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# Chemopreventive effect of dimethyl dicarboxylate biphenyl on malignant transformation of WB-F344 rat liver epithelial cells[1]

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## Key words

dimethyl dicarboxylate biphenyl; liver neoplasm; cell transformation; chemoprevention; epithelial cell; gap junctions

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## Abstract

**Aim:** To study the potential chemopreventive effect of dimethyl dicarboxylate biphenyl (DDB), an anti-hepatitis drug, on hepatocarcinogenesis *in vitro*. **Methods:** The anti-carcinogenesis effect of DDB was assessed on a two-stage chemical oncogenesis model induced by 3-methylcholanthrene and 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) with WB-F344 rat liver epithelial cells (WB-F344 cells) *in vitro*. A soft-agar colony formation assay was used to determine the tumorigenic potential of the transformed WB-F344 cells. The gap junctional intercellular communication (GJIC) was detected using the scrape loading/dye transfer technique. **Results:** DDB at 1 μmol/L, 2 μmol/L, and 4 μmol/L significantly prevented the malignant transformation of WB-F344 cells induced by 3-methylcholanthrene and TPA. The average number of transformed foci decreased dramatically by 10.0%, 37.2%, and 47.4%, respectively. In soft agar, a remarkable decrease in colony numbers was observed in transformed cells treated with 2 μmol/L and 4 μmol/L DDB. DDB at 1 μmol/L, 2 μmol/L, and 4 μmol/L inhibited the downregulation of GJIC induced by TPA in a dose-dependent manner. The GJIC recovered to 25.6%, 34.6%, and 44.9%, respectively, of the control WB-F344 cells by DDB. **Conclusion:** DDB has a potential chemopreventive effect on hepatocarcinogenesis induced by carcinogens *in vitro*.

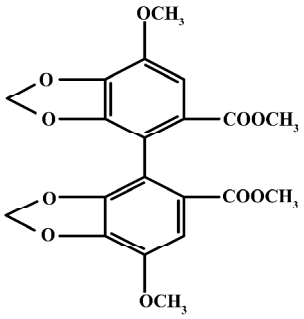
## Introduction

Human hepatocellular carcinoma (HCC) is one of the most frequent malignant cancers. The carcinogenesis of HCC is a multifactorial event. Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the most frequent causes of HCC[1]. Approximately 80% of human HCC are attributable to HBV infection[2]. Chronic HBV carriers are 100–400 times more likely to develop HCC than non-carriers[3]. HCV is the second most common cause of HCC after HBV[4]. Currently, HCC represents more than 4% of all cancer cases worldwide and causes at least 315 000 deaths every year[5]. Although early HCC can be cured by surgical resection, many HCC are asymptomatic, so most HCC patients are not diagnosed in time.

An effective approach to cancer control is chemoprevention. It is known that the therapy of both chronic HBV and HCV generally involves a long-term course. An anti-hepatitis drug with an inhibiting or a suppressing effect on the development of hepatocarcinogenesis, besides its improvement of abnormal liver function, would be of great clinical value.

Dimethyl dicarboxylate biphenyl (DDB) is a synthetic analogue of Schizandrin C, which was isolated from Fructus Schizandrae chinensis[6]. Since 1983, DDB has been widely used to treat hepatitis B patients in China and is exported to Korea, Egypt, Vietnam, Indonesia, Pakistan, and Burma for the treatment of HBV and HCV. The results of the clinical application indicated that DDB markedly improved impaired liver functions, such as the elevated serum transaminase, bilirubin, α-fetal protein, and symptoms of the patients. Pharmacologically, DDB has a protective action against

experimental liver injury in mice and rats[7,8,9]. DDB also had anticancer activity and differentiation-inducing effect on cancer cells[10]. In the present paper, the chemoprevention effect of DDB on hepatocellular carcinogenesis *in vitro* is studied.




## Materials and methods

**Chemicals** DDB with 99% purity was provided by the Beijing Union Pharmaceutical Plant. As DDB is not water-soluble, it was dissolved in dimethyl sulfoxide ($Me_2SO$) for *in vitro* use. 3-Methylcholanthrene (3MC), 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), MTT, and Lucifer yellow CH were obtained from Sigma Chemical Company. Other chemicals were of analytical grade and purchased from Beijing Chemical Company.

**Cell culture** WB-F344 cells were grown in DMEM (GIBCO) media containing 10% newborn calf serum, 100 kU/L penicillin, and 100 mg/L streptomycin in a 37 °C humidified incubator containing 5% $CO_2$ and 95% air, and passaged using 0.25% trypsin plus 0.02% EDTA treatment. The culture medium was changed every other day.

**MTT assay** Cytotoxicity was determined by MTT assay according to the method of Mosmann[11]. WB-F344 cells ($3\times10^3$cells per well) were plated on 96-well plates, and 24 h later various concentrations of DDB were added (0.5 μmol/L–100 μmol/L). The cells were incubated at 37 °C in a $CO_2$ incubator for 72 h. The culture supernatant was sucked out and MTT 0.5 g/L stock solution was added to each well. After 4 h of incubation, $Me_2SO$ was added. The optical density of each well was determined by a microplate reader at a wavelength of 570 nm. The values of absorbance were expressed as relative viable cell number.

***In vitro* transformation of WB-F344 cells** WB-F344 cells were seeded on 25-$cm^2$ tissue culture flasks containing the complete medium at a density of $4\times10^3$cells per flask. The medium was replaced with the complete medium containing 3MC (2 mg/L) or 0.1% $Me_2SO$ 24 h after seeding, and the cells were incubated for another 72 h. After the removal of the medium, the cells were washed twice with sterile phosphate-buffered saline (PBS) and incubated in fresh medium for 4 d. The cells were then incubated with medium containing 100 μg/L TPA. The TPA-containing medium was changed every 2–3 d for 14 d. After sucking out the TPA-containing medium, the cells were washed twice with sterile PBS and then incubated in fresh medium containing 10% newborn calf serum. The fresh medium was changed every 2 d until d 30. DDB was added to the medium from 24 h after cell seeding until the end of the experiment. At d 30, three of these flasks from each group were stained with Wright-Giemsa, and scored for transformed colonies. The remainders were used for soft-agar assay.

**Soft-agar colony formation assay** Cells derived from each group were seeded separately. Agar (0.6%) in the complete medium was kept at 44 °C and poured into 6-well plates (2 mL per well) as to form the lower layer. After the agar medium had set, $1\times10^4$cells per well in 2 mL of 0.3% agar (44 °C) were layered onto the gelled agar as the form of the upper layer. The cells were incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C. On d 9 and d 18, 1 mL of 0.3% agar in the complete medium was added. After 28 d, colonies of more than 20 cells were counted under contra-phase microscope.

**Cell-cell communication assay** The scrape loading/dye transfer (SL/DT) technique was used to detect GJIC according to the method of E1-Fouly *et al*[12]. WB-F344 cells were pretreated with various concentrations of DDB for 1 h at 24 h prior to the addition of TPA (100 μg/L) for 1 h. The other cells were pretreated with 4 μmol/L DDB for 24 h, 48 h, and 72 h before treatment with TPA. Following incubation, the cells were washed twice with PBS. Then Lucifer yellow CH (a fluorescent dye permeating gap-junctional channels) was added and several scrapes were made with a surgical steel-bladed scalpel at low light intensities. These scrapes were performed to ensure that the scrape traversed a large group of confluent cells. After 3 min incubation, the cells were washed with PBS again. Dye migration was observed and photographed with an inverted fluorescent microscope (Olympus, Japan) at ×200 magnification. The number of dyed cells represents the ability of cells to communicate via GJIC. GJIC data are reported as a percentage of the corresponding mean control value. The data are obtained from 3 views per plate, pooled 4 separate plates for each point.

**Statistical analysis** Results are expressed as mean±SD. To compare mean values between 2 groups, the Student's *t*-test was used. $P<0.05$ was considered statistically significant.

## Results

**Cytotoxicity of DDB to WB-F344 cells** To select the appropriate doses of DDB for the present study, the cytotoxicity of DDB to WB-F344 cells was assessed using the MTT assay. No significant cytotoxic effect on the cells was observed when the concentrations of DDB were below 4 μmol/L (Table 1). Therefore, 1 μmol/L, 2 μmol/L, and 4 μmol/L of DDB were used in the subsequent experiments.

**Table 1.** Cytotoxicity of dimethyl dicarboxylate biphenyl (DDB) to WB-F344 rat liver epithelial cells. $n$=9. Mean±SD. $^bP<0.05$ *vs* control group.

| Group | Dose/μmol·L$^{-1}$ | Survival rate/% |
|---|---|---|
| Control | 0 | 100.0±0.0 |
| DDB | 0.5 | 97.9±13.6 |
| | 1 | 105.1±9.3 |
| | 2 | 104.9±12.4 |
| | 4 | 90.2±4.7 |
| | 5 | 79.4±6.9$^b$ |
| | 10 | 78.4±17.6$^b$ |
| | 50 | 79.2±5.1$^b$ |
| | 100 | 61.8±5.4$^b$ |

**Effect of DDB on two-stage transformation of WB-F344 cells** A two-stage (initiation and promotion) chemical induction oncogenesis model with WB-F344 cells was established. The WB-F344 cells became transformed after 3-MC (2 mg/L) initiation for 72 h and then TPA (100 μg/L) promotion for 14 d. The transformed cells were grown in a disorganized multilayer instead of in a monolayer (Figure 1). DDB at concentrations of 1 μmol/L, 2 μmol/L, and 4 μmol/L markedly inhibited transformation of WB-F344 cells in a dose-dependent manner. The average number of transformed foci decreased dramatically by 10.0%, 37.2%, and 47.4%, respectively, after DDB treatment (Table 2).

**Table 2.** The inhibitory effect of dimethyl dicarboxylate biphenyl (DDB) on transformed foci in WB-F344 rat liver epithelial cells undergoing initiation with 3-methylcholanthrene (3-MC) followed by promotion with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). $n$=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* the model group. Transformed foci/flask: colonies containing >~100 cells were scored positive.

| Group | 3-MC+TPA | Transformed foci/plate | Inhibitory rate/% |
|---|---|---|---|
| Control | – | 4.5±1.3 | – |
| Model | + | 53.8±8.2 | – |
| DDB 1 μmol/L | + | 48.4±13.4$^b$ | 10.0 |
| 2 μmol/L | + | 33.8±5.1$^c$ | 37.2 |
| 4 μmol/L | + | 28.3±3.3$^c$ | 47.4 |

**Effect of DDB on colony of transformed WB-F344 cells in soft agar** To evaluate the tumorigenic potential of the treated WB-F344 cells, the efficiency of their soft-agar colony formation was determined. As shown in Table 3, no colony formed in soft agar in untreated WB-F344 cells, whereas the cells initiated with 3-MC and promoted with TPA developed the transformed phenotype of colony formation in soft agar. A remarkable increase in colony numbers was observed. The cells treated with 2 μmol/L and 4 μmol/L DDB also developed the transformed phenotype of colony formation, but the colony numbers significantly decreased compared with

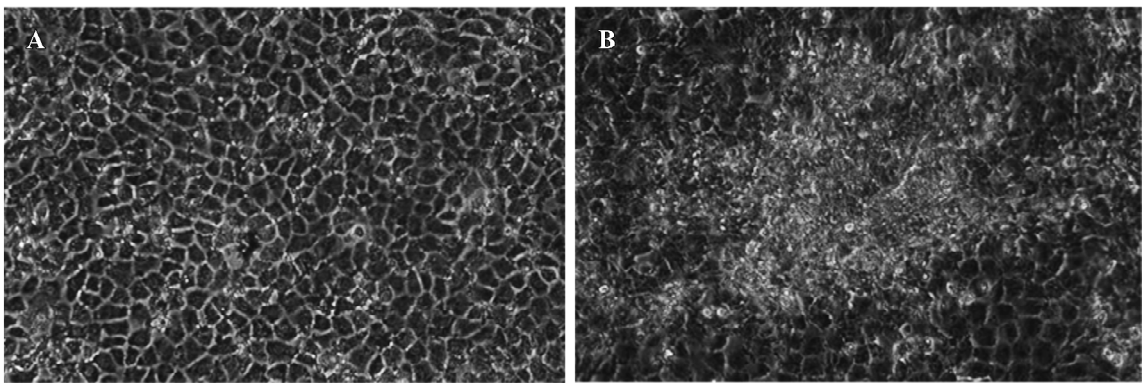



**Figure 1.** Morphology of WB-F344 cells treated with 3-methylcholanthrene (3-MC)/12-*O*-tetradecanoyl phorbol 13-acetate (TPA) or the vehicle dimethyl sulfoxide. (A) control cells, (B) cells treated with TPA 100 μg/L plus 3-MC 2 mg/L. At d 30 of incubation, cell morphology was examined under a microscope (×200). Cells treated with 3-MC and TPA were highly transformed in appearance with the formation of foci.

**Table 3.** Effect of dimethyl dicarboxylate biphenyl (DDB) on tumorigenicity of transformed WB-F344 rat liver epithelial cells using the soft agar culture assay. $n$=3. Mean±SD. $^cP$<0.01 *vs* the model group. Colony forming rate/1×10$^4$cells: colonies containing >~20 cells were scored positive. 3-MC, 3-methylcholanthrene; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

| Group | 3-MC+TPA | Colony forming rate/1×10$^4$cells | Inhibitory rate/% |
|---|---|---|---|
| Control | – | 0.0±0.0 | – |
| Model | + | 231.0±17.1 | – |
| DDB 2 μmol/L | + | 136.0±19.1$^c$ | 41.1 |
| 4 μmol/L | + | 85.0±7.9$^c$ | 63.2 |

the model group.

**Effect of DDB on GJIC** The GJIC of normal WB-F344 cells was well- characterized and did not decrease during the experimental incubation period (Figure 2Aa). After exposing the cells to TPA (100 μg/L) for 1 h, over 85% inhibition of GJIC was detected. The Lucifer yellow CH only stayed at the incision sites or artificially damaged cells (Figure 2Ab). When the cells were pretreated with DDB 1 μmol/L, 2 μmol/L, and 4 μmol/L, respectively, for 24 h, a dose-dependent inhibition of TPA-induced downregulation of GJIC was observed. The GJIC recovered to 25.6%, 34.6%, and 44.9% of the control group, respectively (Figure 2B). The time-dependent inhibitory effect of 4 μmol/L DDB on TPA-induced downregulation of GJIC is shown in Figure 3. By the addition of 4 μmol/L DDB for 24 h, 48 h, and 72 h, TPA-induced downregulation of GJIC was markedly reversed in a time-dependent manner.

## Discussion

WB-F344 cells have often been used in the study of hepatocarcinogenesis[13]. In the present study, we found that the anti-hepatitis drug DDB at non-toxic doses markedly prevented the transformation of WB-F344 cells induced by 3-MC and TPA *in vitro*, which expressed as significant decrease of the number of transformed foci and the malignant degree of transformed cells.

It is well known that carcinogenesis is a multistage and multimechanism process, involving the irreversible alteration of a stem cell (the initiation phase), followed by the clonal

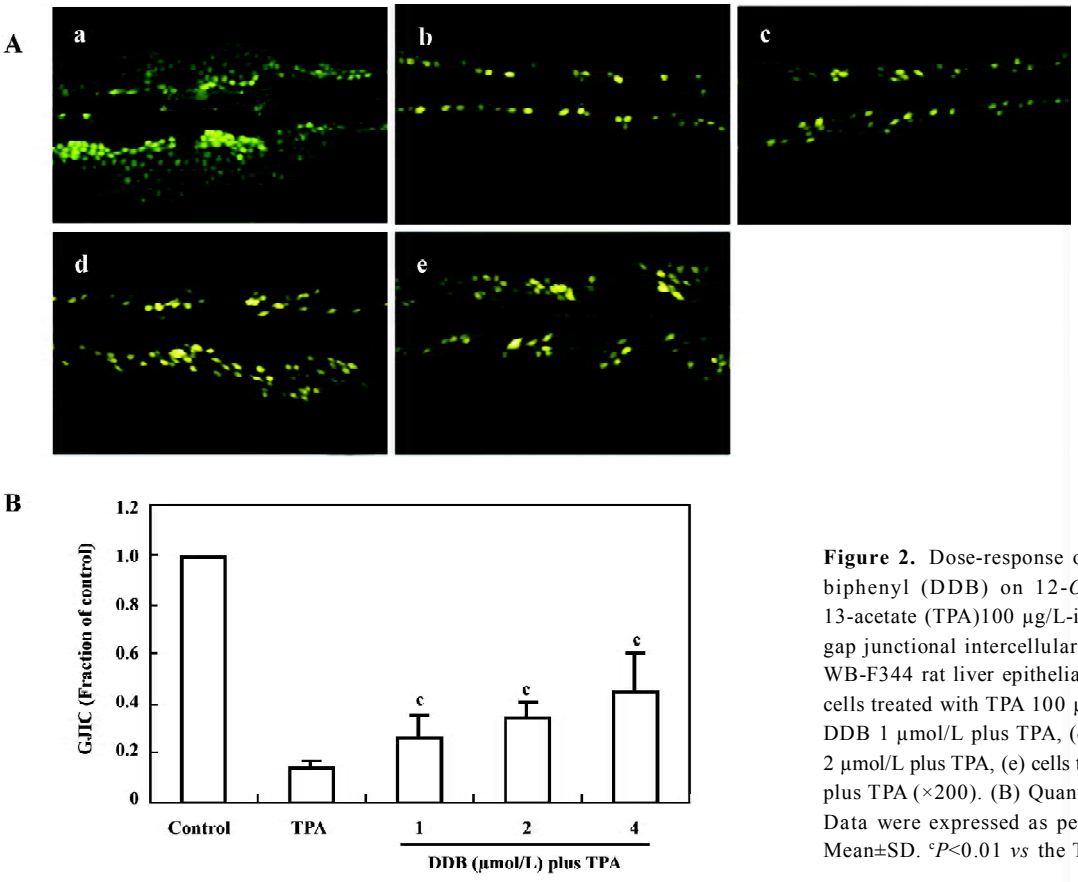



**Figure 2.** Dose-response of dimethyl dicarboxylate biphenyl (DDB) on 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)100 μg/L-induced downregulation of gap junctional intercellular communication(GJIC) in WB-F344 rat liver epithelial cells. (A)(a) control, (b) cells treated with TPA 100 μg/L, (c) cells treated with DDB 1 μmol/L plus TPA, (d) cells treated with DDB 2 μmol/L plus TPA, (e) cells treated with DDB 4 μmol/L plus TPA (×200). (B) Quantification of recovery rate. Data were expressed as percentage of control. $n$=4. Mean±SD. $^cP$<0.01 *vs* the TPA model group.

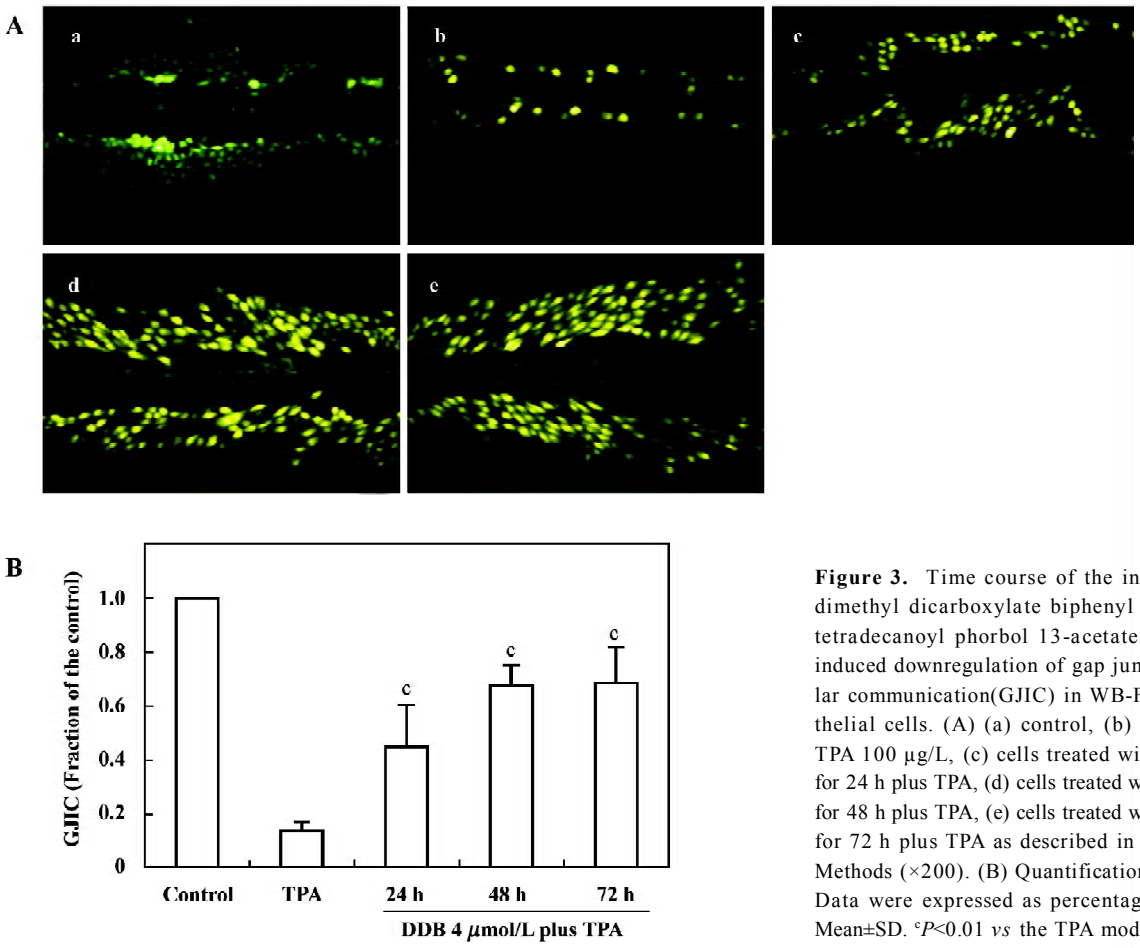



**Figure 3.** Time course of the inhibitory effect of dimethyl dicarboxylate biphenyl (DDB) on 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)100 μg/L-induced downregulation of gap junctional intercellular communication(GJIC) in WB-F344 rat liver epithelial cells. (A) (a) control, (b) cells treated with TPA 100 μg/L, (c) cells treated with DDB 4 μmol/L for 24 h plus TPA, (d) cells treated with DDB 4 μmol/L for 48 h plus TPA, (e) cells treated with DDB 4 μmol/L for 72 h plus TPA as described in the Materials and Methods (×200). (B) Quantification of recovery rate. Data were expressed as percentage of control. $n$=4. Mean±SD. $^cP$<0.01 *vs* the TPA model group.

proliferation of the initiated stem cell (the promotion phase), from which the acquisition of the invasive and metastasis phenotypes are generated (the progression phase). Intervention to prevent cancer can occur at each step. For chemoprevention of carcinogenesis, the development of anti-tumor promoting agent has been regarded as the most effective pathway.

Intercellular communication is necessary in multicellular organisms to maintain tissue homeostasis and to control cell growth and differentiation. Gap junction channels play an important role in intercellular communication by providing a direct pathway for the movement of molecular information, including ions, polarized and non-polarized molecules up to a molecular mass of 1 kDa between adjacent cells[14,15]. Much evidence has been documented to support the hypothesis that the downregulation of GJIC is a cellular event underlying the tumor promotion process, and that any treatment to prevent downregulation of GJIC is important in prevention of tumor promotion[16,17]. Many tumor promoters have been shown to inhibit gap junctional communication *in vitro*[18,19]. TPA is a well-known classical inhibitor of cell communication in most cells, including the WB-F344 cell[20]. In the present study, the underlying mechanisms of DDB against hepatocarcinogenesis were investigated during the promotional phase using TPA to inhibit GJIC. The WB-F344 cells are known to have high GJIC. The treatment with TPA significantly inhibited GJIC, as was determined using the SL/DT assay. The counteracting effect of DDB on GJIC inhibition caused by TPA suggests that DDB has a significant action in maintaining GJIC function, and that it might be beneficial in preventing tumor promotion.

In summary, the results of the present study suggest that DDB can prevent the malignant transforming of WB-F344 cells induced by 3-MC and TPA *in vitro*. The restoration of GJIC in the promotion phase should contribute, at least in part, to the anti-hepatocarcinogenic property of DDB. We conducted other experiments and found that DDB significantly inhibited liver carcinogenesis induced by DEN/PB in mice; the data from these experiments will be published in another paper soon. Both *in vitro* and *in vivo* experiments

demonstrated that DDB had a chemopreventive effect on hepatocarcinogenesis. It is worthy to pay attention to whether DDB potentially prevents liver carcinogenesis in patients with chronic viral hepatitis.

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