Regulation of Phenobarbital-Mediated Induction of CYP102 (Cytochrome $P450_{BM-3}$) in *Bacillus megaterium* by Phytochemicals from Soy and Green Tea

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Cytochrome P450 102 (CYP102 or Cytochrome $P450_{BM-3}$) is induced in *Bacillus megaterium* by barbiturates, perioxisome proliferators, estrogen, and nonsteroidal antiinflammatory drugs. We have previously demonstrated that a CYP102 construct (BMC 143) coupled with a luciferase reporter gene can be used to identify the inducers of CYP102. We now describe the effect of added phytochemicals on the induction of CYP102 by phenobarbital (PB) in *B. megaterium*. The isoflavones genistein, biochanin A, coumestrol, and equol, the green tea flavanoid epicatechin, and the fungal toxin zearalenone inhibit the induction of CYP102 by PB in a dose-dependent manner. However, the isoflavone daidzein, the phytoalexin glyceollin, and catechin, an epimer of epicatechin, failed to exhibit a similar inhibitory effect on PB-mediated CYP102 induction.

Keywords: *P450; phytochemical; Bacillus megaterium; phenobarbital; soy; green tea*

INTRODUCTION

CYP102 in *Bacillus megaterium* is a catalytically self-sufficient enzyme, which contains a cytochrome P450 reductase domain, containing both FMN and FAD bound, and a monooxygenase domain in a single polypeptide chain (*1*−*3*). CYP102 is inducible by barbiturates, fatty acids, perioxisome proliferators, estrogen, and nonsteroidal antiinflammatory drugs (*4*−*9*). The X-ray structure of the CYP102 monooxygenase domain has been resolved and used as a model for the structure of mammalian cytochrome P450 dependent enzymes (*10*, *11*).

The ability of PB to induce P450s and to modulate the metabolism of drugs and other xenobiotics has been known for several years (*12*). Despite its ubiquity, the mechanism of barbiturate-mediated induction has not been elucidated completely. Several studies on PB-mediated induction in mammals indicate that the increase in P450 is primarily due to increase in gene expression. The requirement of near millimolar concentrations for maximal responses and the lack of a suitable in vitro model for studying the regulation of gene expression are major reasons for the slow progress (*13*). Barbiturate-mediated induction is known in bacteria and fungi in addition to mammals (*14*). Several reports suggest that aspects of bacterial induction mechanism are conserved in mammals (*15*). Studies by Fulco and his associates unraveled the major aspects of the mechanism by which induction of the CYP102 gene is regulated by barbiturates in *B. megaterium* (*16*). BM3R1, a helix-turn-helix DNA-binding repressor protein participates directly in the mechanism by binding to a palindromic operator site upstream of the CYP102 operon and preventing transcription (repression). In the presence of barbiturates, BM3R1 dissociates from its operator DNA and allows transcription to proceed through BM3R1 and CYP102 coding sequences (derepression) (*7*, *8*). In addition to this critical repressor protein several cis- and trans-acting elements have been reported to participate in the induction mechanism (*15*). BM3R1 also binds to a 17 base pair regulatory sequence, which is located within the BM3R1 protein-coding region upstream of the BM3 gene. This 17 base pair sequence is highly conserved in phenobarbital-responsive genes (*17*, *18*) and has been called the "Barbie box" (*14*). It has been proposed that the overall mechanism for the repression of the CYP102 gene involves the binding of trans-acting protein factors (BM3R1, BM3P1, and BM1P1) to one or more cis-acting sites (O_{III}, the operator site, and Barbie box) (*19*−*21*).

We have reported previously that a CYP102 construct truncated at C143 and coupled with luciferase reporter gene (BMC 143) interacts with inducers as does the normal gene, and that the luciferase-linked sequence permits a determination of CYP102 induction spectrofluorometrically (*22*). We have used this technique to probe the effect of various phytochemicals on the PB-mediated induction of CYP102 in *B. megaterium* cultures.

Dietary estrogens are produced by either plants themselves (phytoestrogens) or fungi that infect plants (mycoestrogens). Soybeans and clover, as well as other legumes, are the most significant sources of isoflavones and coumestans (*23*). The isoflavones genistin, daidzin, and their aglycons daidzein and genistein (Figure 1) constitute 90−95% of the isoflavone content of soybeans (*24*). In response to pathogens and other stimuli, soybean tissues accumulate the phytoalexin glyceollin, which shares structural similarities with the isoflavones (*25*). Mycoestrogens include primarily zearalenone and its derivatives (*26*). Dietary intake of phytoestrogens is significantly higher in countries where the incidence of breast and prostate cancers is low, suggesting that they

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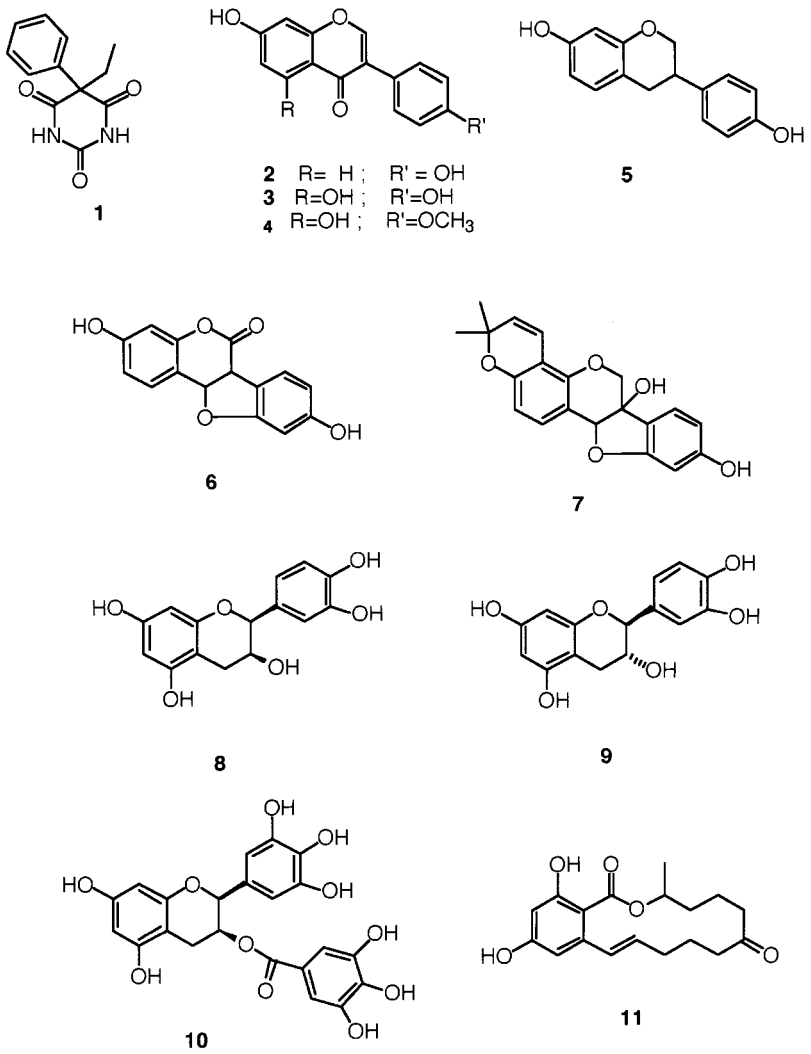

Figure 1. Structures of the compounds used in the study: (1) phenobarbital, (2) daidzein, (3) genistein, (4) biochanin A, (5) equol, (6) coumestrol, (7) glyceollin, (8) epicatechin, (9) catechin, (10) epigallocatechin gallate, and (11) zearalenone.

may act as chemopreventive agents (*27*, *28*). Isoflavones may act as estrogenic or anti-estrogenic depending upon the dose (*29*−*34*). Equol, a metabolic product of daidzein, is reported to have estrogenic activity (*35*). Phytoestrogens have antioxidant properties and inhibit enzymes such as DNA topoisomerase (*36*, *37*) and several kinases (*38*). Because natural estrogen was found to be an inducer of CYP102 in *B. megaterium* (*22*), the effects of phytoestrogens and mycoestrogens on the PB-mediated induction of CYP102 was investigated.

MATERIALS AND METHODS

The phytoestrogens genistein (4′,5,7-trihydroxyisoflavone), daidzein (4′,7-hydroxyisoflavone), coumestrol [2-(2-,4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylicacid-δ-lactone], and zearalenone, catechin, epicatechin, and epigallocatechingallate were purchased from Indofine Chemical Company, Inc. (Belle Mead, NJ). The daidzein metabolite equol was synthesized using a previously reported procedure (*39*). Glyceollin was obtained from Dr. Stephen Boue of the U.S. Department of Agriculture, Southern Regional Research Center, New Orleans, LA.

The *B. megaterium* C 143 clone (BMC 143) was grown overnight under the conditions described for the wild-type bacteria (*8*, *9*) in media supplemented with 0.5% (w/v) glucose. The culture was incubated 14−18 h at 30 °C while being shaken at 150 rpm, and 10-mL aliquots of the culture were transferred to T-25 culture flasks with vented caps (Corning). Test compounds were added in dimethyl sulfoxide (DMSO), and DMSO alone was added to the control flask. In no case did the concentration of DMSO exceed 0.25% (v/v). The cultures were incubated at 30 °C with aeration; and the aliquots were withdrawn at 2, 4, and 6 h intervals after the introduction of the test compounds for the measurement of luciferase activity. Luciferase assays were carried out as reported earlier (*22*). Assays were carried out in duplicate and each experiment was performed at least three times. Statistical analysis (ANOVA and Dunnett test) was carried out using Instat analysis software (GraphPad, Inc., San Diego, CA).

RESULTS

PB induced CYP102 in BMC 143 in a dose-dependent manner at concentrations up to 3 mM at 4 h after addition to the culture. We find that PB is not as potent as the previously reported inducers, estradiol (E2) and 4-*sec*-butylphenol (4sBP). The lower potency of PB is reflected by the requirement of relatively high concentrations of PB for a significant induction of wild type *B. megaterium*. Figure 2 shows the dose-dependent induction of CYP102 in BMC143 by PB at 4 h post addition.

To investigate the effect of phytochemicals on the PB-mediated induction, the test compounds were added along with 3 mM PB to the cultures. Genistein and biochanin A inhibited the PB-mediated induction of

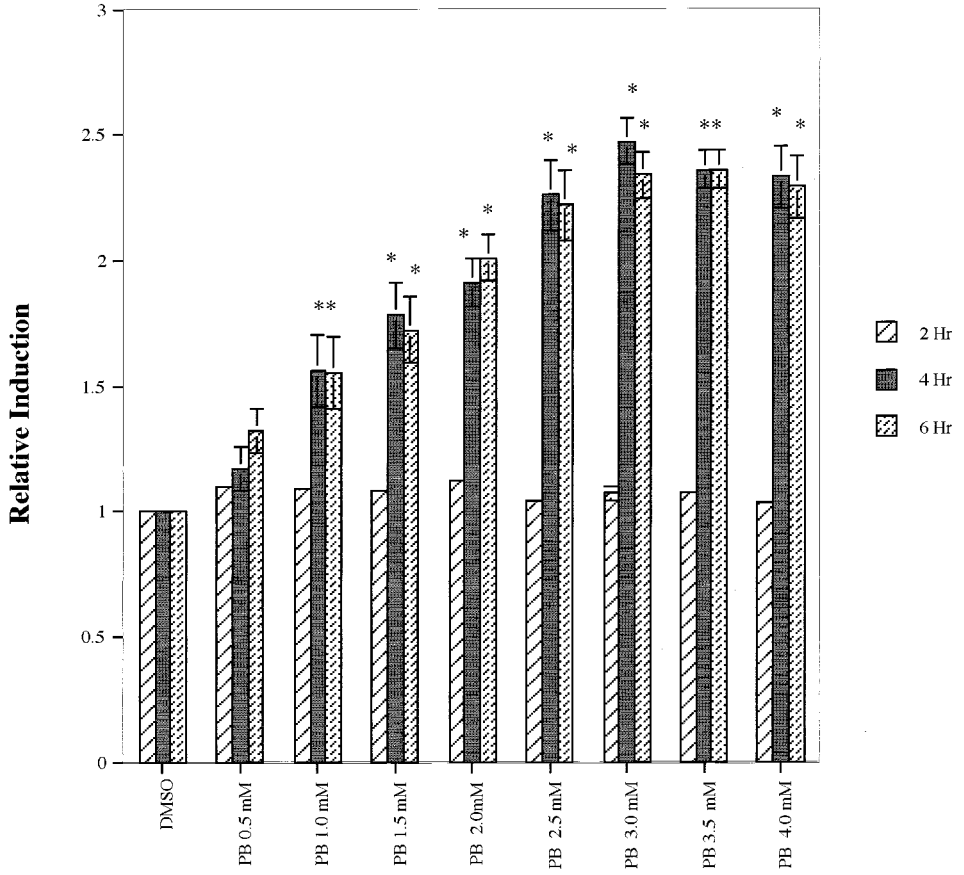

Figure 2. Induction of CYP102 in BMC143 by PB. Cultures of BMC143 were incubated with varying concentrations of PB. A 200-μL aliquot of culture was withdrawn after 2, 4, and 6 h intervals and diluted with 1.7 mL of water in a 1-cm plastic cuvette for analysis. The luciferease reaction was initiated by the addition of 100 μL of 0.1% decanal, and the light emitted at 474 nm was measured for 30 s in a spectrofluorometer. Chemiluminescence was normalized by dividing the intensity of the emitted light by the optical density of the sample at 500 nm. Induction was calculated by dividing the chemiluminescence of each sample by that of the culture incubated with vehicle alone. Assays were done in duplicate and the entire experiment was repeated three times. Statistical analysis (ANOVA and Dunnett test) was carried out using Instat analysis software. Asterisks indicate statistical significance. (P values >0.05 were not significant).

CYP102 in BMC143 in concentration range 25−150 μM (Figure 3a and b). Coumestrol and epicatechin exhibited a dose-dependent inhibition at a similar concentration range (Figure 4a and b). Equol was a more potent inhibitor than daidzein, its metabolic precursor (Figure 5a). Epigallocatechin gallate, the potent green tea polyphenol, inhibits PB-mediated induction (Figure 6). Among the chemicals tested, we find that zearalenone displays the most potent dose-dependent inhibition of PB-mediated induction (Figure 5b). Daidzein, glyceollin, and catechin failed to inhibit PB-mediated induction at all concentrations. Figure 6 shows the inhibitory activities of all the phytochemicals tested.

DISCUSSION

Often biological activity can be correlated with the structure of ligands with respect to the biological system under concern. All the phytochemicals tested have a common phenolic moiety. The hydroxyl group at 5th position of the isoflavone molecules appears to be critical for the inhibition of phenobarbital-induced gene transcription. The isoflavones genistein and biochanin A (possessing a phenolic group at C-5), and zearalenone (possessing a hydroxyl group at the corresponding C-4 position), exhibit inhibitory activity. The hydroxyl groups at 4′ and 7 positions of the isoflavone molecules are also important. Biochanin A with a methoxy group at the 4′ position is less active than genistein, whereas the isoflavone daidzein, which has hydroxyl groups at position 4′ and 7 but lacks a hydroxyl group at the 5th position, was not active. Introduction of structural flexibility increases the inhibitory activity. Equol, a reduction product of the enone moiety of daidzein, is an active inhibitor. The structurally flexible zearalenone and equol were the most potent inhibitors of PB-mediated induction detected. Coumestrol, with hydroxyl groups at 4′ and 7 positions and a relatively rigid ring system, inhibited the PB-mediated induction, but its close structural analogue glyceollin, with one of its hydroxyl groups blocked by an internal ether linkage, was not active. Epicatechin, which has hydroxyl groups at both C-5 and C-7 positions and whose structure resembles that of equol in some respects, was found to inhibit the induction. However its epimer, catechin, possessing the same structural features but opposite chirality, failed to inhibit the induction.

Phytochemicals form a class of phenolic compounds reported to have multiple biological effects, including antioxidant activities (*40*). The presence of more phenolic hydroxyl groups generally is found to result in stronger antioxidant activity (*41*). Catechin and epicatechin both possess four phenolic hydroxyl groups and have very similar antioxidant potential, but only epicatechin inhibits the PB-mediated induction of CYP102. These results indicate that the antioxidant potential of

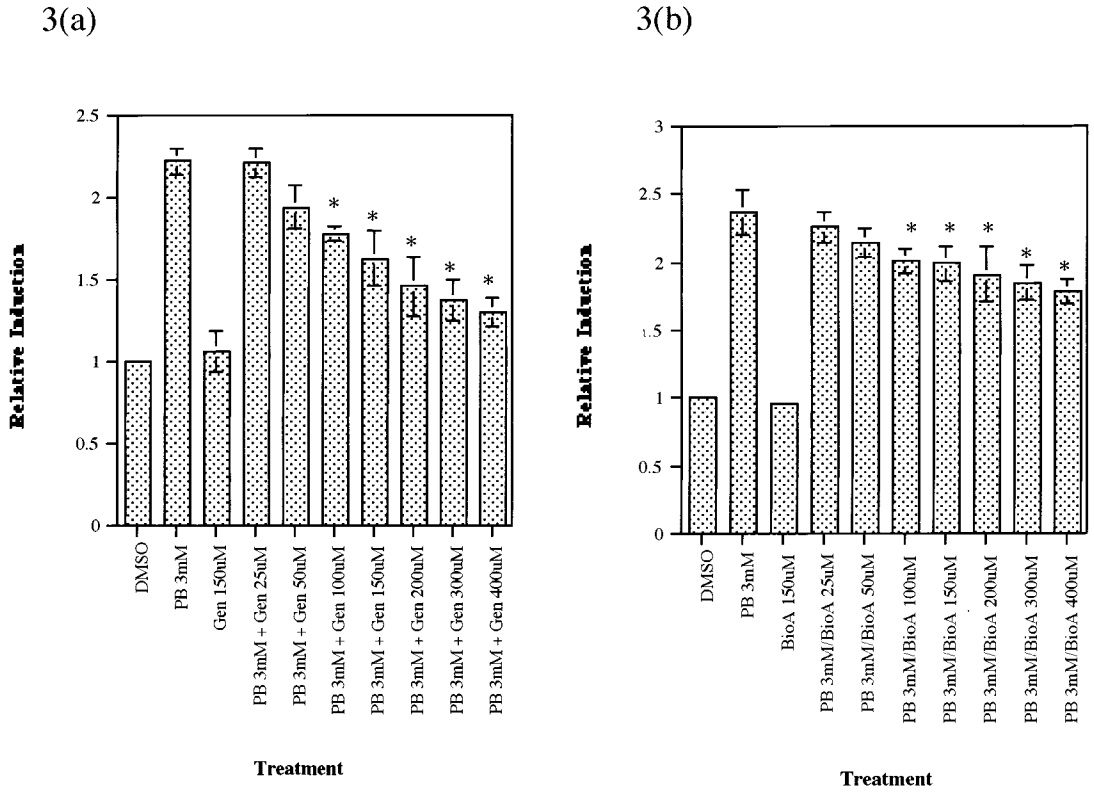

Figure 3. Inhibition of PB-mediated induction of CYP102 in BMC143 by (a) genistein and (b) biochanin A at 4 h post addition to cultures. The phytochemicals (25−400 μM) were incubated along with 3 mM PB and samples were withdrawn and assayed as described in Figure 2. Asterisks indicate statistical significance.

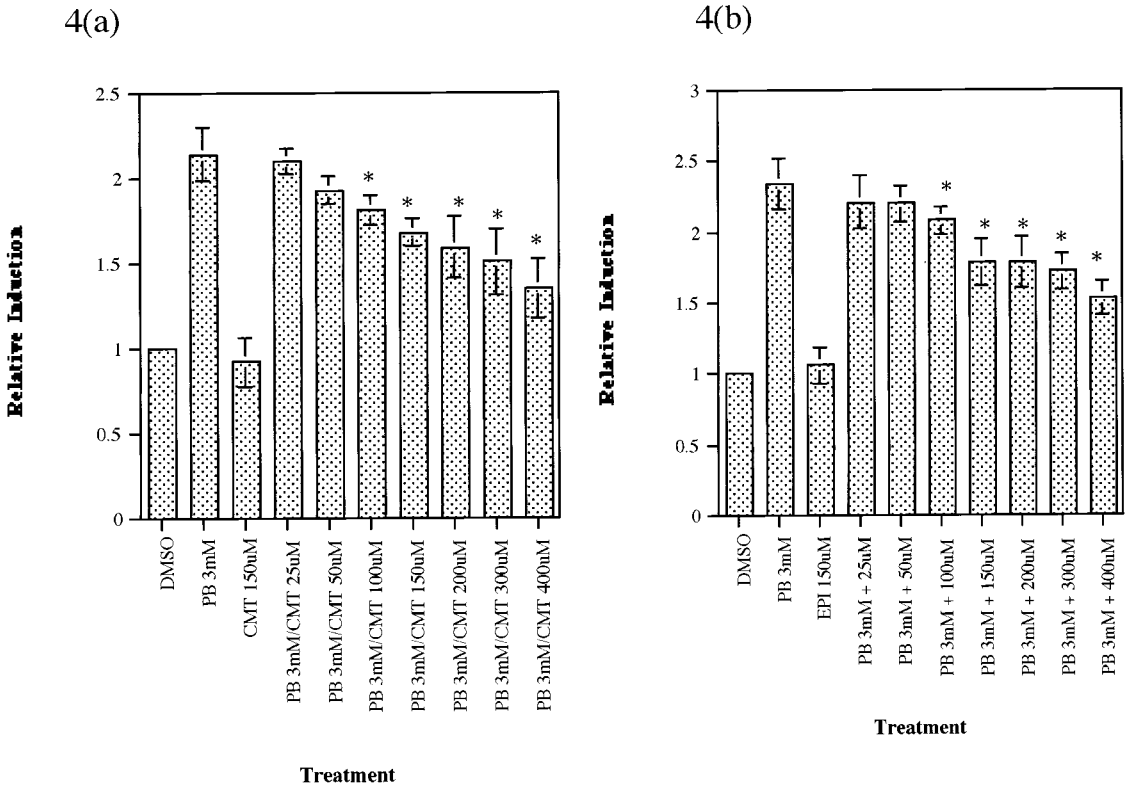

Figure 4. Inhibtion of PB-mediated induction of CYP102 in BMC143 by (a) coumestrol (25−400 μM) and (b) epicatechin (25−400 μM) at 4 h post addition to cultures and assayed as described in Figure 2. Asterisks indicate statistical significance.

the modifying phytochemical does not determine the inhibition of the PB-mediated induction.

The present study demonstrates the use of a bacterial reporter gene assay for the rapid determination of inhibition of phenobarbital-induced gene transcription by phytochemicals from soy and green tea. Although the presence of more phenolic groups on the phytochemicals seems to be associated with greater inhibitory activity,

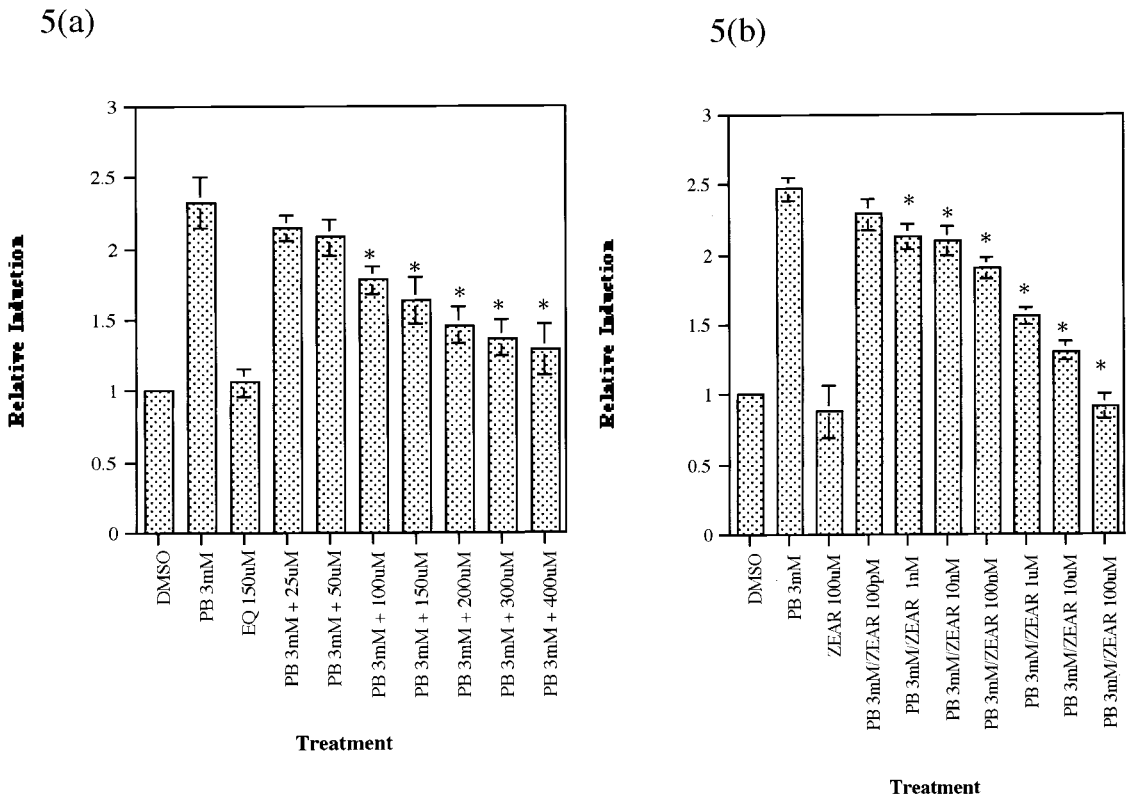

Figure 5. Inhibition of PB-mediated induction of CYP102 in BMC143 by (a) Equol (25−400 μM) and (b) Zearalenone (0.1 nM−100 μM) at 4 h post addition to cultures and assayed as described in Figure 2. Asterisks indicate statistical significance.

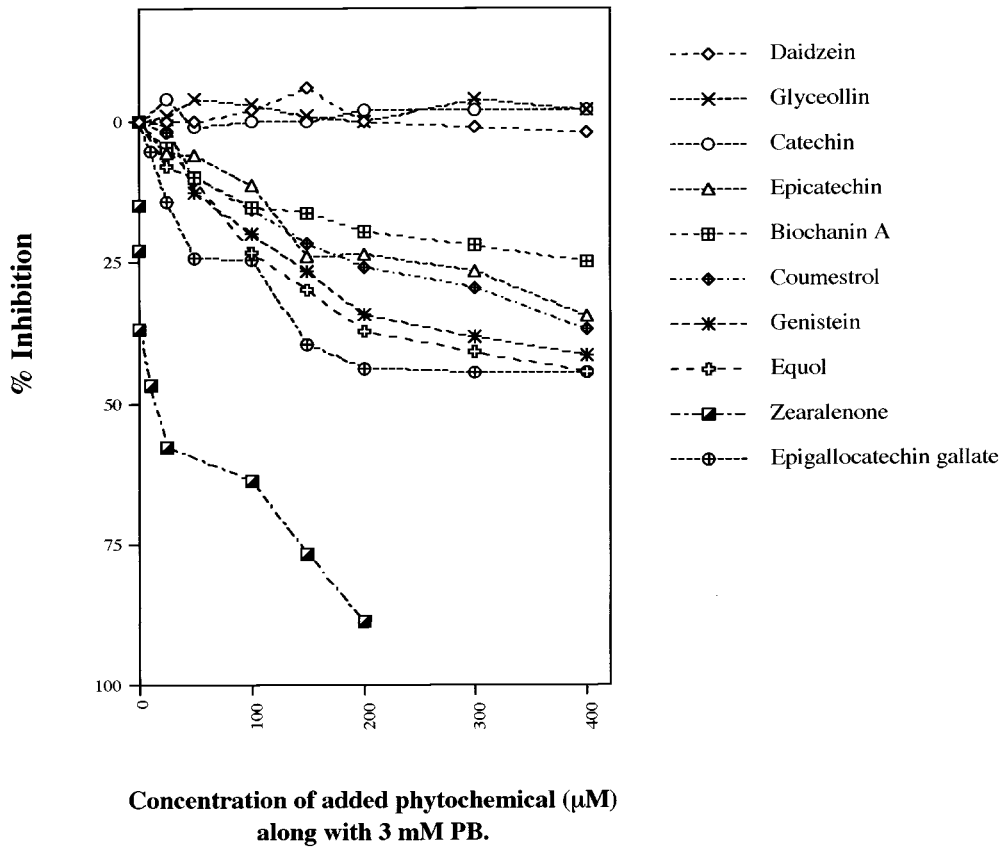

Figure 6. Inhibition of PB-mediated induction of CYP102 by phytochemicals. Percent inhibition was calculated by dividing the relative induction of cultures containing 3 mM PB along with the inhibitor (0.1 nM − 400 μM) by the induction of cultures incubated with 3 mM PB alone.

the inhibitory activity of zearalenone, equol, and catechin are clear exceptions to this trend. It seems possible that phytochemicals which inhibit the phenobarbital-induced transcription of a bacterial gene may also modify phenobarbital-induced gene transcription in mammals. Further studies have been initiated to identify the structural features required to inhibit phenobarbital-induction.

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