

## Induction of apoptosis and caspase-3 activation by chemopreventive [6]-paradol and structurally related compounds in KB cells

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### Abstract

[6]-paradol, a pungent phenolic substance found in ginger and other Zingiberaceae plants, has been demonstrated to be an effective inhibitor of tumor promotion in mouse skin carcinogenesis. In the present study, we found that [6]-paradol and other structurally related derivatives, [10]-paradol, [3]-dehydroparadol, [6]-dehydroparadol, and [10]-dehydroparadol, with the exception of [3]-paradol induce apoptosis in an oral squamous carcinoma cell line, KB, in a dose-dependent manner. [10]-paradol and [10]-dehydroparadol exhibited a similar extent of cytotoxicity to that of [6]-paradol. [6]-Dehydroparadol and [3]-dehydroparadol appeared to be more potent, with an IC<sub>50</sub> less than 40 μM. Treatment of KB cells with an apoptosis-inducing concentration of [6]-dehydroparadol caused induction of proteolytic cleavage of pro-caspase-3. These results suggest that [6]-paradol and structurally related derivatives induce apoptosis through a caspase-3-dependent mechanism. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** [6]-paradol; Apoptosis; KB cells; Caspase-3

### 1. Introduction

Many natural compounds are known to be effective and versatile chemopreventive agents in a number of animal tumor models. Some chemopreventive agents derived from the dietary condiment, such as [6]-gingerol from ginger and organosulfur compounds

in garlic induce apoptosis in several kinds of tumor cells [1–4]. Thus induction of apoptosis may be a good implication for the mechanism of chemopreventive agents [5–7].

Ginger (*Zingiber officinale*) is one of the most frequently and heavily consumed spices throughout the world. In addition to its extensive use as a seasoning ingredient, the rhizome of ginger is used in traditional oriental herbal medicine for the management of symptoms such as common cold, digestive disorders, rheumatism, neuralgia, colic and motion sickness. The rhizome of ginger contains [6]-gingerol as the

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main pungent and pharmacologically active principle [8–11]. [6]-Paradolol (1-[4'-hydroxy-3'-methoxyphenyl]-3-decanone) is the minor constituent of ginger, and this compound can be obtained from gingerol by successive dehydration and hydrogenation [12]. Both [6]-gingerol and [6]-paradol have exhibited a substantial cytotoxicity against human promyelocytic leukemia HL-60 cell [13]. It has been reported that some of the paradol derivatives have a wide spectrum of antimicrobial activity [14,15] and anti-tumor activity of [6]-paradol [12]. However structurally related compounds on solid tumor cells have not yet been investigated. Therefore our present study was designed to examine the effect of [6]-paradol and its derivatives on growth of oral squamous carcinoma cell line KB.

## 2. Material and methods

### 2.1. Materials

[6]-Paradol was synthesized by catalytic hydrogenation of [6]-dehydroparadol according to the procedure previously reported by Locksley et al. [14]. [3]-Paradol and [10]-paradol were prepared in the same manner from [3]-dehydroparadol and [10]-dehydroparadol, respectively. The structures of these compounds are illustrated in Fig. 1.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ethylenediaminetetra-acetic

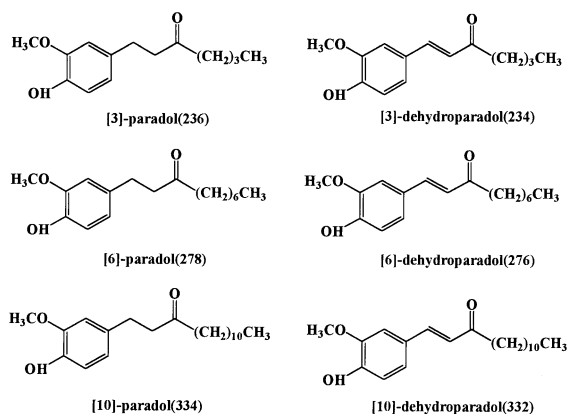


Fig. 1. Chemical structures of [6]-paradol and its derivatives tested in this study.

acid (EDTA), Tris-HCl, sodium dodecyl sulfate (SDS), proteinase K, propidium iodide, DNase free RNase, phenol-chloroform-isoamyl alcohol, pyrrolidine dithiocarbamate (PMSF), aprotinin, dimethylsulfoxide (DMSO) and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), F-12, Fetal bovine serum (FBS), trypan blue and trypsin-EDTA were obtained from GIBCO-BRL (Grand Island, NY, USA). Other solvents and chemicals were of the highest analytical grade.

### 2.2. Cell culture

KB, human oral epidermoid carcinoma cell line (ATCC CCL-17; American Type Culture Collection, Rockville, MD, USA) were grown in DMEM and F-12 media with the ratio of 3:1 supplemented with 10% FBS, 100 IU/ml penicillin, 10  $\mu$ g/ml streptomycin and 0.25  $\mu$ g/ml fungizone. Cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### 2.3. Growth inhibition of KB cells by [6]-paradol and structurally related derivatives

Cells were plated at a density of  $5 \times 10^3$  cells/200  $\mu$ l/well into 96-well plate. After an overnight growth, the cells were treated with a series of paradol derivatives. All of the derivatives of paradol tested were dissolved in DMSO. The final concentration of DMSO in the culture medium was kept below 0.1% and the controls were treated with DMSO alone. Cell viability was assessed using MTT assay. In brief, after the cells were grown in the media in the absence or presence of the test compounds for 48 h, they were then replaced to a 200  $\mu$ l culture medium containing 0.5 mg/ml MTT for 3 h. The resulting MTT-formazan product was dissolved by an addition of the same volume of DMSO. The amount of formazan was determined by measuring the absorbance at 570 nm.

### 2.4. Fluorescent staining of apoptotic nuclei

KB cells were plated at the density of  $3 \times 10^5$  cell/ml/well into 3-well chamber slides (Nunc, USA). After an overnight growth, cells were treated with 75  $\mu$ M of [6]-dehydroparadol for 4, 8 and 24 h. The cells were washed with PBS and fixed in 50% solution

of methanol-acetic acid (3:1, v/v) for 10 min. Apoptotic cells with condensed or fragmented nuclei were stained with 50  $\mu\text{g/ml}$  propidium iodide for 20 min. The nuclear morphology was observed under a fluorescent microscope, and at least  $1 \times 10^3$  cells were counted for assessing the apoptotic death.

### 2.5. Flow cytometric analysis

When grown in confluency, cells were treated with 75  $\mu\text{M}$  of [6]-dehydroparadol for 2, 4, 8 and 24 h. Cells were then harvested through trypsinization, washing once in PBS and fixing with ice-cold ethanol at 4°C for 30 min. Fixed cells were stained with propidium iodide (50  $\mu\text{g/ml}$ ) in PBS containing 0.1% Triton X-100, 0.1 mM EDTA, and 50  $\mu\text{g/ml}$  of DNase free RNase. The DNA content was measured by EPICS profile flow cytometer (Coulter, Hialeah, FL, USA) and all histograms were analyzed by Multi-cycle software (Advanced version, Phoenix flow systems, San Diego, CA, USA). Both G0/G1 and G2/M peaks were determined by Gaussian curve.

### 2.6. DNA fragmentation analysis

Following the treatment of 75  $\mu\text{M}$  of [6]-dehydroparadol for 6, 12 and 24 h, approximately  $5 \times 10^6$  cells were collected in lysis buffer containing 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, and 0.5 mg/ml proteinase K. DNA was extracted with an equal volume of a mixture of phenol-chloroform-isoamylalcohol (25:24:1, v/v) and precipitated with pure ethanol. DNA was resuspended in Tris-EDTA buffer (pH 8.0) containing 5  $\mu\text{g/ml}$  DNase free RNase and incubated at 37°C for 2 h. Fragmented DNA was visualized on 1.8% agarose gels in the presence of 0.5  $\mu\text{g/ml}$  of ethidium bromide.

### 2.7. Western blotting analysis of caspase-3 activation

After the treatment, KB cells were washed twice with ice-cold PBS, lysed in buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 1% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5  $\mu\text{g/ml}$  aprotinin, 5  $\mu\text{g/ml}$  leupeptin) on ice for 30 min and vigorously vortexed before centrifugation at  $12,500 \times g$  for 20 min. Fifty micrograms of the total protein, as determined by BioRad Protein Assay, was resolved on 10% SDS-PAGE, and then transferred to a microcellulose

membrane using the semi-dry transfer system (BioRad). The membrane was blocked with 5% non-fat dry milk in TBS (20 mM Tris-HCl pH7.4, and 8 g/l NaCl) for 1 h at room temperature. The membrane was incubated with anti-caspase-3 (1:1000, Cat. No. 65906E, PharmMingen, CA) antibody in PBS for 2 h after washing three times in TBS. Then, they were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:5000, Zymed biotechnology, San Francisco, CA, USA) antibody for 1 h. The protein was visualized using the chemiluminescence ECL (Amersham, USA) system.

## 3. Results

### 3.1. [6]-Paradol and other structurally related derivatives induced dose-dependent reduction of cell survival.

In order to analyze the effects of paradol derivatives on the viability of KB cells, we initially conducted the MTT assay. As illustrated in Fig. 2, KB cells showed different cytotoxic profiles when treated with each paradol derivative. After 48 h of treatment, all the paradol derivatives except [3]-paradol inhibited the proliferation of KB cells in a time- and dose-dependent manner. Incubation of cells in the presence of [6]-paradol at a concentration higher than 50  $\mu\text{M}$  produced significant growth inhibition. [6]-Dehydroparadol and [3]-dehydroparadol appeared to be more potent, at  $\text{IC}_{50}$  values below 45  $\mu\text{M}$ . [3]-Paradol was not effective in terms of suppressing proliferation at all concentration tested. Treatment with either [10]-paradol or [10]-dehydroparadol inhibited cell proliferation at 100  $\mu\text{M}$ .

### 3.2. DNA fragmentation after [6]-dehydroparadol treatment

To characterize the cell death induced by paradol derivatives, we examined the nuclear morphology of cells by using a fluorescent DNA-binding agent, propidium iodide. We focussed more on [6]-dehydroparadol to further assess the pattern of cell death, which was more potent when compared with other compounds. Within 8 h of treatment with 75  $\mu\text{M}$  [6]-dehydroparadol, tumor cells clearly exhibited condensed and fragmented nuclei, indicative of apop-

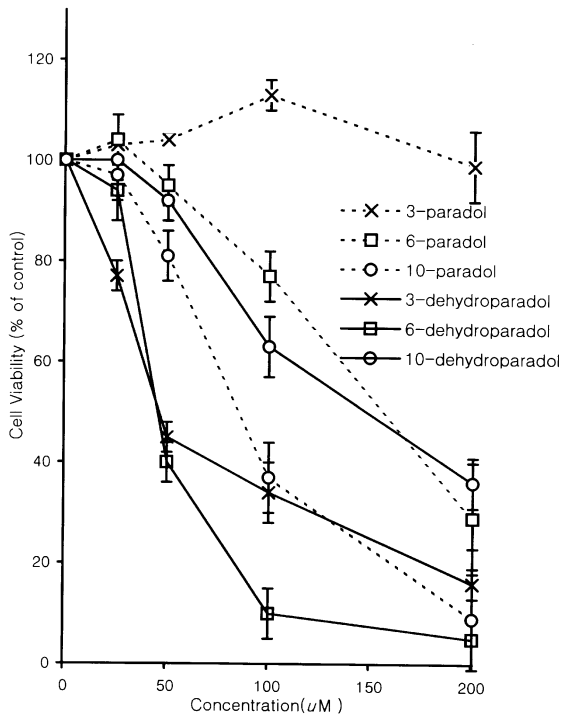


Fig. 2. Comparison of the viability of KB cells treated for 48 h with [6]-paradol and structurally related compounds as determined by the MTT assay. The percentage of cell viability was calculated as a ratio of  $A_{570\text{nm}}$  of treated cells and control cells. Each point is the mean  $\pm$  SD for eight wells.

otic cell death (Fig. 3A). The number of fragmented nuclei determined by fluorescent microscopy significantly increased in a time-dependent manner within 12 h, while there was no increase after 24 h due to the detachment of the apoptotic cells (Fig. 3B). Altered nuclear morphology was not evident in control cells treated with DMSO alone.

### 3.3. [6]-Dehydroparadol induced apoptosis of KB cells

To investigate if [6]-dehydroparadol has an effect on the cell cycle regulation, we incubated [6]-dehydroparadol with KB cells and monitored the change of cell cycle profiles using a fluorescent activated cell sorter. Typical changes in the cell cycle induced by [6]-dehydroparadol are shown in Fig. 4. The control cells incubated solely with vehicle showed a subdiploid population of less than 3.0% of the total

cells. On the contrary, after an 8 h treatment with 75  $\mu\text{M}$  [6]-dehydroparadol, subdiploid cells increased rapidly to a level of 44.7%. The population of subdiploid cells maintained at this level throughout the 24 h treatment. Cell cycle arrest following the treatment was not found.

Internucleosomal DNA fragmentation is a hallmark of apoptosis. The cell death in response to [6]-dehydroparadol treatment was rapid when measured by the cell death-associated genome digestion. At 75  $\mu\text{M}$

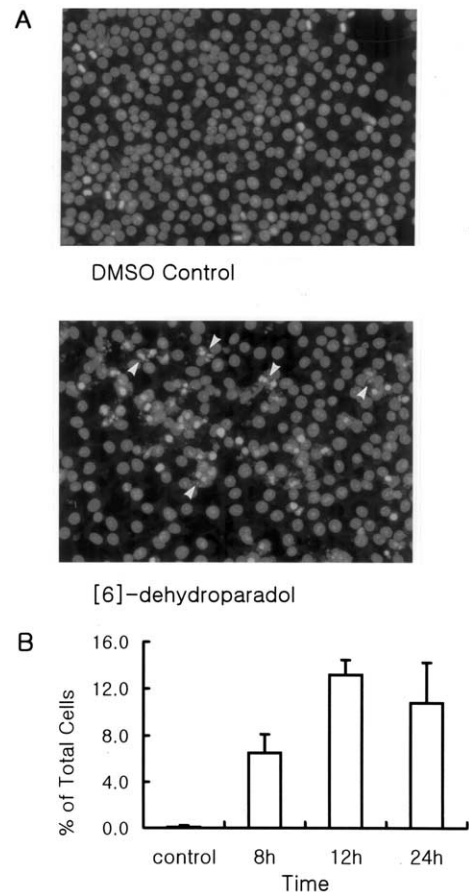
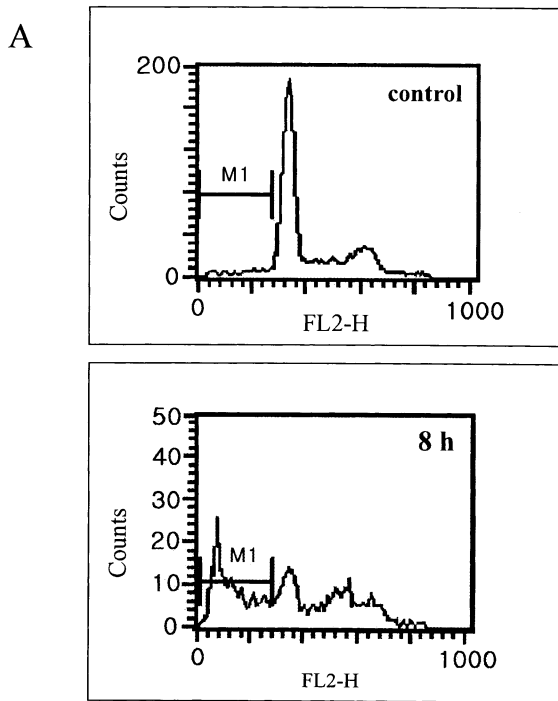


Fig. 3. Morphologic changes (A) and the number of fragmented nuclei (B) formed in KB cells after treatment of [6]-dehydroparadol. KB cells were incubated with 0.1% DMSO or 75  $\mu\text{M}$  [6]-dehydroparadol for 12 h. Arrows, apoptotic cells with condensed or fragmented nuclei. The fixed cells were incubated with propidium iodide, and apoptotic fragments were counted by fluorescence microscopy for at least  $1 \times 10^3$  cells. Error bars represent the mean of triplicate determinations  $\pm$  SE.



B

Control	3.0
2 h	7.59
4 h	19.47
8 h	44.73
24 h	45.49

Fig. 4. Detection of [6]-dehydroparadol-induced apoptotic DNA fragments in KB cells by flowcytometric analysis. KB cells were treated with vehicle or with 75  $\mu$ M [6]-dehydroparadol for 8 h (A). The percentage of cells containing sub-G1 amount of DNA was determined by fluorescence flow cytometry. Region M1 represents the cell population containing sub-G1 levels of DNA.

[6]-dehydroparadol, fragmentation of genomic DNA was evident at as early as 12 h (Fig. 5).

#### 3.4. [6]-Dehydroparadol activated caspase-3

Caspase-3 is considered as a key protease that is activated during the early stages of apoptosis. Caspases are activated in a sequential cascade of cleavages from their inactive forms and the active caspase-3 proteolytically cleaves and activates other caspases and other

relevant target molecules in the cytoplasm or nucleus. To determine whether caspase-3 is involved in apoptosis induced by [6]-dehydroparadol, we examined the cleavage of procaspase-3 by Western blot analysis. Treatment of KB cells with 75  $\mu$ M [6]-dehydroparadol caused a time-dependent proteolytic cleavage of procaspase-3, with accumulation of 17 kDa fragment and the concomitant disappearance of the full size 32 kDa molecule (Fig. 6). The cleavage was apparent within 8 h after treatment of [6]-dehydroparadol, whereas the expression of pro- and active caspase-3 significantly decreased after 24 h due to the apoptotic death of cells.

#### 4. Discussion

Many substances derived from dietary or medicinal plants are known to be effective and versatile chemopreventive agents in a number of experimental models of carcinogenesis [3,8,13,16]. It has been postulated

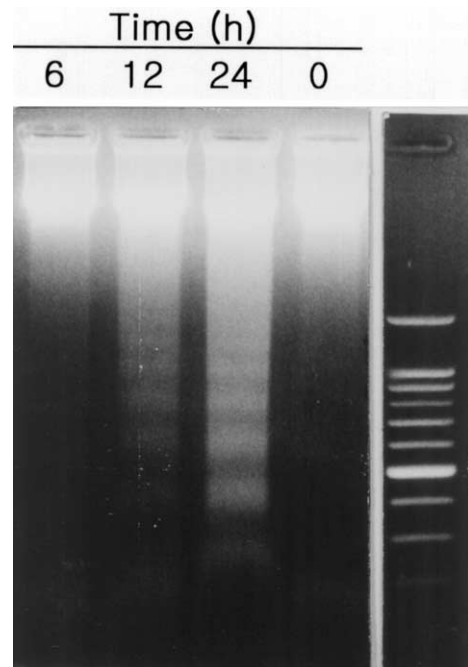


Fig. 5. Fragmentation of internucleosomal DNA by [6]-dehydroparadol. KB cells were treated with [6]-dehydroparadol at concentrations of 75  $\mu$ M for indicated time periods and nuclear DNA was subjected to agarose gel electrophoresis as described in Section 2.6.

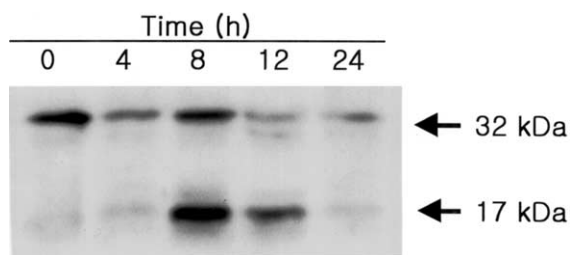


Fig. 6. Cleavage of pro-caspase-3 by [6]-dehydroparadol treatment in KB cells. Cells were treated with 75  $\mu$ M of [6]-dehydroparadol. Cell lysate was prepared and analyzed by Western blot as described in Section 2.7.

that chemopreventive candidates from Zingiberaceae family such as gingerol, paradol, yakuchinone and curcumin share structural similarities [2,3], possessing both the vanillyl (4-hydroxy-3-methoxyphenyl) moiety and the ketone functional group in their structure, which may contribute to the suppression of proliferation of human cancer cells through the induction of apoptosis. In this study, treatment of KB cells with [6]-paradol induced growth inhibition, which accompanied cytoplasmic blebbing and nuclear fragmentation suggesting apoptotic cell death. The cytotoxic activity of [10]-dehydroparadol was similar to that of [6]-paradol. [6]-Dehydroparadol and [3]-dehydroparadol appeared to be more potent with regards to growth inhibition of KB cells, and the  $IC_{50}$  values for these derivatives were around 40  $\mu$ M. A number of recent studies indicate that induction of apoptosis is closely related to the anticarcinogenic activity [8,13,17]. Therefore, we speculate that the [6]-dehydroparadol and [3]-dehydroparadol have a greater chemopreventive effect than naturally occurring [6]-paradol. In addition, [3]-paradol is known to have an antimicrobial effectiveness [15], however we could not find cytotoxic activity of [3]-paradol to KB cells up to 200  $\mu$ M.

During the isothiocyanate inducing apoptosis in HeLa cells, the alkyl carbon chain is known to be important in its cytotoxic effect on tumor cells [18,19]. Through this study we also came across that paradol compounds with saturated side chains may have a close association between the cytotoxicity and alkyl chain length. However, the alkyl chain length does not appear to have any influence on the cytotoxicity of dehydroparadol compounds that have  $\alpha,\beta$ -unsaturated ketone functional group in the chain.

The induction of apoptosis by [6]-paradol and its derivatives occurred rapidly along with chromatin condensation and nuclear fragmentation when observed under a phase contrast microscope [2,20]. Agarose gel electrophoresis with nuclear extract of treated tumor cells clearly showed internucleosomal DNA fragmentation of KB cells at as early as 12 h. Flow cytometric analysis and fluorescent staining of tumor cells showed a more clear apoptotic cell death in KB cells. Sub-G1 peaks, which represent the cell population containing apoptotic nuclear fragments dramatically increased after 8 h treatment, and gradually increased in a time-dependent manner.

The activation of a family of intracellular cysteine proteases, called caspases, is known to play a pivotal role in the initiation and execution of apoptosis induced by various stimuli [21,22]. Among the ten different members of caspases identified in mammalian cells, caspase-3 may serve as a general mediator of apoptosis. When cells are undergoing apoptosis, executioner or effector caspase-3 triggers cellular proteins such as poly (ADP-ribose)polymerase and DNA fragmentation factor, resulting in the characteristic changes of apoptosis [21–23]. Caspase-3 is synthesized as a 33 kDa inactive proenzyme that requires proteolytic activation. Our results showed that high level of proenzyme of capase-3 was present in untreated tumor cells, and active capsase-3 gradually increased after [6]-dehydroparadol treatment, suggesting that this synthetic vanilloid induce apoptosis through a caspase-3-dependent mechanism.

Furthermore, the ability of [6]-dehydroparadol to induce apoptosis implies their potential as chemotherapeutic agents because many anticancer drugs are known to achieve their antitumor functions by inducing apoptosis in the tumor cells. A further challenge is to study whether these derivatives also induce apoptosis in the tissues which they have been proven to be effective anticarcinogenic agents.

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