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[Original Article]

# Preventive Enhancement of Hepatic Phase I and Phase II Enzyme in the Carcinogenesis Prevention by an Antiinflammatory Sesquiterpene α-Humulene

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Cancer chemoprevention relies on the use of naturally occurring or synthetic chemicals, known as xenobiotics, to suppress carcinogens, through a mechanism via induction of phase I and phase II drug-metabolizing enzymes. In the present study the effects of α-humulene (HUM) on the expression and activities of hepatic phase I and phase II enzymes were investigated. HUM is a sesquiterpene compound found in the essential oils of various plant species, previously suggested to be anti-inflammatory and anticarcinogenic. We first confirmed the antitumor effect of HUM by using two-stage mouse skin carcinogenesis test. Skins were exposed to the tumor initiator 7,12-dimethylbenzo[a]anthracene for one week, then were treated repeatedly with HUM followed by a tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate for over 20 weeks: HUM suppressed tumor formation by 75%. We next analyzed hepatic enzymes by using the tissues from mice treated orally with HUM once a day for 4 successive days. The contents of total cytochrome P450 and cytochrome $b_5$ increased by up to 54% and 32% due to HUM. The activities of hepatic phase I enzymes 7-ethoxycoumarin *O*-deethylase, 7-ethoxyresorufin *O*-deethylase and 7-pentoxyresorufin *O*-dependentylase increased by up to 92, 47 and 885%, respectively. As to hepatic phase II enzymes, glutathione *S*-transferase activities toward 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene increased by up to 67% and 178%, respectively. Moreover, HUM significantly elevated the GST activity toward potent carcinogen 4-nitroquinoline 1-oxide, NAD(P)H:quinone reductase activity and hepatic glutathione contents. Taken together, HUM has been demonstrated to be a bifunctional inducer, which enhances both the phase I and phase II enzymes of the liver. The present results suggest that the carcinogenesis suppression by such anti-inflammatory or anti-carcinogenic molecules as HUM involves modulations of the phase I and/or II drug-metabolizing enzymes.

## Ⅰ. INTRODUCTION

Carcinogenesis is closely related to exposure to environmental carcinogens. As to the biological activities of antitumor chemicals, either synthetic or naturally occurring, they have been strongly suggested to act through modulations of drug-metabolizing enzymes affecting their expression or activation[1,2)]. Hepatocytes play a primary role in carcinogen detoxification through the functions of "phase I" and "phase II" drug-metabolizing enzymes (Fig. 1). The phase I enzymes, consisting of a superfamily of cytochrome P450 (CYP), are involved in both bioactivation and detoxification of carcinogens, the phase II enzymes, containing multiple detoxifying enzymes, such as glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase (QR), are known to play essential roles in the detoxification and elimination of activated carcinogens during tumor initiation. Several studies have reported induction of detoxification enzymes by sesquiterpenes[3-5)]. For instance, zerumbone, a sesquiterpene from *Zingiber zerumbet* Smith, has recently been found to exert cancer chemoprevention mainly by affecting phase II enzymes[4,5)]. HUM (Fig. 2) is a sesquiterpene compound lacking only the α,β-unsaturated carbonyl group present in ZER, first found in the essential oils of Hops (*Humulus lupulus* L.) and later also found in herbs and spices such as cannabis,

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clove and ginseng[6]. In the present study the effects of HUM on two-stage carcinogenesis tests *in vivo* and the activities of phase I and phase II enzymes have been analyzed in mouse liver.

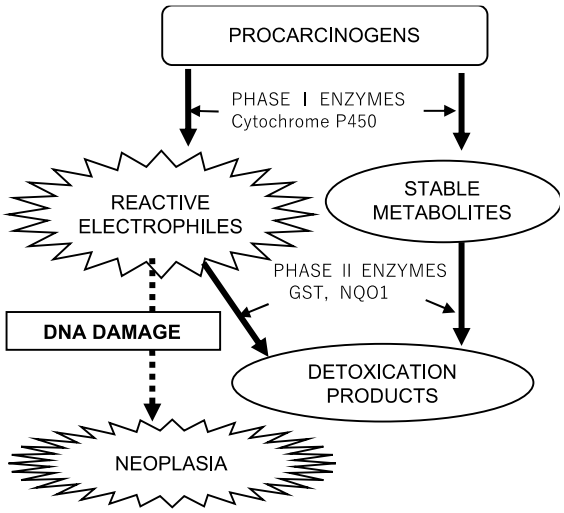


**Fig. 1. Roles of phase I and phase II enzymes in the metabolism of carcinogenic substances**

## Ⅱ. MATERIALS AND METHODS

### 1. Materials

α-Humulene was obtained from Sigma-Aldrich (St Louis MO, USA). Chemicals and reagents were of analytical grade and obtained commercially from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise stated.

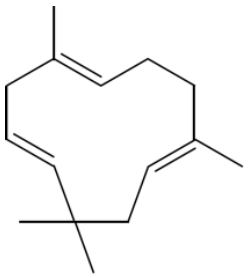

**Fig. 2. Structure of humulene (HUM)**

### 2. Two-stage mouse skin carcinogenesis test

Antitumor activities of HUM were confirmed by two-stage mouse skin carcinogenesis test. To test the effect of topically applied HUM on 12-*O*-tetradecanoylphorbol 13-acetate (TPA) promotion, groups of 11 mice each were initiated with a single dose of 7,12-dimethylbenzo[a]anthracene (DMBA, 200 nmol). One week later initiation one group received twice weekly topical applications of HUM (160 nmol) followed by TPA (1.6 nmol) and the other group received TPA alone for 20 weeks. The incidence of papillomas was determined weekly for 20 weeks.

### 3. Animal care and treatment

Four-week-old mice (ddY strain, male) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Principles in good laboratory animal care were followed and animal experimentation was in compliance with the Guidelines for the Care and Use of Laboratory Animals in the Health Sciences University of Hokkaido. Mice were maintained under a controlled environment (23 ± 1 ˚C with constant humidity of 55 ± 5% and a 12 h light/dark cycle) and provided with water and diet *ad lib*. Chemical samples were administered to mice orally once a day as a 2% gum Arabic suspension. Animals received either a vehicle (control group) or HUM (experimental group; 100, 200 and 300 mg/kg/day) orally for the period of 4 days. The doses used in the present study were chosen based on the action of HUM in preliminary experiments. Twenty-four hours after the last treatment the mice were sacrificed by decapitation. Tissues were removed, washed with ice-cold 1.15% potassium chloride and blotted briefly.

They were then weighed and subjected to the preparation of cytosolic and microsomal fractions as described[7]. Briefly, livers were homogenized with Potter-Elvehjem homogenizers in 5 volumes of ice-cold 1.15% potassium chloride. The homogenates were first centrifuged at 9,000 x g for 20 minutes and the resultant supernatants were centrifuged at 105,000 x g for 60 minutes at 4 ˚C. The supernatants and pellets from the second centrifugation were referred to as cytosolic and microsomal fractions, respectively, and stored at -80 ˚C until use. Protein contents in the samples were determined by the Lowry-Folin method.

### 4. Measurement of enzyme activities

Measurement of enzyme activities were determined as described[5]. Briefly, Cytosolic GST activity with 1-chloro-2,4-dinitrobenzene (CDNB; Kanto Kagaku, Tokyo, Japan), 1,2-dichloro-4-nitrobenzene (DCNB; Kanto Kagaku, Tokyo, Japan) and 4-nitroquinoline 1-oxide (4NQO; Sigma, MO, USA) as a substrate were determined. Cytosolic QR activity with menadione (Sigma, MO, USA) as a substrate was determined.

Microsomal activities of 7-ethoxycoumarin *O*-deethylase (ECOD), 7-methoxyresorufin *O*-demethylase (MROD), 7-ethoxyresorufin *O*-deethylase (EROD) and

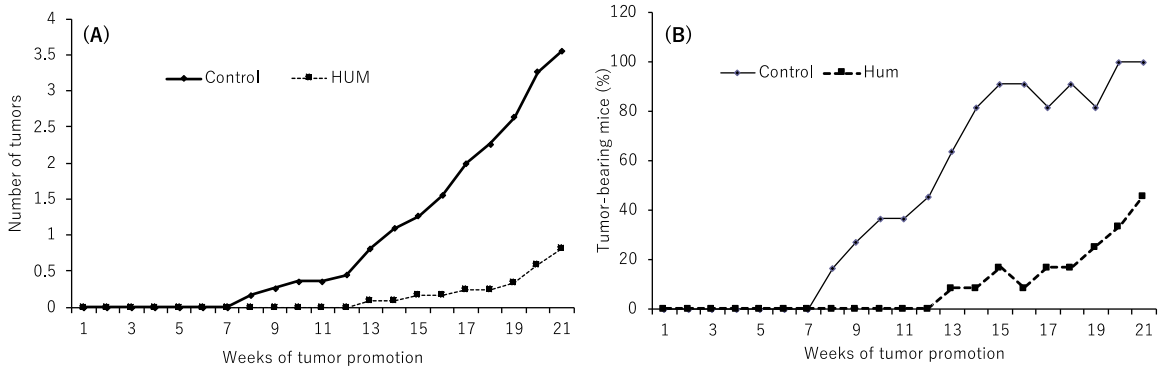


**Fig. 3. Inhibitory effect of HUM on tumor promotion in DMBA-initiated mice**

Initiation was carried out by a single application of DMBA on the back skin of mice. TPA (1.6 nmol), as promoter, was applied on the same place twice a week for 20 weeks from 1 week after the initiation. HUM was applied simultaneously with TPA at the dose of 160 nmol. Each experimental group contained of 11 mice. Data expressed as the average number of papillomas per mouse (A) and the percentage of mice bearing papillomas (B).

7-pentoxyresorufin *O*-depentylase (PROD) were determined.

**5. Measurement of glutathione contents**

Contents of glutathione species in mouse livers were determined as described[5]. Briefly, livers were perfused with ice-cold 1.15% potassium chloride, homogenized and immediately precipitated with 25% metaphosphoric acid. After centrifugation, the resultant supernatant was subjected to determination of the whole glutathione (GSH) content. Oxidized glutathione (GSSG) was analyzed similarly except for preincubating the above supernatant with 2-vinylpyridine and triethanolamine for 1 h in the dark. The amount of reduced GSH was obtained as the difference between the whole glutathione and GSSG.

**6. Measurement of cytochrome P450 and cytochrome $b_5$**

Contents of the cytochromes in the liver were determined as described[5]. Briefly, CYP was determined by measuring the difference in absorbance between 450 and 490 nm and using an absorption coefficient of 91 $mM^{-1}cm^{-1}$. Cytochrome $b_5$ was determined by measuring the difference in absorbance between 409 and 424 nm with an absorption coefficient of 185 $mM^{-1}cm^{-1}$.

**7. Statistical analysis**

Statistical analyses were performed by Dunnett test. Differences with $P$ values < 0.05 were considered statistically significant.

## Ⅲ. RESULTS

**1. Anti-tumor promoting effect on two-stage mouse skin carcinogenesis**

To determine whether HUM affects skin tumorigenesis, we performed a two-stage mouse skin carcinogenesis test with application of DMBA as an initiator and TPA as a tumor promoter. HUM suppressed the average number of tumors (Fig. 3A) and the incidence (Fig. 3B). The first papillomata arose 7 weeks after DMBA treatment in the group treated with DMBA and TPA, all mice in the group developed tumors at week 19, and the average number of tumors per mouse in this group was 3.6 at the end of experimental period. In the group treated with DMBA and TPA and HUM, the first tumor appeared at week 12, and only 45.5% of the mice developed tumors, and the average number of tumors per mouse in this group was 0.8 at the end of experimental period.

**2. Effects of HUM on phase I enzymes**

The contents of total cytochrome P450 and cytochrome $b_5$ showed a significant increase as compared to the controls in a dose-dependent manner (Fig.4). Cytochrome P450 and cytochrome $b_5$ showed a significant elevation of 1.5-fold and 1.3-fold, respectively 4 days after the administration of 300 mg/kg HUM. The activities of hepatic phase I enzymes ECOD (marker of total CYP), EROD (marker of CYP1A1/2) and PROD (marker of CYP2B1/2) also increased in a dose-dependent manner (Fig. 5). The extents of elevation were markedly greater with PROD than EROD. The activity of PROD showed a 9.85-fold increase at 300 mg/kg HUM compared to the control levels. At this dose, the increase in

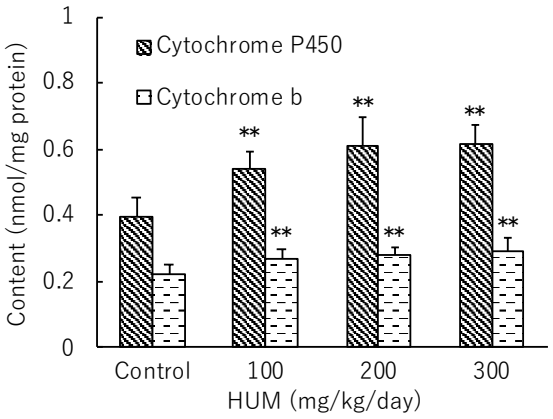


**Fig. 4. Effect of HUM on contents of total cytochrome P450 and cytochrome $b_5$ in mouse liver**
Data represent means ± S.D. (n = 6-7), *: P < 0.05, **: P < 0.01, Significantly different from control.

EROD activity was 1.47-fold, compared to the control level. On the other hand, HUM caused no change in MROD (marker of CYP1A2) activity.

### 3. Effects of HUM on phase II enzymes

The activities of various phase II enzymes were significantly increased by HUM (Fig. 6). GST comprises several isozymes with varying characteristics and mouse liver is known to possess α, μ and π classes as main isozymes. Oral treatment of HUM significantly enhanced GST activities in a dose-dependent manner with various substrates, including CDNB (for the whole isozymes), DCNB (mainly for μ class) and 4NQO (for μ and π classes). At the dose of 300 mg/kg HUM, GST activities using substrate CDNB, DCNB and 4NQO were elevated by 1.67-fold, 2.78-fold and 2.10-fold, respectively. The degree of extention was greater for μ class substrate than other classes.

We also analyzed HUM effects on another major phase II enzyme, QR. Basically, the results paralleled to those for GST. Thus, QR showed a dose dependent induction and the increase of 2.19-fold was seen at 300 mg/kg as compared to control value.

In addition, the hepatic GSH content, an important parameter in the action of GST, was evaluated. Significant elevation of the reduced form of GSH, was found following the HUM treatment, with maximum increase of 1.27-fold seen at the dose of 200 mg/kg（Fig.7）. This increase certainly would contribute to the detoxifying capacity of GST enzymes. On the other hand, HUM caused little effect on the content of hepatic GSSG.

## Ⅳ. DISCUSSION

The present study demonstrates that oral administration of HUM inhibited tumor promotion by TPA following initiation with DMBA in mouse skin. These effects suggested that HUM suppress the promotion and progression of carcinogenesis. In skin carcinogenesis, prostaglandin $E_2$ ($PGE_2$) is known to be involved in the progression of pathogenesis due to promotion of inflammation. The anti-inflammatory activities of HUM have been demonstrated through the inhibitory effects of HUM in the production of $PGE_2$ as well as inducible nitric oxide synthase and cyclooxygenase expression[8]. Thus, it was suggested that anti-tumor activity of HUM could be due to $PGE_2$ suppression, at least partially. However, such a possibility has not been confirmed in a two-stage carcinogenesis model.

The important strategy of cancer chemoprevention aims at reducing the risk of cancer through modulation of detoxification enzymes involved in metabolic activation (Fig. 1). Therefore, inducibility of the phase I and phase II drug metabolizing enzymes is one of the reliable markers for evaluating the chemopreventive potential of the test materials in murine model system[9].

The present study demonstrates that dietary administration of HUM increases the activities of both phase l enzymes, *viz.*, ECOD, EROD and PROD excluding MROD (Fig. 5), and phase II enzyme, *viz.*, GST and QR (Fig. 6), in mouse liver. As for the phase I enzymes, HUM increased CYP concentrations and the activities of various CYP enzymes, especially PROD, marker for a CYP2B enzyme in the liver in a dose-dependent manner. CYP2B gene induction is known to be regulated by the constitutive androstane receptor (CAR)[10]. It is likely that HUM may induce certain drug-metabolizing enzymes through activation of the nuclear receptor CAR.

There have been several lines of evidence suggesting that phase II enzymes such as GST and QR play essential roles in the detoxification and elimination of activated carcinogens during tumor initiation while phase I enzymes are involved in both bioactivation and detoxification of carcinogens. The present study revealed that hepatic activities of GST and QR were significantly increased in HUM-treated mice when compared with the control (Fig. 6). Similar to those previously reported for other known chemopreventive agents[11]. GST belongs to a superfamily of multifunctional isoenzymes categorized into the three major classes, α, μ and π, and all

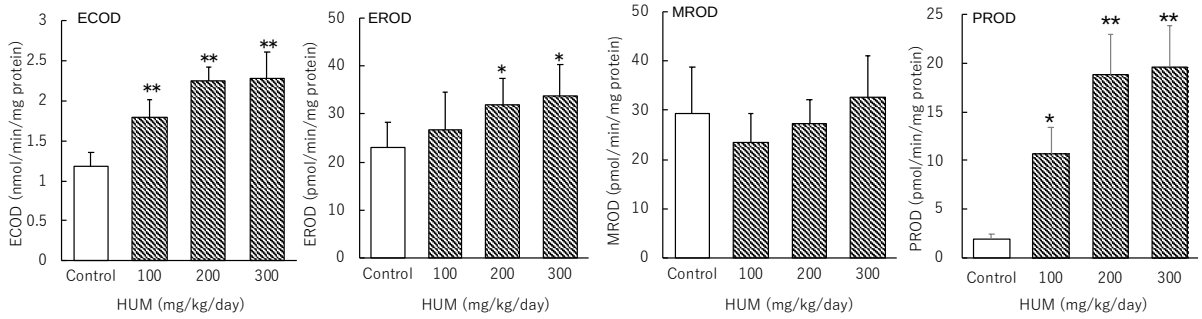


**Fig. 5. Effect of HUM on activities of phase I enzymes (ECOD, EROD, MROD and PROD) in mouse liver**
Data represent means ± S.D. (n = 6-7), *: P < 0.05, **: P < 0.01, Significantly different from control.

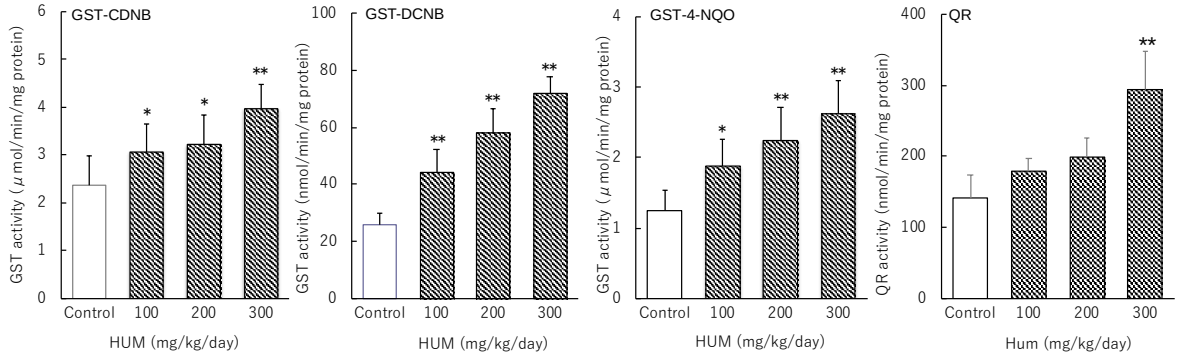


**Fig. 6. Effect of HUM on activities of phase II enzymes in mouse liver**
Data represent means ± S.D. (n = 7-8), *: P < 0.05, **: P < 0.01, Significantly different from control.

three have overlapping substrate specificities and physiological functions[12]. As the GST activation was most apparent with DCNB as substrate, HUM seems to mainly induce the activity of class μ GST, which is known to contribute to detoxification of the carcinogen 4NQO[13] and aflatoxin $B_1$[14]. In addition, reduction of electrophilic quinones by QR is an important detoxification pathway, which converts quinones to hydroquinones and reduces oxidative cycling[15]. HUM also elevated the liver content of the reduced form of GSH, the GST substrate (Fig. 7). This change may also contribute to the augmentation of the phase II enzyme functions.

Taken together, we demonstrated here that HUM acted as a bifunctional inducer, enhancing both the phase I and phase II enzymes of the liver. The results suggested that the carcinogenesis suppression by HUM involves modulations of the phase I and/or II drug-metabolizing enzymes and that HUM may be a viable candidate for cancer chemoprevention.

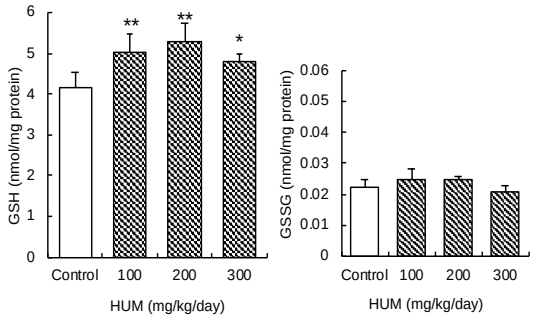


**Fig. 7. Effect of HUM on mouse liver glutathione contents**
Data represent means ± S.D. (n = 5-7), *: P < 0.05, **: P < 0.01, Significantly different from control. GSH, reduced glutathione; GSSH, oxidized glutathione.

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