Acta Biochimica Polonica

Vol. 49 No. 4/2002

917–925

QUARTERLY

Effect of natural phenols on the catalytic activity of cytochrome P450 2E1

Renata Mikstacka✉, Jerzy Gnojkowski and Wanda Baer-Dubowska

Department of Pharmaceutical Biochemistry, University of Medical Sciences, Grunwaldzka 6, 60-780 Poznań, Poland

Received: 30 July, 2002; revised: 12 November, 2002; accepted: 21 November, 2002

Key words: polyphenols, tannic acid, protocatechuic acid, resveratrol, CYP2E1, inhibition kinetics, mechanism-based inhibition

The effect of protocatechuic acid, tannic acid and *trans*-resveratrol on the activity of *p*-nitrophenol hydroxylase (PNPH), an enzymatic marker of CYP2E1, was examined in liver microsomes from acetone induced mice. *trans*-Resveratrol was found to be the most potent inhibitor (IC_{50} = 18.5 ± 0.4 μM) of PNPH, while protocatechuic acid had no effect on the enzyme activity. Tannic acid with IC_{50} = 29.6 ± 3.3 μM showed mixed- and *trans*-resveratrol competitive inhibition kinetics (K_i = 1 μM and 2.1 μM, respectively). Moreover, *trans*-resveratrol produced a NADPH-dependent loss of PNPH activity, suggesting mechanism-based CYP2E1 inactivation. These results indicate that *trans*-resveratrol and tannic acid may modulate cytochrome P450 2E1 and influence the metabolic activation of xenobiotics mediated by this P450 isoform.

Tannic acid, protocatechuic acid and *trans*-resveratrol are naturally occurring phytophenols present in edible fruits and vegetables. A number of dietary polyphenols were shown to modulate the process of multistage carcinogenesis in animal models (Newmark, 1996). Protocatechuic acid, a simple phenolic acid, and a constituent of apples, green and black tea and herbal medicines, was reported to be an efficacious agent in reducing the carcinogenic action of nitrosoamines and related amino derivatives as well as dimethylbenz[a]anthracene in rats (Ohnishi *et al.,* 1997; Mori *et al.,* 1999; Nakamura *et al.*, 2000). Tannic acid, a mixture of digallic acid esters of glucose, mainly present in tea, cocoa, beans, grapes, strawberries and persimmon, was shown to inhibit the mutagenicity of polycyclic aromatic hydrocarbons in *Salmonella typhimurium* and Chinese hamster V79 cells as well as the tumorigenicity of polycyclic aromatic hydrocarbons and *N*-methyl nitrosourea in mouse skin, lung and forestomach (Nepka *et al.,* 1999; Chen &

✉Corresponding author: tel:. (48 61) 869 9181; e-mail: rmikstac@mail.am.poznan.pl

Abbreviations: CYP2E1, cytochrome P450 2E1; Me_2SO, dimethylsulfoxide; K_i, equilibrium dissociation constant for enzyme-inhibitor complex; PNPH, *p*-nitrophenol hydroxylase.

Chung, 2000). Moreover, tannic acid applied to mouse skin caused the inhibition of covalent benzo(a)pyrene-diol-epoxide binding to epidermal DNA (Baer-Dubowska *et al.,* 1997), which is considered a critical event in the initiation stage of carcinogenesis. Resveratrol (3,5,4′-trihydroxystilbene) occurs in peanuts, grapes and herbal remedies used in Japan and China (Ignatowicz & Baer-Dubowska**,** 2001). It can be found in the *cis* and *trans* configurations, either free or in a glycosylated form (Soleas *et al.*, 1997; Burns *et al.*, 2002). Significant concentrations (1–10 μM) of the aglycons *cis*- and *trans*-resveratrol are present in red wine (Soleas *et al.*, 2002). *trans*-Resveratrol has been reported to inhibit dimethylbenz[a]anthracene induced preneoplastic lesion formation in mouse (Jang *et al.,* 1997). The suppression of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol was recently reported (Li *et al.*, 2002). The antimutagenic activity of resveratrol was demonstrated against a foodborne heterocyclic amine, 3-amino-1,4-dimethyl-5H-pyrido-[4,3-b]indole (Trp-P-1) and 2-aminofluorene in *Salmonella* bacterial tester strains (Uenobe *et al.,* 1997; Gusman *et al.,* 2001).

Most chemical carcinogens require metabolic activation catalyzed by cytochrome P450 in order to exert their genotoxic and carcinogenic effects. Thus one possible mechanism by which phenolic compounds might exert anticarcinogenic effects is through an interaction with the cytochrome P450 system, either by the inhibition or activation of certain forms of this enzyme, leading to a reduced production of the ultimate carcinogen (Guengerich & Shimada, 1998; Hursting *et al.*, 1999; Sporn & Suh, 2000). Our previous, and other authors' studies have shown that these structurally diversified phenolics inhibit cytochromes P450 1A1, 1A2 and 2B (Baer-Dubowska *et al.,* 1998; Das *et al.,* 1999; Chang *et al.,* 2001).

In this paper, we have evaluated the effect of a simple phenolic acid, protocatechuic acid; a polyphenol, tannic acid and a trihydroxystilbene, *trans*-resveratrol (Fig. 1) on

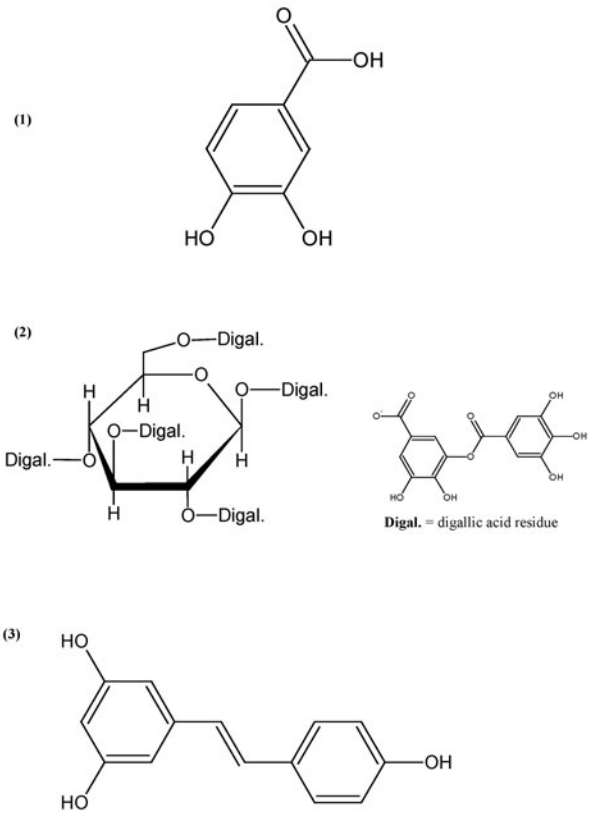

Figure 1. The structures of protocatechuic acid (1), tannic acid (2) and *trans*-resveratrol (3).

murine hepatic hydroxylation of *p*-nitrophenol, which represents a selective substrate for cytochrome P450 2E1. CYP2E1 is vital in catalyzing the activation of nitrosodimethylamine, alkanes, halogenated hydrocarbons, and many other low molecular mass environmental chemicals (Guengerich *et al.,* 1991). Inhibition of CYP2E1 is expected to block the toxicity and carcinogenicity of these compounds.

MATERIALS AND METHODS

Chemicals. Protocatechuic acid (purity 97%), *trans*-resveratrol (purity 99%), *p*-nitrophenol, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO, U.S.A.). Tannic acid (purity 97%) was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other compounds were commercial products of the highest purity available.

Animals, treatment and microsome preparation. Female Swiss mice (7–9 weeks old, 25 g) were used in all experiments. The animals were housed in polycarbonate cages containing hardwood chip bedding at 21°C and with a relative humidity of 40–70%. Commercial mouse food and distilled water were available *ad libitum.* The mice were treated intragastrically with 0.5 ml of 50% acetone in water for 4 consecutive days. Twenty four hours after the last treatment, the mice were killed by cervical dislocation, their livers were removed and homogenized in 0.25 M sucrose/0.05 M Tris buffer (pH 7.5), containing 0.025 M KCl and 0.003 M $MgCl_2$. The livers from 6 animals were pooled and microsomes were prepared by differential centrifugation as described by Gnojkowski *et al.* (1984). Protein concentrations were determined by the method of Lowry *et al.* (1951).

PNPH assay. The PNPH activity was measured by determining *p*-nitrocatechol formation, according to the procedure described by Reinke & Moyer (1985). The reaction mixtures (2 ml total volume) contained acetone induced microsomes (0.7 mg protein), *p*-nitrophenol (0.2 mM), an NADPH generating system (7.5 mM glucose-6-phosphate, 2 units/ml of glucose-6-phosphate dehydrogenase, 0.4 mM $NADP^+$, 5 mM $MgCl_2$), 100 mM potassium phosphate buffer (pH 6.8) and various concentrations of the tested compounds. Tannic acid was added as a water solution, whereas *trans*-resveratrol was dissolved in 10% Me_2SO. The final concentration of Me_2SO in the preincubation mixture did not exceed 0.1%. At this concentration Me_2SO did not affect CYP2E1 catalytic activity. Control incubations did not contain the tested compounds. The reaction was initiated by the addition of $NADP^+$ and terminated after 30 min incubation at 37°C by the addition of 0.5 ml of trichloroacetic acid. Samples were centrifuged for 15 min at 13 000 × ***g*** and 0.1 ml of 10 M sodium hydroxide was added to 1.5 ml of supernatant. Formation of the product (*p*-nitrocatechol) was measured at 526 nm, using the molar absorption coefficient of *p*-nitrocatechol equal 9.53 $mM^{-1}cm^{-1}$. The percent inhibition of the enzyme activity by the phenolics was calculated and IC_{50} values were determined graphically, using the Microsoft Excel software program. IC_{50} values represent an average of two separate experiments.

Inhibition kinetics. The enzyme assay was as described in the preceding section. Inhibition constants (K_i) were determined for two concentrations of the tested compounds and the substrate range from 0.02–0.2 mM. The K_i value was determined from double reciprocal plots of the enzyme activity *versus* substrate concentration (Voet & Voet 1995).

Inactivation of PNPH. A two-stage incubation procedure was used to examine the time-dependent inactivation of PNPH. *trans*-Resveratrol and tannic acid (at the concentrations indicated in each figure legend) or the appropriate vehicle (control) were preincubated (first stage incubation) with mouse liver microsomes (3.5 mg protein/ml)

and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 6.8) at 37°C. At various time points, 0.2 ml aliquots were removed and added to 1.8 ml of the secondary reaction mixture to determine the hydroxylase activity. The second-stage incubation was carried out according to the procedure of the PNPH assay. All the assays were run in triplicate and the coefficient of variation was usually less than 10%.

RESULTS AND DISCUSSION

This study was designed to evaluate selected, naturally occurring phytophenols for their ability to modulate cytochrome P450 2E1 mediated enzyme activity. For this purpose, we have assumed that the predominant cytochrome P450 mediating PNPH activity is CYP2E1 (Koop *et al.,* 1989, Kobayashi *et al.,* 2002). This P450 isoform is involved in the metabolism of several xenobiotics, including chemical carcinogens. Therefore, its modulation can dramatically affect the compound's toxicity and carcinogenesis.

The effects of protocatechuic acid, tannic acid and *trans*-resveratrol on hepatic PNPH in mouse microsomes are presented in Fig. 2. The inhibition curves for each compound and IC_{50} values were determined within different concentration ranges depending on the compound. Protocatechuic acid within the concentration range 0.05 to 5 mM did not inhibit the activity of PNPH. At a lower concentration (< 1 mM), even a slight stimulation of PNPH activity was observed (Panel A). Tannic acid was more potent (IC_{50} = 29.6 ± 3.3 μM) and *trans*-resveratrol was the most potent inhibitor of PNPH activity (IC_{50} = 18.5 ± 0.4 μM).

These two compounds were further studied for both the mode and the type of inhibition. For this purpose various concentrations of the substrate were examined at two inhibitor concentrations. Lineweaver-Burk plots were then generated from the resulting data sets. The results of these analyses are presented in Fig. 3.

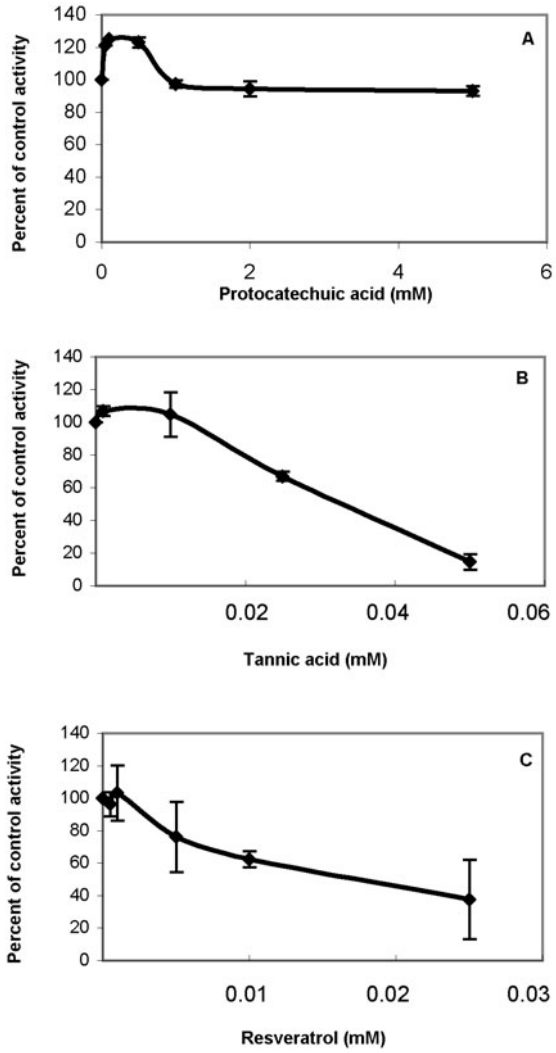

Figure 2. Concentration-dependent inhibition of *p*-nitrophenol hydroxylation by: protocatechuic acid (A), tannic acid (B) and *trans*-resveratrol (C).

Data are expressed as mean ±S.D. percentage of control enzyme activity for three independent experiments. PNPH activity for the control experiment was 0.0017 ± 0.0003 μmoles *p*-nitrocatechol/min per mg protein.

Tannic acid is a mixed (K_i = 1 μM) and *trans*-resveratrol a competitive PNPH inhibitor (K_i = 2.1 μM).

A two-stage incubation procedure (Cai *et al.*, 1993) was utilized to examine the ability of these phenolics to inactivate the enzymatic activities associated with hepatic microsomal PNPH. These results are presented in Fig. 4 and show that only *trans*-resveratrol (panel B) possessed the ability to inactivate microsomal

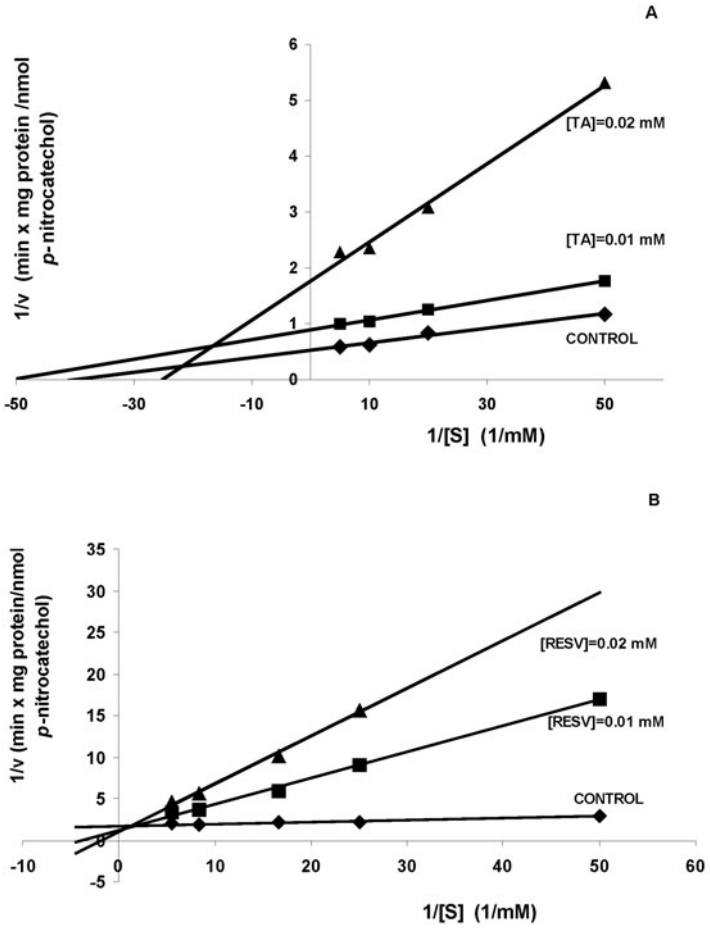

Figure 3. Inhibition of mouse liver CYP2E1 activity by tannic acid (A) and *trans*-resveratrol (B).

Lineweaver-Burk plots for the inhibition of *p*-nitrophenol hydroxylase in mouse liver microsomes. *p*-Nitrophenol was used in the range of concentration 0.02–0.2 mM. Values are means of triplicate determinations. Coefficient of variation did not exceed 6.6%.

PNPH with $k_{\text{inactivation}}$ equal 0.06 min^{-1}. No inactivation of PNPH activity was detectable in the absence of NADPH (data not shown).

Until now, a number of compounds have been reported as selective inhibitors of cytochrome(s) P450, including both reversible and mechanism-based inactivators. For example, naturally occurring coumarins, found in human diet, demonstrated a structure-activity relationship for P450 selectivity and the type or mode of inhibition (Cai *et al.,* 1993). Among them, coriandrin was found to be a potent inhibitor and inactivator of murine and purified human P450 1A1 (Cai *et al.*, 1993). The selective inhibition of P450 2E1 by diallyl sulfide and phenethyl isothiocyanate, the components of *Allium sp.* and *Brassicaceae* vegetables, was also reported (Guyonnet *et al.*, 2000; Nakajima *et al.*, 2001; Yang *et al.*, 1994). On the other hand, effective inhibitors of carcinogenesis are known which are not selective inhibitors of cytochromes P450. An example of this group is chlorophyllin, which non-specifically inhibits several CYP-mediated activities (Yun *et al.*, 1995).

The phenolic compounds examined in the present study rather represent the non-selective group of P450 inhibitors. In our previous studies, tannic acid inhibited mouse hepatic cytochromes P450 1A1, 1A2 and 2B mediated enzyme activities *in vitro* even to a greater extent (IC_{50} 2.6–7 μM) than CYP2E1 in the current study (Baer-Dubowska *et al.,* 1998; Das *et al.,* 1987). As in this report, tannic acid was also a mixed inhibitor of pentoxyresorufine O-dealkylase (CYP 2B), suggesting that tannic acid may bind not only to the substrate binding site of both enzymes but also to an additional site that causes a loss of enzyme activity. Protocatechuic acid, which did not show any effect on the activity of PNPH in the current study, was shown to be an efficient inhibitor of methoxy- and pentoxyresorufin O-dealkylases, the enzy-

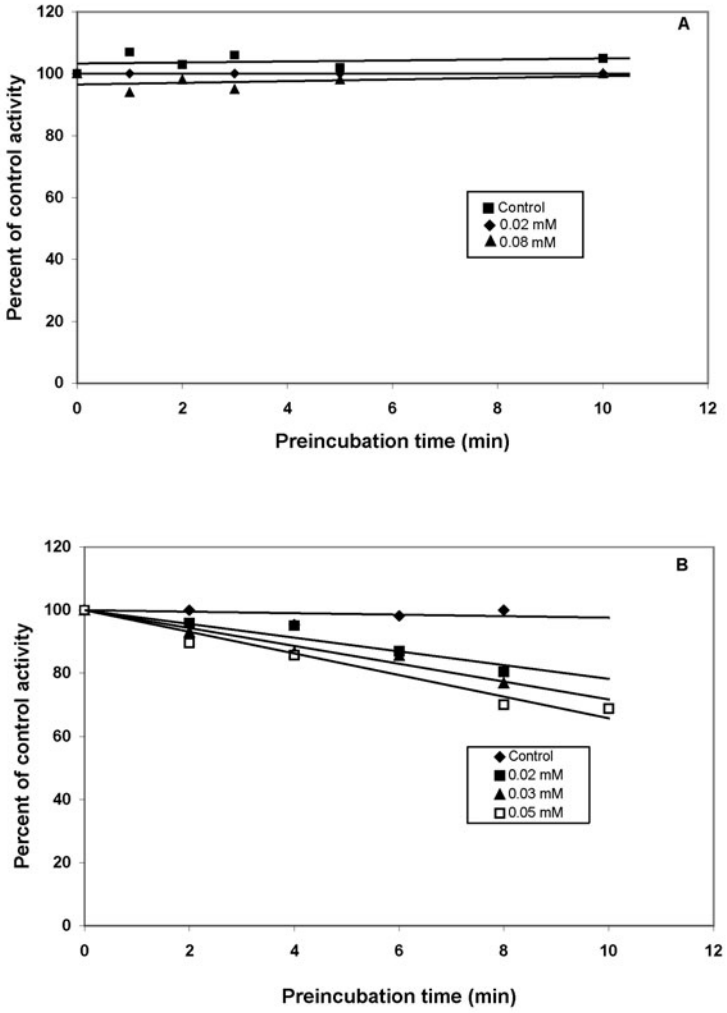

Figure 4. Inactivation of CYP2E1 by tannic acid (A) and *trans*-resveratrol (B) in mouse liver microsomes.

Mouse liver microsomes were preincubated with varying concentrations of inactivators as described under Materials and Methods. Each point represents the mean percentage of control activity of three experiments. Coefficient of variation did not exceed 9.2%.

matic markers of CYP1A2 and 2B, respectively (Baer-Dubowska *et al.,* 1998). *In vivo,* induction of CYP1A is controlled by aryl hydrocarbon receptor (AhR). *trans*-Resveratrol, the most potent inhibitor of PNPH in the present study, was shown to block *CYP1A1* transcription in human HepG2 hepatoma cells by preventing the receptor from binding to the enhancer sequences of the *CYP1A1* promoter, that regulate the transcription of the gene (Ciolino *et al.*, 1998). Moreover, inhibition of human CYP1A1 and the other AhR gene battery products, cytochromes 1B1 and 1A2 by resveratrol was also demonstrated (Chun *et al.,* 1999; Chang *et al.*, 2000). Thus AhR might be a potential target for all the phytophenols examined in this paper study for modulating carcinogen activation. However, the results of this study indicate that, especially for *trans*-resveratrol, an inhibition of P450 2E1 might be equally important. Moreover, *trans*-resveratrol was also reported to inhibit human recombinant CYP3A4 and CYP3A5 *in vitro* (Chang & Yeung, 2001). In our studies, *trans*-resveratrol was a competitive inhibitor of PNPH. This is in contrast to the observation of Piver *et al.* (2001), who demonstrated that *trans*-resveratrol and red wine solid components act as non-competitive inhibitors of CYP2E1 activity in rat and human liver microsomes. In their studies, however, chlorzoxazone 6-hydroxylation was used as an enzymatic marker of the catalytic activity of CYP2E1, so the difference in the mechanism of inhibition may be explained by the existence of more than one active site specific for different substrates.

Our present study also showed the ability of *trans*-resveratrol to inactivate cytochrome P450 2E1 in a mechanism-based manner. The

requirement for NADPH in the *trans*-resveratrol inactivation of CYP2E1 indicates that it is not *trans*-resveratrol, but a reactive intermediate that is responsible for the inactivation of this enzyme. Mechanism-based inactivation of CYP1A2 by *trans*-resveratrol has also been reported (Chang *et al.,* 2001). More detailed studies, which are underway, are necessary to explore the mechanism(s) of the inactivation of specific cytochromes P450 by this naturally occurring polyphenol. The nature of the metabolite formed in the biotransformation of *trans*-resveratrol catalysed by CYP2E1 remains to be identified as well. The metabolic hydroxylation of *trans*-resveratrol by CYP1B1 recently reported by Potter *et al.* (2002) demonstrates that *trans*-resveratrol can be converted to piceatannol, a tyrosine kinase inhibitor and a compound of known anticancer activity (Geahlen & McLaughlin, 1989).

In conclusion, our studies show that tannic acid and *trans*-resveratrol are potent inhibitors of CYP2E1 activity in mouse liver microsomes, but they affect this cytochrome in different ways. Future studies will focus on the mechanism of interaction and modulation of these structurally diverse polyphenols with carcinogen metabolizing enzymes.

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