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Metabolic Pathway of Dibenzoylmethane, a β-diketone Analogue of Curcumin, by NADPH-dependent Cytochrome P450 Enzymes in Mouse Liver Microsomes

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ABSTRACT

Dibenzoylmethane (DBM), a curcumin-related β-diketone analogue, has been reported to exhibit a remarkable inhibitory effect on 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumorigenesis in Sencar mice. Investigation of the underlying mechanisms of DBM in the prevention of mammary tumorigenesis implied its role as an effector of Phase I enzymatic system. In this report, the metabolic fate of DBM by NADPH-dependent cytochrome P450 enzymes in mouse liver microsomes was demonstrated. Isolation of the major reductive metabolites of DBM ($DBMH_2$), together with several minor metabolites identified by NMR, GC and LC-MS, explained the potential role of DBM as a modulator of the cytochrome P450 reductase that is required for the function of oxidase to metabolize DMBA. These might also contribute to the result of the inhibitory effect of DBM on DMBA-induced mouse mammary tumorigenesis.

Key words: dibenzoylmethane, 7,12-dimethylbenz[a]anthracene, reductive metabolite, mouse liver microsomes, NADPH-dependent cytochrome P450 enzymes, mammary tumorigenesis

INTRODUCTION

Curcumin, the major yellow constituent in turmeric (the ground rhizome of *Curcuma longa* Linn) has been used for the treatment of inflammatory-related diseases in traditional medicine. Several studies have shown that dietary curcumin inhibits certain chemically-induced tumorigenesis in animal models, mainly due to its strong antioxidant and antiinflammatory activities[(1)]. Despite of the broad chemopreventive effects on various cancers, its poor absorption and bioavailability precluded curcumin from inhibiting mammary tumor formation[(2-4)]. However, dibenzoylmethane (DBM), a β-diketone structural analogue of curcumin, has received attention in recent years because of its potent anticarcinogenic activity against 7,12-dimethylbenz[a]anthracene(DMBA)-induced mammary tumorigenesis tested in both mice and rats[(4,5)].

DMBA, an effective carcinogenic initiator, is metabolically activated by cytochrome P450 oxidase to electrophilic diol-epoxide intermediates, which subsequently interact with DNA to form DMBA-DNA adducts[(8,13)]. In our previous *in-vivo* and *in-vitro* studies, DBM inhibited DMBA metabolism and formation of DMBA-DNA adducts in a dose-dependent manner[(14,15)]. Investigation of the underlying mechanism regarding the involvement of β-diketone moiety of DBM on mammary tumorigenesis is important. The β-diketone functionality in curcumin has been showed to exhibit antioxidative activity on *tert*-butylhydroperoxide-induced lipid peroxidation of erythrocyte membrane ghosts[(6)]. Talalay also reported the potency of DBM as an inducer of Phase II detoxification enzymes, in part due to the β-diketone functionality[(7)]. However, not any report published so far has concerned the influence of the β-diketone group on cytochrome P450 Phase I metabolizing enzymes, since DMBA needs to be oxidatively metabolized to bioactive carcinogen[(8)].

In the present study, we examined the metabolic fate of DBM by NADPH-dependent cytochrome P450 enzymes in mouse liver microsomes. *In vivo*, curcumin with a β-diketone moiety has been proved to be transformed through reduction as well as glucuronidation to tetrahydrocurcumin found in rat bile excretion[(3)]. The finding of a major reductive DBM metabolite in β-diketone moiety *in vitro* may provide partially the explanation from chemical aspects on the inhibitory of DBM on DMBA-induced tumorigenesis.

MATERIALS AND METHODS

I. *Chemicals*

Dibenzoylmethane, glucose-6-phosphate, glucose-6-

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phosphate dehydrogenase, NADPH, TLC plate (250 μm thickness, 2-25 μm particle size), silica gel (70-230 mesh) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CD_3OD was purchased from Aldrich (Milwaukee, WI). All solvents (HPLC grade) were purchased from Fisher Scientific Co. (Springfield, NJ, USA).

II. *Preparation of Mouse Liver Microsomes*

Seven to 8 weeks old female Sencar mice from Charles River Laboratories (Wilmington, MA, USA) were fed on an AIN 76A diet (control group) or 1% DBM diet for 2-3 weeks. The mice were killed by decapitation and livers were removed, rinsed in cold saline to remove excess blood. The livers were then homogenized in 2 volumes (w/v) of 0.05 M Tris-HCl (pH 7.5 at 4˚C), 0.15 M KCl and the microsomal fraction was isolated by differential centrifugation. Briefly, the homogenates were centrifuged at 11,000×g for 20 min; the supernatant fraction was re-centrifuged for 1 hr at 105,000×g. The microsomal fraction was suspended in 0.25 M sucrose and stored in small aliquots at -80˚C. The concentration of protein was determined by a Bio-Rad protein assay kit with BSA as a standard.

III. *Incubation of DBM with Mouse Liver Microsomes*

The reaction mixture contained 0.5 mg liver microsomes, 100 mM KH_2PO_4 (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA, and a NADPH regeneration system, which consisted of 20 mM glucose-6-phosphate, 1 unit G-6-P dehydrogenase, 5 mM NADPH and 100 μM of DBM in a final volume of 100 mL. The reaction was incubated at 37˚C for several hours. After the incubation, the reaction was stopped by adding 1 mL of 5 N HCl and extracted with 2-3 volume folds of ethyl acetate. The organic layer was dried over $MgSO_4$, concentrated under reduced pressure to about 200 μL and then subjected to TLC and HPLC analysis.

IV. *Procedure for Separation and Identification of Major DBM Metabolite*

Analysis of DBM metabolites was performed by using a Varian 5000 HPLC system equipped with a Varian 2050 detector and a normal phase column (α-chrom, 3 mm × 25 cm, Upchurch Scientific, Inc.) The solvents used for separation of DBM and its metabolites consisted of *n*-hexane/ethyl acetate. The solvent gradient programmed in HPLC was as follows: 0 min isocratic at 100/0; 5 min of gradient to 95/5; 1 min of gradient to 92/8; 14 min of gradient to 80/20; 5 min of gradient to 70/30; 5 min of gradient to 60/40. The HPLC metabolite peaks were detected by UV at 265 nm. Isolation of the major metabolite was performed by silica gel column chromatography using *n*-hexane/ethyl acetate (80/20) as eluent. The spots shown on TLC corresponded to DBM (R_f 0.9), major product (R_f 0.5) and minor products (R_f 0.1). The fractions with major metabolite were collected and concentrated to afford a colorless liquid (about 20% yield). Structural confirmation of the major metabolite was achieved by NMR and identified as 1,3-diphenyl-3-hydroxyl-1-propanone. ^{1}H-NMR (200 MHz, CD_3OD): 7.98 (2H, d, J = 7.2 Hz, H-2'), 7.60-7.25 (8H, m, H-2", H-3', H-3", H-4', H-4"), 5.31 (1H, dd, J = 8.4, 4.8 Hz, H-3), 3.52 (1H, dd, J = 8.4,16 Hz, H-2a), 3.29 (1H, dd, J = 4.8, 16 Hz, H-2b).

V. *Analysis of the Minor DBM Metabolites by GC-MS and LC-MS*

Analysis of the minor metabolites of DBM was achieved by the GC-LC and LC-MS system consisting of Hewlett-Packard 5989A mass spectrometer interfaced with a 5890 Series II gas chromatograph or HP59980 particle beam. The gas chromatograph was equipped with a 30 m DB-1 capillary GC column (0.32 mm I.D., 0.3 μm film thickness) and a MS detector. The injector port and detector temperatures were 250˚C and 280˚C, respectively. The column temperature was programmed from 70˚C to 250˚C at a rate of 4˚C/min with helium as the carrier gas. Injections were made in splitless mode at a column head pressure of 14 psi. The retention time and mass fragmentation pattern for each metabolite peak were compared with those of standards searched by GC-MS spectral database (Wiley138 library). LC-MS was performed by using a Supelco reverse-phase column (15 cm × 2 mm). An isocratic mobile phase consisting of acetonitrile/water (7/3) at a flow rate of 0.4 mL/min was used. Chemical ionization spectra were obtained using ammonium as the reagent gas.

RESULTS AND DISCUSSION

I. *Identification of A Major Metabolite of DBM from Incubation with Mouse Liver Microsomes In Vitro*

The HPLC profile for DBM metabolism in a NADPH-dependent mix function oxidase system by liver micro-

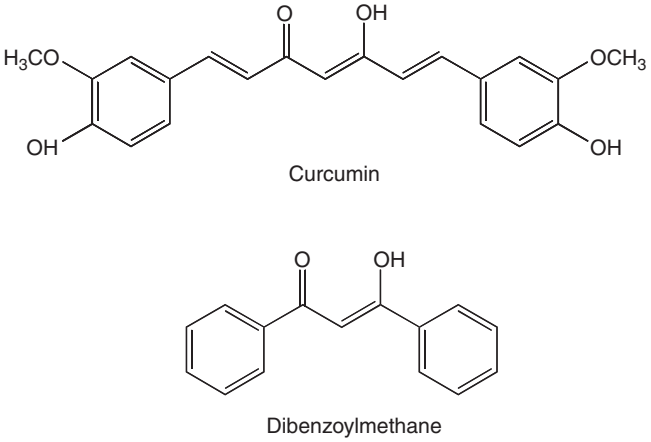

Figure 1. Structures of curcumin and dibenzoylmethane.

Table 1. Identification of the metabolites of DBM by GC-MS and LC-MS

Metabolite	Method	Retention time (min)	Characteristic MS fragments
1,3-diphenyl-3-hydroxyl-1-proopanone[a]	LC-MS	0.81	244 (M+18), 227 (M+1), 226 (M), 209 (M-17)
Benzoic acid	GC-MS	16.71 (trace)	122, 106, 105 (base peak), 77, 51
	LC-MS	0.47	123 (M+1)
Chalcone	GC-MS	37.66	208, 207 (base peak), 131, 105, 103, 77
Dihydro-chalcone	GC-MS	38.92	210, 105, 104 (base peak), 79, 77

The condition for incubation was the same as described in assay for *in-vitro* DMBA metabolism except that DBM (200 μM) without DMBA was the only substrate added to the reaction. The reaction mix was subjected to GC-MS and LC-MS analysis as described in "MATERIALS AND METHODS". The retention time and mass fragmentation pattern for each metabolite peak in GC-MS spectra were compared with those of reference standards searched by GC-MS spectral database (Wiley138 library).

[a]The reductive metabolite was further characterized by NMR and compared with synthesized authentic standard.

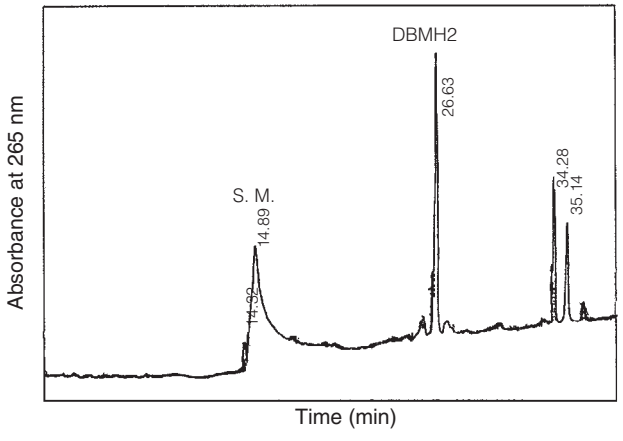

Figure 2. HPLC profile for analysis of metabolites of DBM by mouse liver microsomes. Seven to 8 weeks old female Sencar mice were fed with AIN 76A diet or 1% DBM diet for 2-3 weeks and the mice were sacrificed for the preparation of liver microsomes. Reaction mixture consisted of 200 mM DBM, 0.5 mg liver microsomes, 100 mM KH_2PO_4 (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA, and a NADPH regeneration system, which consisted of 20 mM glucose-6-phosphate, 1 unit G-6-P dehydrogenase, 5 mM NADPH in a final volume of 100 mL. After 5 hr of incubation at 7°C, the organic components were extracted and subjected to TLC and HPLC analysis as described in "MATERIALS AND METHODS".

somes from Sencar mice fed on a control AIN-76A diet or 1% DBM diet is shown in Figure 2. One major and two minor peaks were observed in the cases of both control and 1% DBM microsomes after 5 hr of incubation. We have previously examined the effect of incubation time on the formation of metabolites by HPLC analysis (data not shown). It seemed that the major product reached its maximum quantity after 3 hr and two minors reached the maximum after longer incubation time in both cases. Isolation of the major metabolite was performed by silica gel column chromatography using *n*-hexane/ethyl acetate (4/1) as eluent. The fractions were concentrated and monitored by thin-layer chromatography (TLC). A colorless liquid corresponding to R_f 0.5 was identified as the major metabolite of DBM by HPLC analysis. The structure was deduced by ^1H NMR spectrum as 1,3-diphenyl-3-hydroxyl-1-propanone, a reductive form of DBM ($DBMH_2$), which was further confirmed by comparing with the synthesized authentic compound. The NMR spectrum of DBM metabolite clearly showed the characteristic ABX absorption pattern of β-hydroxyl ketone moiety, where two methylene protons (C-2a, C-2b) near a chiral center (C-3) are not chemical shift equivalent and these three protons are splitted by each other (J_{gem} = 16 Hz, J_{vic} = 8.4 Hz and 4.8 Hz). Two conclusions can be addressed from this finding: (1) The formation of reductive metabolite that is more polar than DBM will facilitate the conjugative metabolism by Phase II enzymes and then excretion; (2) The cytochrome P450-dependent drug metabolism system has been considered consisting of two protein and a lipid components, cytochrome P450, NADPH cytochrome P450 reductase and phosphatidylcholine[9]. Therefore, it is reasonable to suggest that DBM may be an inhibitory factor interferring with the electron supply to the hemoprotein by accepting electrons directly from the cytochrome P450 reductase.

II. *Identification of Minor Metabolites of DBM by GC-MS and LC-MS*

For further understanding of DBM metabolic pathway by mouse liver microsomes, the incubation mix was examined by GC-MS or LC-MS to detect other possible minor and small molecular metabolites. The identified structures with their MS spectral information are listed in Table 1 and their spectra are shown in Figure 3. Several secondary metabolites of DBM, including chalcone, dihydrochalcone and the oxidative cleaved product, benzoic acid (further characterized by LC-MS with pseudomolecular ion peak at *m/z* 123), were identified and the possible metabolic pathway of DBM by mouse liver microsomes was proposed (Figure 4). It is worth mentioned that injection of $DBMH_2$, the primary metabolite of DBM, into GC-MS resulted in several thermal degradation products, including benzaldehyde, 1-phenyl-ethanone and traces of chalcone. Therefore, the identity of chalcone as a metabolite of DBM by liver microsomes *in vitro* remained questionable and we still cannot tell whether the reduction come from $DBMH_2$ directly or from chalcone, the product resulted from dehydration of $DBMH_2$. In addition, the

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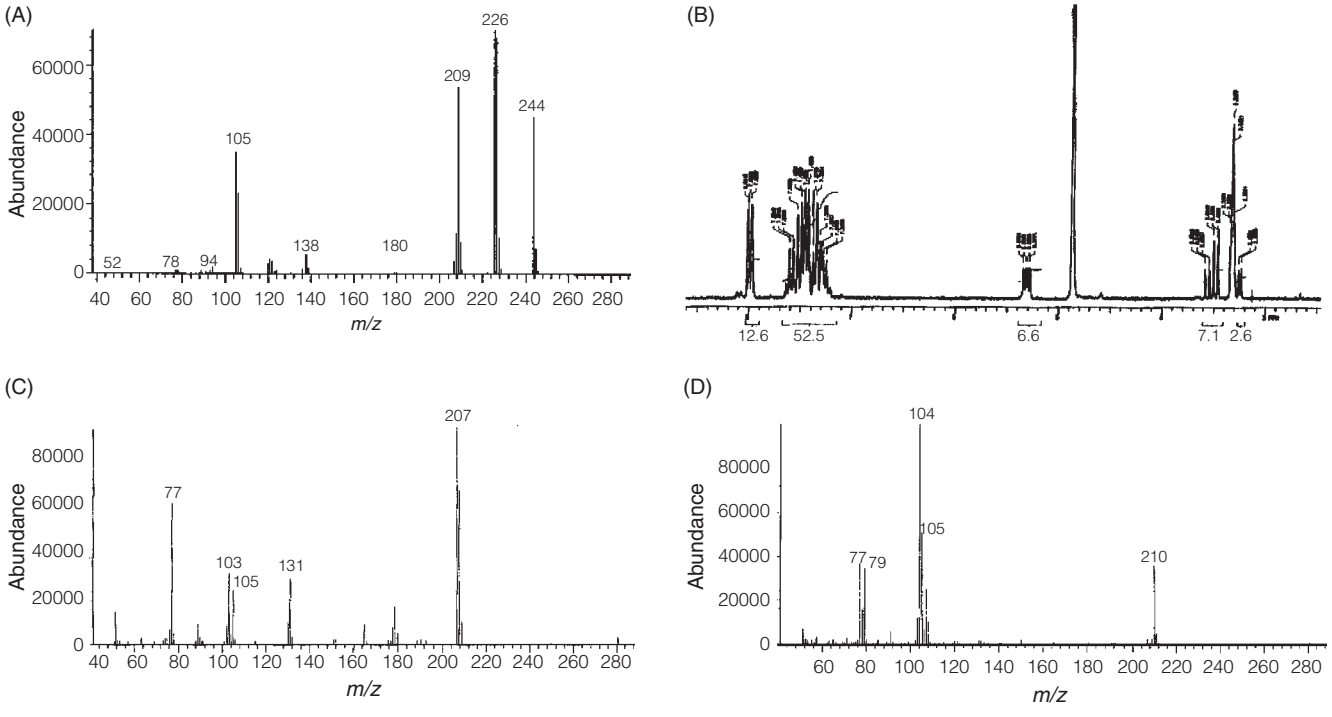
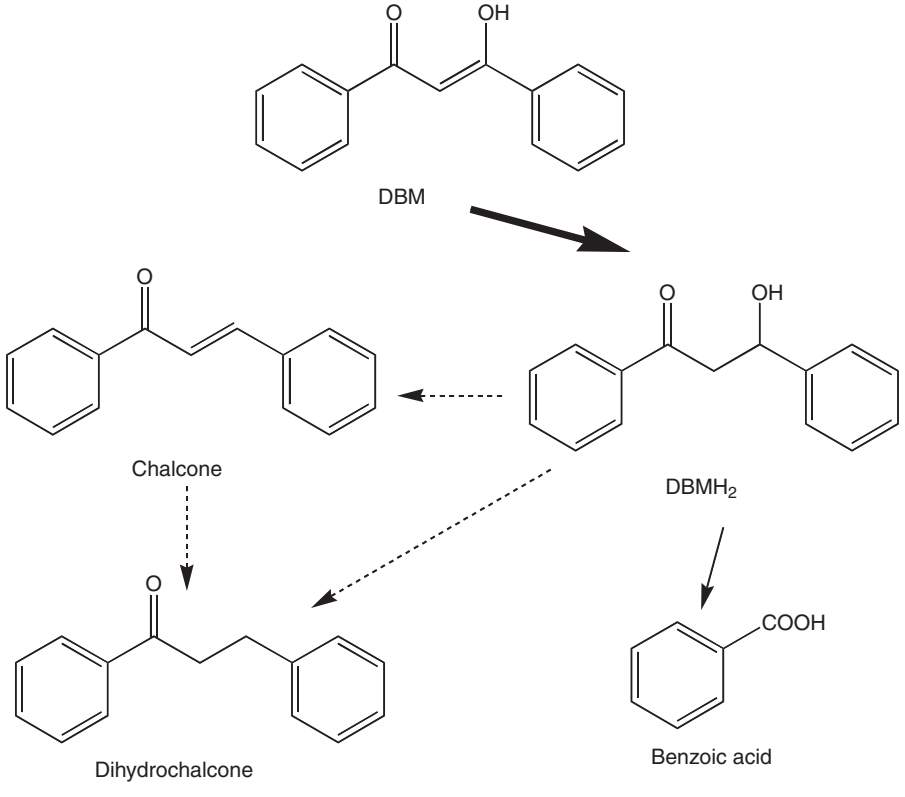
identification of benzoic acid by LC-MS suggested the possible important role of oxidative cleaved acid in biochemical and pharmacological aspects *in vivo*.

The metabolic fate of curcumin *in vivo* has been studies back in 1970's[3]. The major glucuronidated tetrahydro- and hexahydrocurcumin as well as minor dihy-



Figures 3. The spectra of identified DBM metabolites. (A) LC-MS of $DBMH_2$ (B) NMR of $DBMH_2$ (C) GC-MS of chalcone (D) GC-MS of dihydrochalcone.



Figures 4. Proposed metabolic pathway of DBM by mouse liver microsomes.

droferulic acid were identified in rat biles. Several possible routes of reduction has been suggested, including transformation during intestinal absorption[10], gut flora, NADPH-linked α,β-ketoalkene double bond reducatse from rat liver cytosol[11]. However, several studies also suggested that most of the administered curcumin was reduced by an endogenous reductase system[12]. In our studies, DBM, a β-diketone analogue of curcumin, was used to demonstrate that the β-diketone functionality in DBM is reduced through the action of liver microsomes, in which cytochrome P450 reductase is thought to be responsible for the reduction. Therefore, the previous conclusion regarding the location of the reductive metabolic action on curcumin needs to be re-considered.

In conclusion, the metabolic fate of DBM by NADPH-dependent cytochrome P450 enzymes in mouse liver microsomes was demonstrated. The isolated reductive metabolites of DBM would imply the potential role of DBM as an inhibitor of cytochrome P450 reductase that is required for the function of oxidase to metabolize DMBA, resulting in the inhibitory effect on DMBA-induced mouse mammary tumorigenesis.

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