 assay kit was purchased from KeyGEN Biotech Co., Ltd. (Nanjing, China). A genomic DNA extraction kit for cultured cells was the product of Tiangen Biotech Co., Ltd. (Beijing, China). All other reagents and chemicals used were of analytical grade.

Cell culture

The SL2 cell line was obtained from the China Center for Typical Culture Collection (CCTCC) and cultured in Schneider's insect medium supplemented with 10% fetal bovine serum and grown at 27˚C. The cultures were sub-cultured every 5 days.

Cell cytotoxicity assay

Cytotoxic effects were assayed using 24-well flat-bottomed plastic microplates. Cells with a density of 1×10^5 cells per milliliter (0.5 mL) were seeded into each well. After overnight incubation, the cells were exposed to compound C (dissolved in DMSO) with a series of concentrations (10, 20, 30, 40, 50, and 100 μM) for 48 h. The cell viability was analyzed by the MTT colorimetric assay method[24] and calculated as the percent of absorbance in the control (0.1% DMSO treated cells).

Morphological changes

Compound C was added to the culture at a final concentration of 50 μM and the morphological characteristics of SL2 cells were recorded with an inverted phase contrast microscope (Lecia, GER) following 48 h of treatments. Cells cultured with 0.1% DMSO were used as the control group.

DNA fragmentation assay

SL2 cells were treated with 50 μM of compound C for either 24 h or 48 h. Cells cultured with 0.1% DMSO were used as the control group. Then the cells were harvested by centrifugation at 1000 rpm for 5 min at 4˚C and washed twice with PBS. The genomic DNA was isolated by TIANamp genomic DNA extraction kit and analyzed via DNA electrophoresis on a 1.5% agarose gel[25].

Measurement of caspase-3 activity

The activity of caspase-3 was evaluated on the basis of spectrophotometric detection with the Capase-3 assay kit. After treatment with 0.1% DMSO and compound C (50 μM) for 0, 4, 8, 12, and 24 h, the cells were collected by centrifugation at 10000 rpm for 10 min at 4˚C and subsequently resuspended in cell lysis buffer and incubated on ice for 1 h; the resulting supernatant (50 μL) was mixed in 2 × Reaction Buffer (50 μL) and Caspase-3 Substrate (5 μL) and incubated at 37˚C for 4 h. Finally, caspase-3 activity was measured at 405 nm with a microplate reader (BioTek, USA).

Mitochondrial membrane potential ($\Delta\Psi m$) analysis

The mitochondrial membrane potential was analyzed by flow cytometry. After treatment with compound C at concentrations of 50 μM and 0.1% DMSO (the control) for 12 h, the SL2 cells (1×10^5 cells per milliliter) were harvested and washed twice with PBS and then incubated with Rh 123 (2 μM for final concentration) in the dark for 60 min at 37˚C. The cells were collected by centrifugation after being washed twice with PBS to remove extracellular Rh 123 and the fluorescent intensity was measured by flow cytometry (Beckman Coulter Inc, USA) at an excitation wavelength of 507 nm and an emission wavelength of 529 nm.

Cell cycle analysis

SL2 cells were treated with 50 μM compound and 0.1% DMSO (the control) for 12 h then subsequently harvested by centrifugation at 1000 rpm for 5 min at 4˚C and washed twice with PBS. The cells were then fixed with 70% ethanol at 4˚C overnight, washed twice with PBS, then incubated with RNAase (20 mg/L for the fi-

nal concentration) for 30 min at 37˚C. Finally, the cells were stained with 50 mg/L propidium iodide (PI) in the dark for 30 min at 4˚C and then filtered with cell strainers (75 μm). Cell cycle analysis was carried out with flow cytometry (Beckman Coulter Inc, USA). The proportion of each phase was calculated using the ModFit LT software (Verity Software House, USA).

Statistical analysis of data

All experiment data were generated from at least three independent experiments and statistical analyses were performed using SPSS V 17. Statistical comparisons were performed by Student's *t*-test (Significant difference was taken as $*P<0.05$ or $**P<0.01$). Error bars represent SEM.

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Conflict of Interest The authors declare no conflict of interest.

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