C_{11} cyclopentenone from the ascidian *Diplosoma* sp. prevents epidermal growth factor-induced transformation of JB6 cells

Sergey N. Fedorov, Sergey A. Dyshlovoy, Larisa K. Shubina, Alla G. Guzii, Alexandra S. Kuzmich, Tatyana N. Makarieva

Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia

Abstract

C_{11} cyclopentenone, 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclopenta[b]pyran-6-one, isolated earlier from the sponges and ascidians, is known as a natural product possessing antimicrobial and cytotoxic properties. However, its cancer preventive activity has not been studied. Cancer preventive and proapoptotic properties of the compound as well as its effect on the main Mitogen-Activated Protein-Kinase (MAPK) signaling pathways were examined by the methods of epidermal growth factor- induced (EGF-induced) JB6 Cl41 P^+ cell transformation in soft agar, flow cytometry, and MTS test of cell viability. Results: the compound inhibits EGF-induced neoplastic JB6 Cl41 P^+ cell transformation in soft agar and induces apoptosis of HL-60 and THP-1 human leukemia cells. Jun N-terminal Kinase and p38 MAPK signaling pathways are involved in the cellular response to the treatment by the compound. Conclusions: 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclopenta[b]pyran-6-one and other related marine C_{11} cyclopentenones have potential for development of a new antitumor agent in cancer prophylactics and should be further investigated.

Introduction

5-Hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one (Figure 1), a representative of C_{11} cyclopentenone compounds, was isolated first from the marine sponge *Ulosa* sp.[1] We isolated it from the didemnid ascidian *Diplosoma* sp.[2] As was shown earlier, 5-Hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one demonstrates antimicrobial and cytotoxic activities.[1,3] However, its cancer preventive properties have not been examined.

The purpose of the present work is to study the cancer preventive and proapoptotic properties of 5-Hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one, as well as its action on Mitogen-Activated Protein-Kinase (MAPK) signaling pathways in mouse JB6 Cl41 cells.

Materials and Methods

General procedures

The analysis of the onset of apoptosis was performed by flow cytometry using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA). Cell colonies in the anchorage-independent phenotype expression assay were scored using the LEICA DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH, Germany) and Image-Pro Plus software, version 3.0 for Windows (Media Cybernetics, Silver Spring, MD, USA). The MTS reduction assay to determine cell viability was carried out using the μQuant microplate reader (Bio-Tek Instruments, Inc, USA).

Reagents

5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one was obtained as described previously[2] and was pure in accordance with NMR, MS, TLC, and HPLC data. Minimum essential medium (MEM) and RPMI medium were from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Calabasas, CA, USA). Penicillin/streptomycin and gentamycin were from Bio-Whittaker (Walkersville, MD, USA), L-glutamine was from Mediatech, Inc. (Herndon, VA, USA). The Annexin V-FITC Apoptosis Detection Kit was from Medical & Biological Laboratories (Watertown, MA, USA). Epidermal growth factor (EGF) was obtained from Collaborative Research (Bedford, MA, USA). The Cell Titer 96 Aqueous One Solution Reagent [5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS)] kit for the cell viability assay were from Promega (Madison, WI, USA).

Cell culture

The JB6 Cl41 P^+ mouse epidermal cell line and its stable transfectants JB6 Cl41 DN-ERK2, JB6 Cl41 DN-JNK1, or JB6 Cl41 DN-p38 cells were cultured in monolayers at 37°C and 5% CO_2 in MEM containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin.[4] The human tumor cell lines, HL-60 and THP-1, were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured at 37°C and 5% CO_2 in RPMI, containing 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Information regarding the genetic background of these cell lines is available online (http://www.atcc.org).

Anchorage-independent transformation assay

The cancer preventive effect of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one was evaluated using an anchorage-independent neoplastic transformation assay. EGF (10 ng/mL) was used for stimulating neoplastic transformation of JB6 Cl41 P^+ cells. The assay was carried out in six-well tissue culture plates. Mouse JB6 Cl41 P^+ cells (8×10^3 per mL) were treated with various concentrations of the substances in 1 mL of 0.33% basal medium Eagle (BME) agar con-

Correspondence: Dr. Sergey N. Fedorov, Pacific Institute of Bioorganic Chemistry, 159 Prospect 100-let Vladivostoku, Vladivostok, 690022, Russian Federation.
Tel. +7.4232.2311.168 - Fax: +7.4232.2314.050.
E-mail: fedorov@piboc.dvo.ru

Key words: C_{11} cyclopentenone, ascidian, *Diploso-ma* sp., cancer preventive activity, apoptosis.

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Contributions: SNF, participated in the study of biological activities of the compound studied and in paper preparation; SAD, participated in the study of biological activities of the compound studied; LKS, ASK, participated in the study of biological activities of the compound studied; AGG, TNM participated in the isolation and purification of the compound studied.

Conflict of interest: the authors declare that there are no conflicts of interest.

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taining 10% FBS over 3 mL of 0.5% BME agar containing 10% FBS and various concentrations of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one. The cultures were maintained in a 37°C, 5% CO_2 incubator for 1 week. Then cell colonies were scored.

Apoptosis assay using flow cytometry

The onset of early and late apoptosis was analyzed by flow cytometry using Annexin V-FITC and propidium iodide (PI) double staining. HL-60 or THP-1 cells, 1×10^6/10 cm dish, in 10% FBS-RPMI were treated with various concentrations of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one for 24 h. After incubation, cells were washed with PBS by centrifugation at 1,000 rpm (170 rcf) for 5 min, and processed for detection of apoptosis using Annexin V-FITC and PI staining according to the manufacturer's protocol. In brief, 1×10^5 to 5×10^5 cells were resuspended in 500 μL of 1× binding buffer (Annexin V-FITC Apoptosis Detection Kit, Medical & Biological Laboratories). Then, 5 μL of Annexin V-FITC and 5 μL of PI were added, and the cells were incubated at room temperature for 15 min in the dark and were analyzed by flow cytometry.

Cell cycle assay using flow cytometry

The cell cycle distribution of THP-1 cells treated with the cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one was analyzed by flow cytometry. The DMSO-treated cells (50 μL of DMSO per 1 mL of the medium) were used as a positive control. THP-1 cells were plated at 2×10^5 cells per well of 6-well plate in 10% FBS-RPMI. Then the cells were incubated with the indicated concentrations of the cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one for 24 h. After treatment with the substance, cells were harvested, centrifuged at 1,000 rpm (170 rcf) for 5 min and washed with PBS followed by centrifugation at the same conditions. Then 1 mL of ice-cold ethanol was added and cells were fixed at -20°C overnight. After 0.5 ml of PBS was added followed by centrifugation, and cells were stained with 20 μg/mL of propidium iodide (PI) and RNAse, 200 μg/mL, for 30 min on ice in the dark. DNA content was analyzed by a Becton Dickinson FACs Calibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). The population of apoptotic cells was determined using Cell Quest Pro software (BD Biosciences, San Jose, CA, USA).

Cell viability assay

The effect of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one on cell viability was evaluated using MTS reduction into its formazan product.[5] The cells were cultured for 12 h in 96-well plates (6000 cells/well) in the corresponding medium (100 μL/well) containing 5% FBS. The medium was then replaced with 5% FBS-medium containing the indicated concentrations of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one, and the cells were incubated for 22 h. Then 20 μL amount of MTS reagent was added into each well, and MTS reduction was measured 2 h later spectrophotometrically at 492 and 690 nm as background.

Statistics

The statistical computer program, Statistica 6.0 for Windows (StatSoft, Inc., Tulsa, OK, USA, 2001) was used for analysis of the obtained data.

Results and Discussion

Natural products having low cytotoxicity but showing anticancer effects are attracting more and more attention as good candidates in chemopreventive strategies. Several of these natural products, for example, resveratrol, a phytoalexin produced in grapewine skin,[6] caffeine and (−)-epi-gallocatechin gallate from tea,[7] flavonoids from berries and fruits,[8,9] terpenoids from marine alga and mollusks,[10] and some others inhibit carcinogenesis and induce apoptosis of tumor cells.

We also showed that 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b] pyran-6-one demonstrates cancer preventive properties at noncytotoxic concentrations

To assess whether C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one possesses cancer preventive properties, we used the well accepted anchorage-independent assay in soft agar and EGF (10 ng/mL) as a promoter of JB6 Cl41 P^+ cells colony formation.[11-15] The JB6 cell system of clonal genetic variants, including promotion sensitive (P^+), promotion resistant (P^-), or malignantly transformed cells, facilitates the search for chemopreventive compounds and helps to determine their cancer preventive properties at the molecular level.[16,17] The JB6 P^+, P^-, and transformed variants are a series of cell lines representing earlier to late stages of preneoplastic to neoplastic progression.[11,12,18] JB6 Cl41 P^+ cells undergo neoplastic transformation when stimulated with tumor promoters such as epidermal growth factor (EGF) or 12-O-tetradecanoylphorbol-13-acetate (TPA) resulting in the formation of colonies in soft agar. The transformation involves the activation of AP-1 nuclear factor which regulates the transcription of various genes related to inflammation, proliferation and metastasis.[4,11,19]

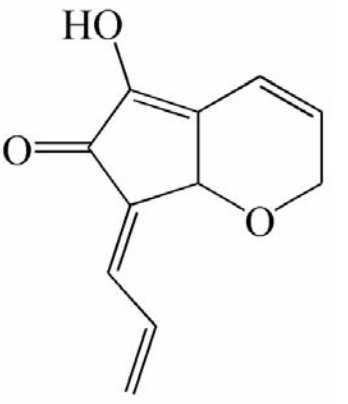

Figure 1. Structure of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one.

The results of our experiments using JB6 Cl41 P^+ cells in soft agar treated with compound 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one are shown in Figures 2A and B as the pictures (A) or as the numbers (B) of transformed JB6 Cl41 P^+ cell colonies in comparison with control. The experiments demonstrated that 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one at 5-10 μM concentrations in a dose-dependent manner inhibited EGF-induced neoplastic transformation of JB6 Cl41 P^+ cells (Figures 2A, B). Specifically, a 50% inhibition of EGF-induced JB6 Cl41 P^+ cells colony formation by 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one was achieved at a concentration of 6.8 μM (Figure 2B). This concentration is 3 times less than the dose that induces cytotoxicity, IC_{50}=21.5 μM (Figure 2C).

C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b] pyran-6-one induced apoptosis in human leukemia HL-60 and THP-1 cell lines

Apoptosis is a general mechanism for removal of unwanted cells from organisms and plays a protective role against carcinogenesis. Evidence from both *in vivo* and *in vitro* experiments shows that apoptosis is involved in successful cancer treatment and prevention using many drugs and cancer preventive natural substances.[20-23]

The ability of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one to induce apoptosis in human tumor cell lines was evaluated by flow cytometry using Annexin V-FITC and propidium iodide double staining. We used flow cytometry since no other method allows such rapid, quantitative and detailed analysis of the cell subpopulations in early, or late apoptosis, or necrosis.[24,25] Either HL-60 or THP-1 cells were treated with increasing concentrations of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one and harvested after 24 h. The results are shown in Figure 3 as the percentage of early (bottom right) or late (top right) apoptosis. Apoptosis was induced by 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one in human leukemia HL-60 (Figure 3A) and THP-1 (Figure 3B) cells in a dose-dependent manner. Furthermore, it was established that 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one is more active inducer of apoptosis in HL-60 than in THP-1 cells. Indeed, 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one induced about 40% of total apoptosis in HL-60 cells at a concentration of 10 μM (Figure 3A), whereas in THP-1 cells compound 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one induced the same per cent of apoptosis only at 30 μM concentration (Figure 3B). To confirm the induction of apoptosis by cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one, we used cell cycle experiments. It is known that apoptotic cells, as compared with necrotic cells, have reduced DNA stainability following staining with a variety of fluorochromes.[24,26] The appearance of sub-diploid DNA peak (sub-G_1 peak) is a specific marker of apoptosis; necrosis induced by cytotoxic drugs or lysis produced by complement do not induce any sub-G_1 peak in the DNA fluorescence histogram.[24,27] In our experiments we used THP-1 cells treated with 12.5, 25, or 50 μM concentrations of cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one. DMSO (50 μL per 1 mL of the medium) was added to the cells in culture as the positive control.[28] As was shown, cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one at concentrations of 25 or 50 μM induced sub-G_1 peak of 17.4% or 40.5%, correspondingly, as compared with sub-G_1 peak of 8.4% in negative control (Figure 4).

Our further experiments using JB6 Cl41 cells expressing the dominant-negative forms of JNK1, p38 or ERK2 allowed us to suggest some peculiarities of the molecular mechanism of cancer preventive action of C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one.

Jun N-terminal Kinase and p38 kinase are involved in C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b] pyran-6-one-induced effects

Three main MAPK signaling pathways have been identified in mammalian cells: through ERKs, JNKs, and p38 kinases. In general, JNKs and p38 are primarily activated by environmental stresses, whereas ERKs respond mainly to mitogenic and proliferative stimuli.[29] To identify which MAPK signaling pathways are involved in the action of compound 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one, we evaluated the role of MAPKs in the effect of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one on viability of JB6 Cl41 cells expressing the dominant-negative forms of JNK1, p38 or ERK2 as indicated by MTS method.[5] Time of incubation of various JB6 Cl41 cells with compound 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one was 24 h. The results are shown in Figure 5 as percent of viable cells in comparison with control. C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one showed dose-dependent effect on JB6 Cl41 cells viability. Expression of the dominant negative JNK1 or

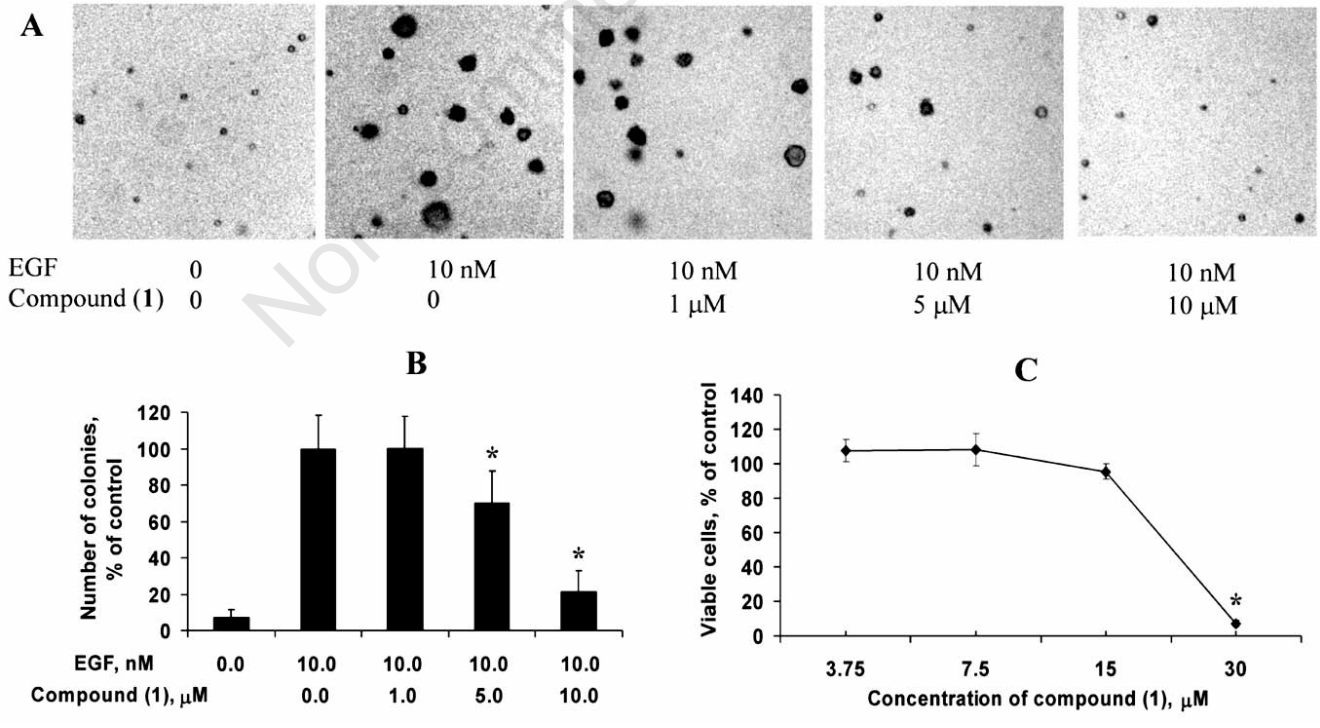

Figure 2. Inhibition of epidermal growth factor -induced neoplastic transformation of JB6 Cl41 P+ cells (A, B) by C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one. Cytotoxic activity of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one against JB6 Cl41 P+ cells (C). Data are shown as means±SD of six samples from two independent experiments. * P <0.05.

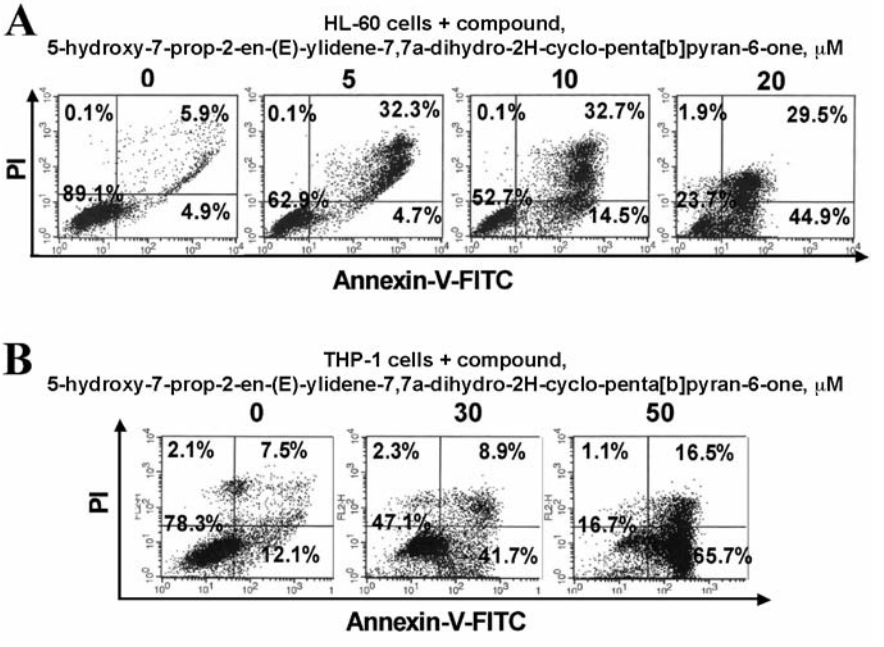

Figure 3. The induction of apoptosis by 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one in human leukemia HL-60 (A) or THP-1 (B) cells as analyzed by flow cytometry. Representative experiments are shown.

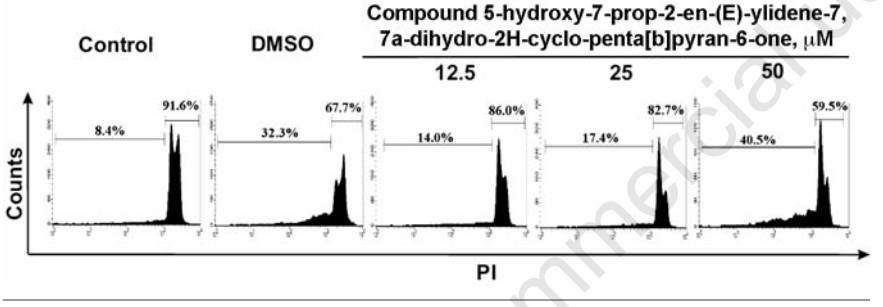

Figure 4. The induction of sub-G_1 peak by 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one in human leukemia THP-1 cells as analyzed by flow cytometry. Representative experiment is shown.

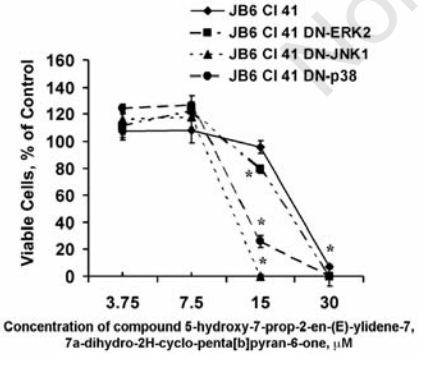

Figure 5. The effect of C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one on the viability of JB6 Cl41, JB6 Cl41 DN-ERK2, JB6 Cl41 DN-JNK1, and JB6 Cl41 DN-p38 cells. Data are shown as means±SD of six samples from two independent experiments. * P <0.05.

p38 dramatically induced the cytotoxic activity of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one in JB6 Cl41 DN-JNK1 or JB6 Cl41 DN-p38 cells, whereas expression of DN-ERK2 had little effect on cytotoxicity induced by 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one in JB6 Cl41 DN-ERK2 cells in comparison with control JB6 Cl41 cells (Figure 4). Therefore, JNKs and p38 are involved in C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one-induced effects in JB6 cells.

In conclusion, 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one, a representative of marine natural C_{11} cyclopentenone compounds showed cancer preventive properties at subtoxic concentrations. This activity of 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one is mediated, at least in part, by induction of apoptosis. Furthermore, MAPK JNKs and p38 signaling pathways are involved in C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one-induced effects in JB6 cells. Based on the obtained data, C_{11} cyclopentenone 5-hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclo-penta[b]pyran-6-one and its analogs may be used for the development of the new promising cancer preventive agents.

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