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Metabolism of 2-hydroxy-4-methoxybenzophenone in isolated rat hepatocytes and xenoestrogenic effects of its metabolites on MCF-7 human breast cancer cells

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Abstract

The metabolism and cytotoxicity of 2-hydroxy-4-methoxybenzophenone (HMB) in isolated rat hepatocytes and the xenoestrogenic activity of HMB and its metabolites in MCF-7 human breast cancer cells and an estrogen receptor competitive binding assay have been studied, respectively. The incubation of hepatocytes with HMB caused a concentration- and time-dependent decrease in cell viability, accompanied by loss of intracellular ATP and adenine nucleotide pools. HMB at a low-toxic level (0.25 mM) in the hepatocyte suspensions was converted enzymatically to 2,4-dihydroxybenzophenone (DHB) and a hydroxylated intermediate, which was tentatively identified as an isomer of 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) as determined by mass spectroscopy coupled with HPLC. Furthermore, the parent compound and both intermediates were rapidly conjugated to glucuronides, whereas free unconjugated DHMB and 2,3,4-trihydroxybenzophenone (THB) were identified

Abbreviations: BPA, bisphenol A; DES, diethylstilbestrol; DHB, 2,4-dihydoxybenzophenone; DHMB, 2,2′-dihydroxy-4-methoxybenzophenone; DMSO, dimethyl sulfoxide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid); HMB, 2-hydroxy-4-methoxybenzophenone; THB, 2,3,4-trihydroxybenzophenone.

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as trace intermediates. In another experiment, DHB and THB displaced competitively 17β-estradiol bound to the recombinant human estrogen receptor α in a concentration-dependent manner: IC_{50} of diethylstilbestrol and bisphenol A, which are known xenoestorogenic compounds, and DHB and THB was $\approx 1 \times 10^{-8}$, 1×10^{-5}, 5×10^{-5} and 5×10^{-4} M, respectively. Further, DHB at concentrations from 10^{-8} to 10^{-6} M caused a concentration-dependent proliferation of MCF-7 cells. DHMB and THB at 10^{-7} and 10^{-6} M also elicited a slight increase in cell numbers, whereas HMB at concentrations from 10^{-9} to 10^{-4} M did not affect the cell proliferation. Based on the relative IC_{50} for the competitive binding and the proliferative effect on MCF-7 cells, it follows that in estrogenic potency, DHB > THB > DHMB. These results indicate that some hydroxylated intermediates such as DHB rather than the parent compound act as a xenoestrogen via biotransformation.

Keywords: 2-Hydroxy-4-methoxybenzophenone; Metabolism; Toxicity; Xenoestrogens; Hepatocytes; MCF-7 cells

1. Introduction

2-Hydroxy-4-methoxybenzophenone (HMB) and eleven analogues have been used commercially as ultraviolet (UV) light stabilizers in plastic surface coatings or polymers and cosmetically in sunscreens for more than 30 years. HMB, 2,2′-dihydroxy-4,4′-dimethoxybenzophenone and 2,2′-dihydroxy-4-methoxybenzophenoene (DHMB) are natural components that can be extracted from certain flower pigments. Since HMB is widely used in sunscreens/UV stabilizers, it has been investigated in vivo and in vitro for its toxicological properties and metabolism [1–8]. Though HMB is not a potent toxicant, the liver and kidney were identified as target organs of toxicity induced by HMB and unsubstituted benzophenone (diphenyl methanone) in rats and mice; treatment-related increases in liver weights were attributed to hypertrophy and/or cytoplasmic vacuolization of hepatocytes and increased kidney weights were associated with a spectrum of renal changes with foci of tubule regeneration [9,10].

HMB is well absorbed via the oral and dermal routes in rats; the compound undergoes extensive metabolism and is excreted in urine and bile [6–8]. HMB is enzymatically converted to at least three intermediates and their glucuronides and/or sulfates. 2,4-Dihydroxybenzophenone (DHB) is a major intermediate formed by *O*-demethylation of the parent compound, which in turn is converted to 2,3,4-trihydroxybenzophenone (THB) by aromatic hydroxylation [7]. HMB is also converted to DHMB by aromatic hydroxylation [7].

Although many natural and synthetic compounds are ubiquitous in the environment, little is known about the potential risks to humans of exposure to known xenoestrogens. Recently, considerable attention has focused on bisphenol A (BPA) as well as other phenolic compounds as endocrine disrupting chemicals having weak estrogenic activity in in vivo [11,12] and in vitro [13] bioassays. Benzophenone is listed among ‘chemicals suspected of having endocrine disrupting effects’ by the World Wildlife Fund, the National Institute of Environmental Health Sciences in

the USA and the Japanese Environment Agency. In previous studies [14,15], we reported that unsubsituted benzophenone at a low toxic level was enzymatically converted to at least three metabolites, benzhydrol, 4-hydroxybenzophenone and a sulfate, in isolated rat hepatocytes and that the effect of 4-hydroxybenzophenone on the proliferation of MCF-7 cells in vitro and uterotorophic activity in immature rats was weakly estrogenic compared to that of 17β-estradiol, whereas the parent compound and benzhydrol were essentially inactive at the concentrations used.

The metabolic pathway and toxicity of HMB have been studied; however until now, the relationship between metabolism and estrogenic activity has not been investigated. In the present study, we investigate the metabolism and action of HMB in isolated rat hepatocytes and assess the potential estrogenic activities of HMB and its intermediates using a competitive binding assay for recombinant human estrogen receptor α and a proliferative assay of MCF-7 cells which are estrogen-responsive human breast cancer cells. The mechanisms of the activities of HMB and its metabolites are discussed.

2. Materials and methods

2.1. Materials

The chemical compounds used were obtained from the following companies: HMB, DHB, 2,2′-dihydroxy-4-methoxybenzophenone (DHMB), THB and BPA (purities of $>97\%$) from Tokyo Kasei Kogyo Co. (Tokyo, Japan); 17β-estradiol (E_2), diethylstilbestrol (DES), β-glucuronidase, sulfatase (type VI, β-glucuronidase-free *Aerobacter aerogenes)*, *N-(*2-hydroxyethyl)-piperazine-*N*-(2-ethanesulfonic acid) (HEPES) and bovine serum albumin from Sigma Chemical Co. (St. Louis, MO); collagenase from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals were of the highest purity commercially available. The chemical structures of benzophenones used in this study are shown in Fig. 1.

2.2. Isolation and incubation of hepatocytes

Male F344/DuCrj (240–260 g) rats were obtained from Charles River Japan Inc. (Yokohama, Japan) and were housed in wire-bottom cages. The rats were allowed food (CE-2, Clea Japan Inc., Tokyo) and water ad libitum before hepatocytes were prepared. The hepatocytes were isolated by collagenase perfusion of the liver, as described previously [16]. Hepatocyte viability was assessed by Trypan blue exclusion, and initial cell viability in each experiment was more than 85%.

Hepatocytes (10^6 cells/ml) were suspended in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM HEPES and 0.1% albumin. All incubations were performed in rotating, round-bottomed flasks at 37 °C, under a constant flow of humidified

carbogen (95% O_2/5% CO_2). Reactions were started by the addition of HMB dissolved in dimethyl sulfoxide (DMSO; final concentration, $< 1\%$). Corresponding control groups received an equivalent volume of DMSO. Aliquots of incubation mixture were taken at intervals to monitor cell death and the concentrations of intracellular adenine nucleotides, and HMB and its metabolites.

2.3. Determination of HMB metabolites by HPLC

An equal volume of chilled methanol was added to the cell suspension and the mixture was filtered through a membrane cartridge (pore size of cellulose membrane, 0.45 μm; 13 mm i.d. × 20 mm; Millex-SLHV, Millipore Co., Bedford, MA). The eluate (20 μl) was injected onto an analytical Capcell pak C18 column (4.6 mm i.d. × 250 mm, 5 μm particle size; Shiseido Co., Tokyo) or Richrosorb RP-18 column (10 mm i.d. × 250 mm, 7 μm particle size; Merck KgaA, Darmstadt, Germany) equipped with a UV absorbance detector (260 nm). The mobile phase was methanol/0.1 M ammonium dihydrogen phosphate (50:50, by volume, pH 5.3), and the flow rate was 1.0 ml/min. HMB and its metabolites (DHB, DHMB and THB) were identified by co-chromatography or by comparison of their HPLC retention times with these authentic compounds. The recoveries for HMB and DHB were checked by the addition of known amounts of authentic compounds to hepatocyte suspensions, and were more than 85%.

Conjugates derived from HMB in the cell suspensions were enzymatically hydrolyzed with β-glucuronidase or β-glucuronidase-free sulfatase and the hydrolytic products were identified and/or determined by HPLC. Briefly, aliquots of cell suspensions were treated with a cell disrupter (Sonifier Branson Sonic Power Co.; Danbury, CT) in ice-water for 20 s and then filtered through an ultrafiltration cartridge (limit of elution; < molecular weight 5000; Ultrafree-MC centrifugal filter unit; Millipore Co.). The eluate adjusted to pH 4.5 with sodium acetate buffer was incubated with β-glucuronidase (about 500 U) or sulfatase (about 2 U) for 3 h at 37 °C and then the mixture (40 μl) was injected into the HPLC system.

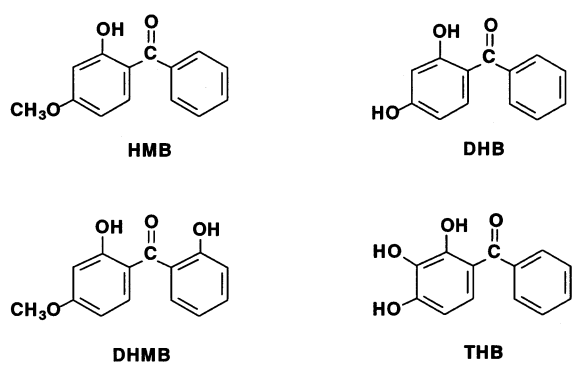

Fig. 1. Chemical structures of 2-hydroxy-4-methoxybenzophenone (HMB), 2,4-dihydroxybenzophenone (DHB), 2,2′-dihydroxy-4-methoxybenzophenone (DHMB) and 2,3,4-trihydroxybenzophenone (THB).

2.4. Identification of a hydroxylated intermediate of HMB by mass spectroscopy coupled with HPLC

A fraction (peak b in Fig. 3) obtained separately by HPLC using the Richrosorb RP-18 column described above was concentrated by solid-phase extraction using C_{18} Sep-Pak cartridges (Waters Co., Milford, MA), and was also subjected to negative electrospray ionization/mass spectroscopy (HPLC/-ESI/MS) analysis. Negative ion mass spectra of HMB metabolites were obtained using a LC/MS system, consisting of a Waters mass spectrometer ZMD and a Waters pump 2690 (Waters Co.). The mobile phase was 10% methanol in water and the flow rate was 0.2 ml/min. The MS spectrum was scanned from 100 to 1000 Da in 1.0 s and produced with a corn voltage of 40 V.

2.5. Competitive binding assay

Competitive binding between 17β-estradiol and various compounds was determined using an estrogen-R(α) competitor screening kit (Wako Pure Chemical Industries Ltd., Osaka); the kit consists of recombinant human estrogen receptor α (ERα) coated on the bottom of 96-well multiwell plates and fluorescein-labeled 17β-estradiol as the competitor for the assay. DES and other compounds dissolved in DMSO were added to a reaction solution containing fluorescein-labeled 17β-estradiol, and the mixture (100 μl) was added into each well. The concentrations of the various compounds used in this study ranged from 5×10^{-10} to 5×10^{-4} M: at more than 5×10^{-4} M, HMB did not dissolve in the mixture. After 2 h of incubation at room temperature, the mixture, which contained free compounds or the 17β-estradiol unbound to ERα, in wells was aspirated and exchanged with the assay solution (100 μl). The concentration of fluorescein-labeled 17β-estradiol bound to ERα on the bottom was measured in a CytoFluor 4000 fluorescence plate reader (PerSeptive Biosystems Inc., Framingham, MA) with filters set for 485-nm excitation and 535-nm emission. The results are expressed as percentages of the fluorescence values for the reaction solution without samples.

2.6. MCF-7 cell proliferation assay

MCF-7 cells (cultured human breast cancer cells) were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in phenol red free-RPMI-1640 medium, supplemented with 5% fetal calf serum (FCS), 15 mM HEPES, 50 U/ml penicillin, 50 μg/ ml streptomycin and 10 ng/ml insulin at 37 °C with 5% CO_2 in air at saturating humidity, and were routinely passed at $\approx 80\%$ confluence. Prior to initiating experiments, cells were seeded and attached in 96 well multiwell plates at 4×10^3/well in 0.3 ml of RPMI-1640 medium, supplemented with 5 estrogen-free FCS. After 24 h, the medium was replaced with the same volume of the above medium containing 17β-estradiol (1 nM) as a positive control, HMB or its metabolites: the concentrations of HMB and its metabolites used in this study ranged from 1 nM to 500 μM. Estrogen-free FCS was prepared using the

dextran-charcoal procedure [17]. The cells were cultured for 5 days and cell numbers in each well were determined using a cell proliferation assay kit (cck-8; Dojindo Laboratories Co., Kumamoto, Japan). Thirty microliters of WST-8 solution, 5 mM 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt and 0.2 mM 1-methoxy phenazinium methylsulfate dissolved in 150 mM KCl, were added to wells containing 0.3 ml of medium with cells. After incubation for 60–90 min at 37 °C in a humidified 5% CO_2 atmosphere, the absorbance of the well was read at 450 nm (reference wavelength, 650 nm) by a microculture plate reader (model 450, Bio-Rad Laboratories, New York, NY). The assay, a modified tetrazolium colorimetric assay, is based on metabolic reduction of WST-8 to its corresponding formazan and the coloration obtained is directly proportional to the cell number. The coloration was found to be linear for up to 120 min after addition of the dye-containing medium, with cell numbers exceeding 3×10^4.

2.7. Statistical analysis

Statistical analysis was performed by one-way analysis of variance, followed by Dunnett's multiple comparison test. Statistical significance was assumed at $P < 0.05$.

3. Results

3.1. Toxic effects of HMB on rat hepatocytes

The incubation of rat hepatocytes with HMB (0.25–1.0 mM) caused a concentration and time-dependent cell killing accompanied by a depletion of intracellular ATP and total adenine nucelotides, and formation of surface blebs, indicating cellular morphological damage (Fig. 2). Although HMB at 0.5 or 1.0 mM elicited the rapid depletion of total nucleotide pools, the loss of ATP was reflected by concomitant increases in the levels of ADP and AMP (data not shown). At a concentration of 0.5 mM, the toxicity of DHB in hepatocytes was more than that of parent compound, HMB; cell death caused by HMB and DHB were 33.2 ± 4.7 and $78.5 \pm 5.2\%$ (means $\pm$ SE) after a 1 h incubation, respectively.

3.2. Metabolism of HMB in isolated rat hepatocytes

To understand the metabolism of HMB, the time courses for the change in the levels of the compound and its metabolites in hepatocytes were investigated at a low toxic concentration (0.25 mM) of HMB. As shown by the HPLC elution profiles (Fig. 3), most of the HMB (retention time; RT, ≈ 16.0 min) was effectively converted to several metabolites during a 3 h incubation. The disappearance of HMB (0.25 mM) in the hepatocyte suspensions was accompanied by a temporal increase in DHB (RT, 9.1 min), whose level reached a maximum concentration 30

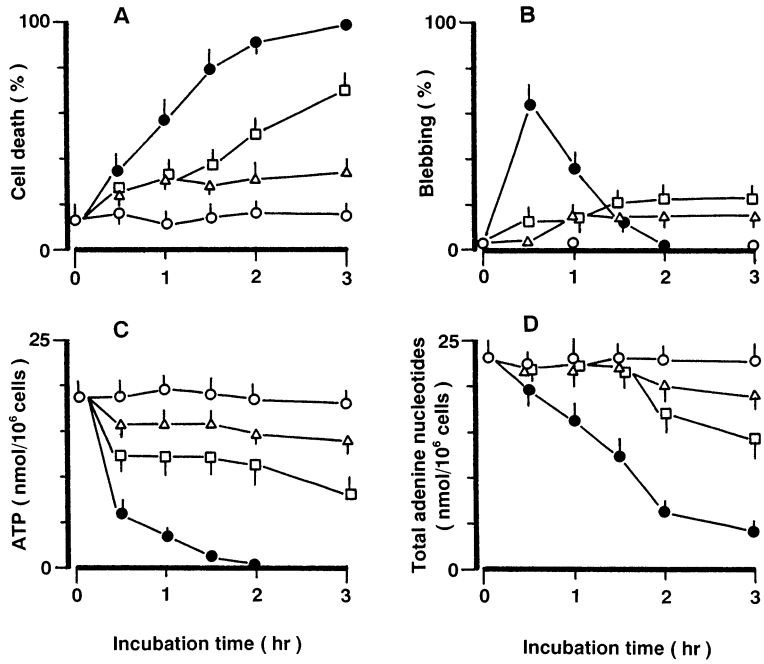

Fig. 2. Effects of HMB on cell death (A), blebbing (B), and intracellular levels of ATP (C) and total adenine nucleotides (D) in isolated rat hepatocytes. Hepatocytes were incubated at 10^6 cells/ml in Krebs-Henseleit buffer, pH 7.4, with no addition (○), 0.25 mM (△), 0.5 mM (□), or 1.0 mM (●) as described in Section 2. Results are expressed as the means ± SE of three experiments.

min later and then decreased with time (Fig. 4). Since both HMB and DHB were stable when incubated under carbogen gas for 3 h at 37 °C in Krebs–Henseleit buffer without hepatocytes, the result indicates that the changes in the level of HMB and DHB depend on an enzymatic reaction by hepatocytes. The material eluted in peak b (RT, 7.5 min) did not correspond to authentic DHMB (RT, 10.0 min) and THB (RT, 5.9 min), which are known as di- or tri-hydroxylated intermediates of HMB in rats [7]. Both DHMB and THB were detected as trace elements, whose levels were less than 0.1 and 0.2 μM in hepatocyte suspensions 1 h later, respectively. The -ESI/MS spectrum of peak b showed a molecular ion at m/z 243, corresponding to the M–H$^{\bullet}$and a base peak at m/z 228, corresponding to a loss of 16 (oxygen; 16 amu) and suggesting incorporation of an oxygen atom into the HMB molecule. Although the position of the oxygen was unclear from the mass spectrum, the material was tentatively identified as an isomer of DHMB; the area corresponding to the amount of peak b reached a maximum 30 min later and then decreased with time. In a preliminary experiment, some HMB added to rat microsomal suspensions with a NADPH-generating system was converted to DHB and the isomer of DHMB (data not shown). On the other hand, the material(s) eluted in peak a (RT, 3.8 min) was enzymatically hydrolyzed with β-glucuronide, not with sulfatase, to yield free unconjugated HMB, DHB and the isomer of

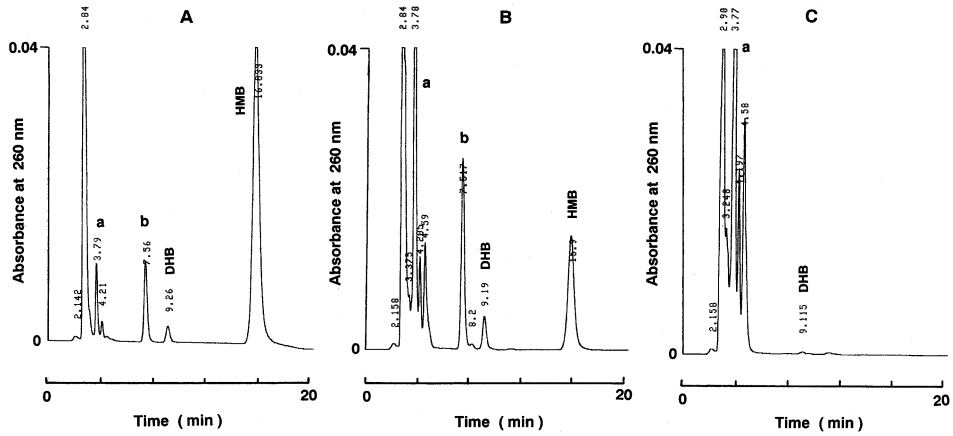

Fig. 3. Typical HPLC elution profiles of metabolites in hepatocyte (10^6 cells/ml) suspensions treated with 0.25 mM HMB; after 5 min (A), 1 h (B), and 3 h (C) incubation. Peaks are glucuronides derived from HMB/its metabolites ((a) retention time (RT), 3.8 min), a hydroxylated intermediate of HMB ((b) RT, 7.5 min), DHB (RT, 9.2 min) and HMB (RT, 16.0 min)

DHMB, indicating that the materials in peak a were hybrids of glucuronides derived from the parent compound and two intermediates. The materials in two peaks (RTs, 4.2 and 4.6 min) followed by the glucuronides (peak a) resisted hydrolysis by sulfatase and/or (β-glucuronidase. Although the metabolic route of benzophenones is thought to be via a reduction of the carbonyl group between aromatic rings to the corresponding secondary alcohol [14,18], no such intermediate was detected in hepatocyte suspensions treated with HMB.

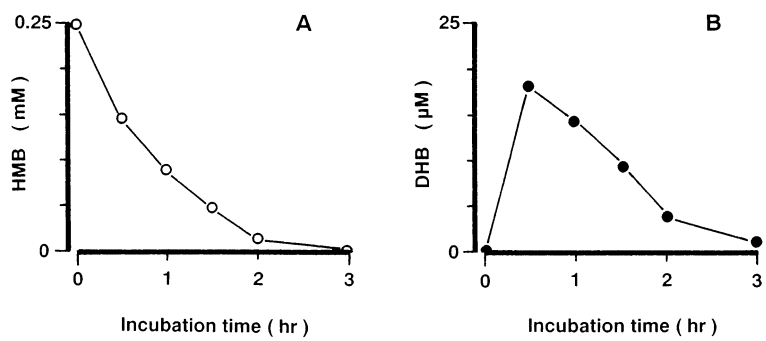

Fig. 4. Changes in the levels of HMB (A) and DHB (B) in rat hepatocyte suspensions. Hepatocytes (10^6 cells/ml) were incubated with 0.25 mM HMB for 3 h. Results are the averages of two experiments.

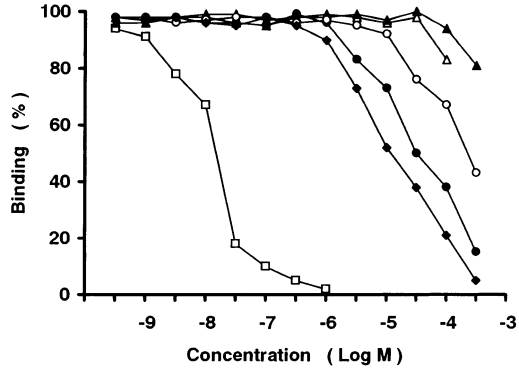

Fig. 5. Competitive binding assay of HMB, its metabolites, BPA and diethylstilbestrol (DES) for human recombinant estrogen receptor α (ERα). Ability of these compounds for binding to ERα was via competition with that of fluorescein-labeled 17β-estradiol. Results are expressed as the means from three determinations. Keys: HMB, △; DHB, ●; DHMB, ▲; THB, ○; BPA, ◆; DES, □.

3.3. Competitive binding assay of HMB and its metabolites for estrogen receptor α (ERα)

To compare affinities among HMB, DHB, DHMB, THM and known xenoestrogenic compounds, DES and BPA, to estrogen receptor, the ability of these compounds to bind to ERα was assayed with that of fluorescein-labeled 17β-estradiol (Fig. 5). DES displaced with very high affinity fluorescein-labeled 17β-estradiol bound to ERα, while BPA and DHB displaced in a competitive manner the 17β-estradiol bound to its receptor. Fifty percent inhibitory values (IC_{50}) of DES, BPA, DHB and THB were $\approx 1 \times 10^{-8}$,1×10^{-5}, 5×10^{-5} and 5×10^{-4} M, respectively. Neither HMB nor DHMB at concentrations from 10^{-9} to 10^{-4} M impaired the binding of 17β-estradiol to ERα.

3.4. Proliferative effects of HMB and its metabolites on MCF-7 cells

Fig. 6 shows the effects of HMB and its metabolites, DHB, DHMB and THB, on MCF-7 cells derived from estrogen-responsive human breast cancer cells. The results were compared with those for untreated control cells. Relative to control ($\approx 10.2 \times 10^3$ cells per well) on day 6, DHM increased cell numbers in a concentration-dependent manner from 10^{-8} to 10^{-6} M and the compound at 10^{-6} M increased cell numbers $\approx$2.7-fold. DHMB and THB at 10^{-7} and 10^{-6} M also caused a slight increase in cell numbers, whereas the parent compound, HMB, used at the concentrations from 10^{-9} to 10^{-5} M did not affect the cell proliferation (tested up to a concentration of 10^{-5} M). The concentration of 17β-estradiol is typical of a positive control in tests evaluating the estrogenicity of the above compounds, and the proliferation induced by estradiol in this study is consistent with previous studies [19,20]. On the other hand, HMB, DHMB and THB at concentrations of $> 10^{-4}$ M elicited a considerable decrease in cell numbers relative to control. This indicates that these compounds at higher concentration were cytotoxic in MCF-7 cells.

4. Discussion

The results obtained in the present study show that in suspensions of hepatocytes, HMB elicited a concentration- and time-dependent loss of cell viability which was accompanied by a decrease in intracellular levels of ATP and total adenine nucleotide pools, and support at least partly that the liver was identified as a target organ by HMB in rats [9]. Although at high concentrations HMB itself was toxic, the compound at a low toxic level (0.25 mM) was rapidly converted to some glucuronides and two major intermediates, DHB and an unknown dihydroxylated intermediate, which was tentatively identified as an isomer of DHMB, since its ESI/-MS spectrum suggests that an oxygen atom was incorporated on an aromatic ring of HMB; the position of the second hydroxy group on the aromatic ring of HMB was not established in this study. Because HMB and DHB were essentially stable in Krebs-Henseleit buffer without hepatocytes under carbogen flow during a

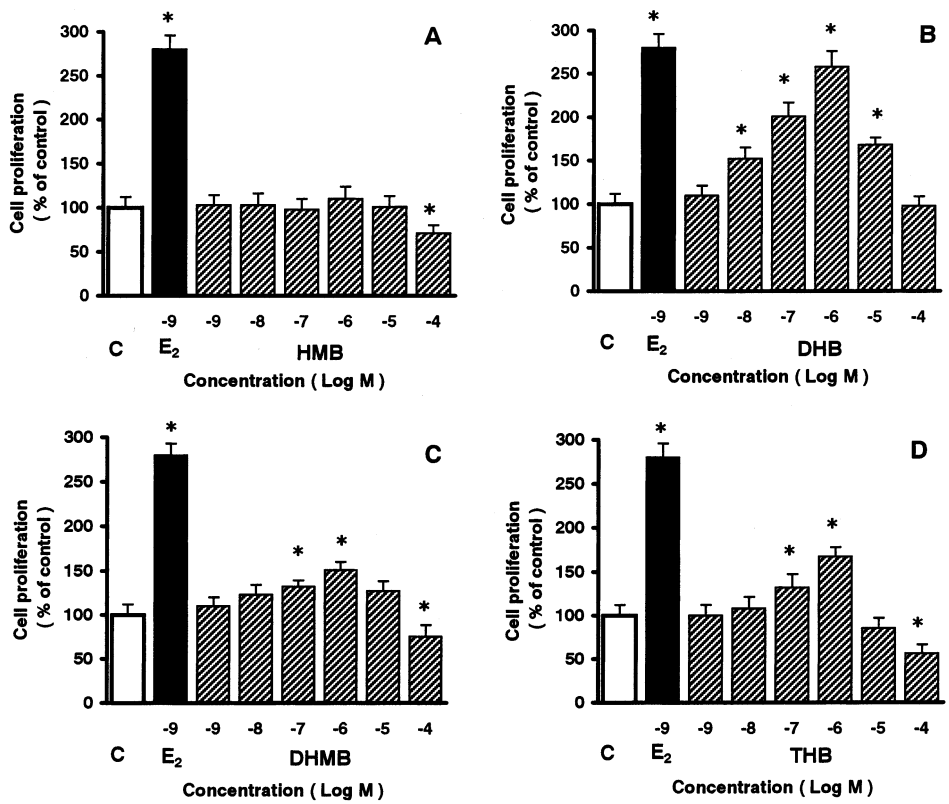

Fig. 6. Effects of HMB (A) and its metabolites, DHB (B), DHMB (C) and THB (D) on the growth of MCF-7 cells. These compounds were tested at concentrations ranging from 10^{-9} M (-9) to 10^{-4} M (-4) for 5 days. Results are the means $\pm$ SE of three or four determinations. C and E_2 indicate untreated control and 17β-estradiol (10^{-9} M)-treated cells, respectively. *Significant differences between the control group and treated groups ($P < 0.05$).

3 h incubation and did not elicit an increase in oxygen consumption (data not shown) which indicates that auto-oxidation through the formation of superoxide anion radicals does not occur readily [21], these results support that the loss of HMB and formation of DHB, which is more toxic than parent compound, are due to enzymatic reactions in rat hepatocytes. In addition, the isomer of DHMB was also stable in Krebs-Henseleit buffer containing an equal volume of methanol, which was used for the deproteinization, under carbogen flow for a 3 h incubation at 37 °C (data not shown). Therefore, DHB and the isomer are formed probably via oxidative *O*-dealkylation of 4-methoxy group and hydroxylation of an aromatic ring, respectively, in the HMB molecule by microsomal cytochrome P-450 monooxygenases. In connection with this result, Okereke et al. have reported that in rats treated by dermal administration of HMB, the formation of DHB and THB was enhanced by pretreatment with phenobarbital, an inducer of the cytochrome P-450 enzyme system [22]. In addition, HMB administered orally to rats is enzymatically converted to at least three intermediates and their glucuronides and/or sulfates: DHB as a major intermediate and DHMB and THM as two trace intermediates are detected in serum, liver and other tissues, and the glucuronides derived from HMB and DHB are excreted in urine [7]. In the present study, however, free unconjugated DHMB and THB in hepatocyte suspensions were detected very trace amounts, whose concentrations were 0.1 and 0.2 μM after a 1 h incubation, respectively. Because glucuronidation and sulfation are major detoxification pathways for metabolism and excretion of phenols in mammals [23], these results indicate that some HMB at a low-toxic level (0.25 mM) is either directly conjugated or indirectly conjugated via these hydroxylated intermediates by glucuronidation, which is a phase II detoxification pathway, in rat hepatocytes.

In chemical structure, bisphenolic compounds are similar to the synthetic estrogens, DES and hexestrol. Recently, the xenoestrogenicity of BPA, alkylphenols and other phenolic compounds has been demonstrated in a number of in vitro and in vivo assays [12,13]. In this study, though the parent compound, HMB, did not exhibit a proliferative effect on MCF-7 human breast cancer cells with estrogen receptor, three known hydroxylated intermediates caused a proliferation of MCF-7 cells at concentrations from 10^{-8} to 10^{-6} M; in turns of proliferative potency, DHB $\gg$ THB $>$ DHMB (Fig. 6). The estrogenic potency of DHB is reproduced by a cell-free assay; DHB as well as BPA elicits a concentration-dependent displacement of 17β-estradiol bound to ERα (Fig. 5). Based on the relative IC_{50}, it follows that in binding potency for ERα, DES $\gg$ BPA $>$ DHB $>$ THB. It is well established that hydrophobicity, as demonstrated by its close correlation with the partition coefficient of xenobiotics, is often associated with biological action, which is expressed as the structure-toxicity or -activity relationship [24]. The *n*-octanol/water partition coefficient ($\log p$), a physical property used extensively to describe a chemical's lipophilic or hydrophobic properties, of DES, BPA, DHB and THB is $\approx$ 5.07, 3.64, 2.96 and 2.90, respectively [24–26]; all of these compounds have a common structure with units of 4-mono- or 4,4′-di-hydroxy group in aromatic rings. Whereas $\log p$ of HMB and DHMB is 3.52 and 3.82, respectively, both compounds with very weak or negligible estrogenic activity (Figs. 5 and 6) do not possess the 4-hydroxy group.

It is well known that alkylphenols with an alkyl group in the *para* (or 4)-position on the phenolic ring are able to stimulate the proliferation of MCF-7 cells [27,28]. Acetamidophenol (acetaminophen) significantly increased the proliferation of MCF-7 cells and in relative potency in stimulating the proliferation of cells, its isomer ranked $p -> m$ (or 3) $-> o$ (or 2)-acetamidophenol, indicating the position of the hydroxy group on the aromatic ring influences the estrogenic effect [29]. This finding is consistent with other studies that have shown *p*-phenols with an alkyl group and/or 4-hydroxybiphenyl have estrogenic activity and are more potent inducer of proliferation in MCF-7 cells than their 3- and 2-isomers [28,30]. In addition, Routledge et al. have reported that alkyl esters of 4-hydroxybenzoic acid, known as parabens, are weakly estrogenic in vitro [31]. On the other hand, the onset of estrogenic effects of biphenyls is associated with some hydroxylated intermediates produced by microsomal cytochrome P-450 monooxygenases. In the previous studies [14,15], we reported that unsubsituted benzophenone was enzymatically converted to at least three metabolites, benzhydrol, 4-hydroxybenzophenone and a sulfate, in rat hepatocytes and that the effects of 4-hydroxybenzophenone on the proliferation of MCF-7 cells in vitro and uterotrophic response in immature female rats were estrogenic, whereas neither the parent compound nor benzhydrol was essentially active. Further, xenoestrogenic activities of methoxychlor, a bis-4-methoxy derivative of DDT, and methoxybisphenol are attributed to the action of its 4-hydroxylated intermediates through biotransformation by oxidative *O*-dealkylation [32]. Although experimental data regarding the estrogenic potential of glucuronides of HMB and DHB are not available, it has reported that these conjugates would not have the capability to interact with the estrogen receptor, because glucuronide and/or sulfate of BPA and alkylphenols did not interact with the estrogen receptor and did not affect estrogenic activity in MCF-7 cells [33,34]. HMB was active when administered by the oral route in an in vivo model for estrogenicity, eliciting dose-dependent increases in uterine weight in immature rats [35]. In addition, rats and/or mice receiving a diet with HMB (5%) showed a marked elongation of the estrous cycle and low epididymal sperm density [9]. Since it is known that differences between the metabolic activation and elimination of compounds vary according to exposure routes, such as oral and subcutaneous administration [36], oral ingestion rather than subcutaneous injection of HMB may induce greater estrogenic activity. Based on the results and findings, it appears that there are at least two mechanisms for the onset of estrogenic activity produced by aromatic compounds such as bisphenols and biphenyls; one is direct interaction between an intrinsic 4-hydroxylated compound and the estrogen receptor, and the other is indirect action by 4-hydroxylated intermediates via biotransformation; either hydroxylation or *O*-dealkylation. Therefore, DHB may be acting as a xenoestrogen in target sites in reproductive organs. Recently, Schlumpf et al. suggest that HMB is a weak endocrine modulator, because level of the lowest-observed uterotrophic effect was more than 1000 mg/kg per day in immature rats that received HMB for 4 days in powdered feed [37]. It is necessary to determine the position of a hydroxy group incorporated in HMB (the isomer of DHMB) and its xenoestrogenic potency.

In conclusion, the present study has shown that HMB at a low toxic concentration in isolated rat hepatocyte suspensions is converted enzymatically to some glucuronide conjugates directly and/or via DHB and a hydroxylated intermediate, which has tentatively been identified as an isomer of DHMB. Despite this, free unconjugated DHMB and THB were identified as trace intermediates. DHB and THB displaced 17β-estradiol bound to ERα in a competitive- and concentration-dependent manner, and the proliferative potency of DHB in MCF-7 cells was greater than that of DHMB or THB at intermediate concentrations. However, the parent compound, HMB, used at concentrations from 10^{-9} to 10^{-4} M did not affect the cell proliferation and competitive binding. Based on the relative IC_{50} and the proliferative effect on MCF-7 cells, it follows that in estrogenic potency, DHB > THB > DHMB.

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