JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Inhibitory Effect(s) of Polymeric Black Tea Polyphenol Fractions on the Formation of [^{3}H]-B(a)P-Derived DNA Adducts

RAJESH KRISHNAN AND GIRISH B. MARU*

Tobacco Carcinogenesis Group, Cancer Research Institute, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai 410 208, India

Five polymeric black tea polyphenol fractions (PBP-1−5) were isolated from a popular brand of black tea. The effect of these PBPs and epigallocatechin gallate (EGCG), a major green tea polyphenol, was studied on the formation of [^{3}H]-B(a)P-derived DNA adducts in vitro, employing rat liver microsomes. PBP-1−3 inhibited microsome-catalyzed [^{3}H]-B(a)P-derived DNA adduct formation in vitro in a dose-dependent manner. This inhibition was further enhanced on preincubation of microsomes with each of the PBPs. PBP-4 was not effective per se and required preincubation with microsomes to exhibit its inhibitory effect, whereas PBP-5 remained ineffective with or without preincubation with microsomes. Further investigations revealed that the observed decrease in [^{3}H]-B(a)P−DNA adduct formation was due to inhibition of isozymes of CYP450s by PBPs. Overall, results suggest that polymeric black tea polyphenol fractions retain one of the chemopreventive effects exhibited by the monomeric green tea polyphenol EGCG in vitro.

KEYWORDS: Benzo[*a*]pyrene; rat liver microsomes; cytochrome P450 isozymes; DNA adducts; polymeric black tea polyphenol fractions; epigallocatechin gallate; inhibition; in vitro

INTRODUCTION

Tea is one of the most widely consumed beverages in the world (*1*, *2*). The vast majority of tea consumed by humans can be classified into three main types (based on manufacturing technique), namely, green tea (20−22% of world tea consumption), oolong tea (2−3% of world tea consumption), and black tea (75−78% of world tea consumption).

Studies on green tea have shown that it possesses antioxidative (*3*), antimutagenic (*4*), and anticarcinogenic activities (*5*). Experimental studies with different animal models have shown protective effects of green tea against carcinogen-induced initiation (*6*, *7*), promotion (*8*, *9*), and progression (*10*). These effects have been attributed mainly to free catechins present in green tea (90% of total polyphenol content). During black tea manufacture, monomeric free catechins are oxidatively polymerized to an entirely new set of compounds, resulting in a significant decrease (∼60%) in the levels of biologically active monomeric free catechins (*11*) (**Figure 1**). Despite the fact that the polymeric polyphenols are the most abundantly occurring oxidation products in black tea, their chemistry is to date the least understood.

It is not known whether protective effects shown by free catechins are retained by newly formed polymeric polyphenols and oligomeric theaflavins (TFs). The present investigation was carried out to compare the chemopreventive effects of polymeric black tea polyphenols (PBPs) and the most potent green tea polyphenol, epigallocatechin gallate (EGCG), by studying their effects on (a) the formation of microsome-catalyzed [^{3}H]benzo[*a*]pyrene [B(a)P]-derived DNA adducts and (b) the activity of CYP450 isozymes in vitro.

* Corresponding author (telephone +91-22-2741-2919; fax +91-22-2741-2894; e-mail gmaru@actrec.res.in).

MATERIALS AND METHODS

Chemicals. Benzo[*a*]pyrene (purity ≥ 98%) [B(a)P], ethoxyresorufin (ER), methoxyresorufin (MR), pentoxyresorufin (PR), resorufin (R), (−)-epicatechin (EC), (+)-catechin (C), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), (+)-gallocatechin gallate (GCG), caffeine, and TLC plates with fluorescent indicator were purchased from Sigma Chemical Co., St. Louis, MO. Aroclor 1254 was from Monsanto, St. Louis, MO; Sephadex LH-20 was from Pharmacia Biotech, Uppsala, Sweden. [^{3}H]-B(a)P (8000 mCi/mmol) was purchased from the Board of Radio Isotope Technology (BRIT), Bhabha Atomic Research Centre (BARC), Mumbai, India, and the desired specific activity was achieved by dilution with unlabeled B(a)P. NADPH was purchased from SRL, Mumbai, India, and other chemicals and solvents were of AR grade and purchased locally.

Isolation/Purification of PBPs and EGCG. A popular brand of black tea powder was purchased from the local market in Mumbai, India. Green tea extract was purchased from Encee Aromatics Ltd., Mettupalayam, India. Earlier attempts at isolation of PBPs employing Sephadex LH-20 column chromatography (*12*) and reverse phase HPLC (*13*) were not successful due to massive interaction of PBPs with solid supports (*14*). Hence, isolation/purification of PBPs was carried out by successive extraction of an aqueous extract of black tea with chloroform, ethyl acetate, and *n*-butanol, followed by acidification and further extraction with *n*-butanol (*15*). The chloroform extract yielded mainly caffeine. The residues from the other three extracts were purified by fractional precipitation employing mixtures of acetone/chloroform, acetone/ether, and methanol/ether. This process yielded five polymeric black tea polyphenol fractions, namely, PBP-1, PBP-2, PBP-3, PBP-4, and PBP-5, which belonged to one general structural type (*15*).

10.1021/jf049979o CCC: $27.50 © 2004 American Chemical Society
Published on Web 05/27/2004

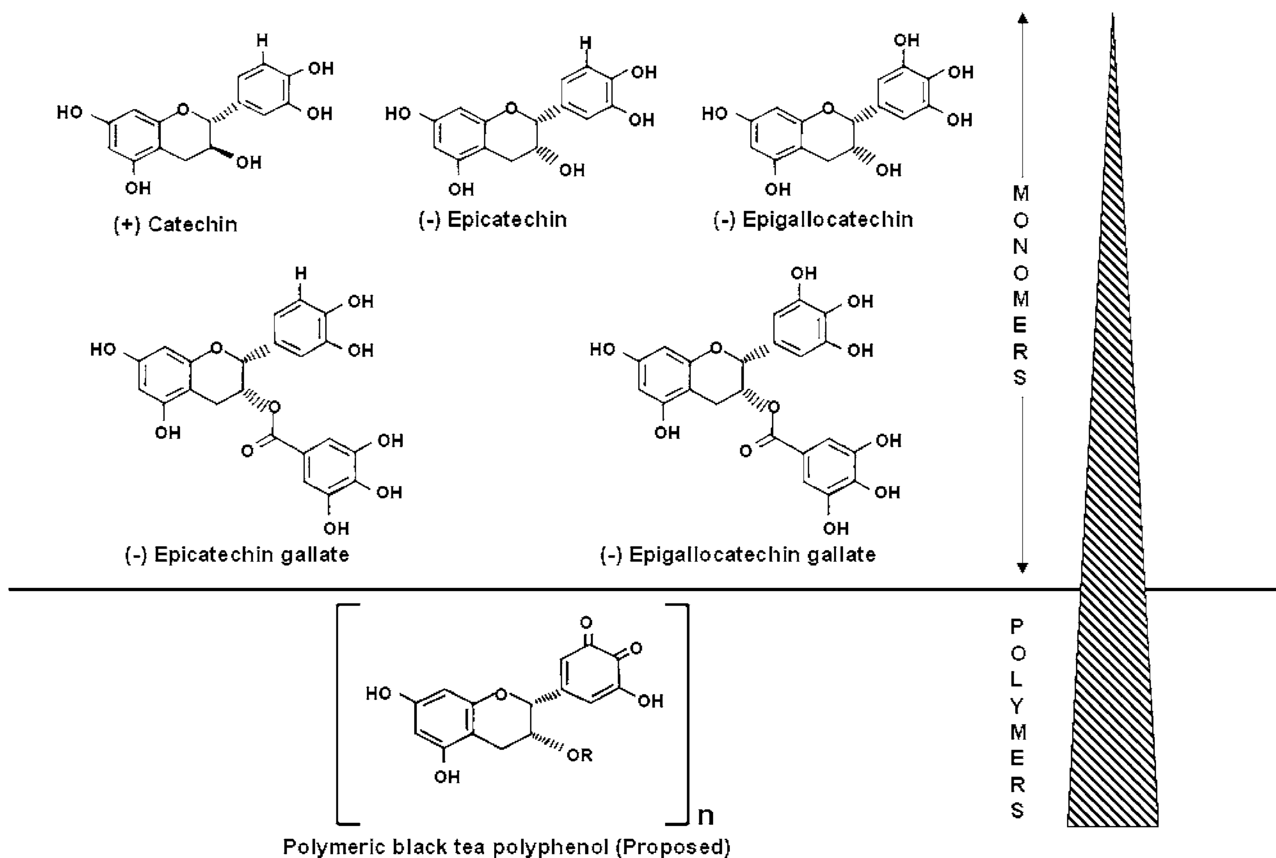


Figure 1. Structures of monomeric free catechins and proposed structure of polymeric black tea polyphenols (PBPs).

Table 1. Physicochemical Propertis of Polymeric Black Tea Polyphenol Fractions PBP-1–PBP-5 Isolated from Black Tea Powder by Solvent Extraction

property	PBP-1	PBP-2	PBP-3	PBP-4	PBP-5	EGCG
color	brown	light brown	light yellow	dark brown	bronish black	orangish brown
pH of 1% aq solution	5.53	5.55	5.40	4.26	4.09	4.81
λ_{max1} (nm)	211	219	217	211	210	215
λ_{max2} (nm)	272	272	268	263	270	273
$\lambda_{max1}/\lambda_{max2}$	2.78	1.68	1.78	3.21	6.28	1.45
% yield	0.97	1.42	1.06	1.42	0.75	-
$FeCl_3$ reactivity[a]	weakly positive	weakly positive	weakly positive	weakly positive	weakly positive	strongly positive
TLC mobility[b]	–	–	–	–	–	+

[a] To distinguish between simple and condensed polyphenols. [b] To demonstrate absence of known biologically active mobile components of black tea in isolated PBPs. Stationary phase: silica gel with 254 nm fluorescent indicator. Mobile phase: chloroform/ethyl acetate/formic acid (6:4:1). Detection method: UV absorption.

The absence of known biologically active components such as caffeine, free catechins (C, EC, ECG, EGC, EGCG, GCG) or TFs in PBPs was confirmed in TLC analysis employing silica gel as the stationary phase and ethyl acetate/chloroform/acetic acid (6:4:1) as the mobile phase. The yields of PBP-1, PBP-2, PBP-3, PBP-4, and PBP-5 were 0.97, 1.42, 1.06, 1.42, and 0.75% (wt %/wt of solids), respectively. Each PBP was dried by employing a rotary flash evaporator and stored at −20 °C for later use.

EGCG was isolated from green tea extract employing Sephadex LH-20 column chromatography (*16*).

Determination of pH and UV Absorption Spectra. The UV absorption spectrum was determined by solubilizing each PBP in 0.1 M sodium phosphate buffer, pH 7.4, whereas 1% aqueous solution of each PBP was taken and the pH of these solutions measured employing a pH-meter.

FT-IR and NMR Analysis. PBPs and monomeric free catechins (C, EC, ECG, EGC, and EGCG) were compared by FT-IR and NMR analysis. FT-IR spectra were recorded on Magna 550 series infrared spectrophotometer (Nicolet). Spectra were taken in transmittance mode at a resolution of 0.4 cm^{-1} and a frequency of 32 scans over a range of 400−4000 cm^{-1}. ^1H NMR spectra were recorded on a Mercury Plus (300 MHz) instrument, Varian, at a rate of 318 completed transients (ct) over an acquisition time (at) of 2 s. DMSO was employed as solvent and also served as an internal standard.

Preparation of Rat Liver Microsomes. Six−eight-week-old male Sprague−Dawley rats were treated intraperitoneally with a single dose of Aroclor 1254 (500 mg/kg of body weight). Animals were fasted for 12−15 h prior to sacrifice on the fifth day postinjection; their livers were perfused, excised, and subjected to differential centrifugation (*17*). The protein content of the microsomes was determined (*18*), and then the microsomes were stored in aliquots at −80 °C.

Determination of [^{3}H]-B(a)P−DNA Adducts. The reaction mixture for the in vitro assay employing rat liver microsomes as metabolic activation system contained calf thymus DNA (1 mg), EDTA (10 mM), $MgCl_2$ (50 mM), sodium phosphate buffer, pH 7.4 (100 mM), and rat liver microsomes (0.72 mg of protein). [^{3}H]-B(a)P, specific activity = 1000 mCi/mmol (10 μCi; 10 nmol), and various concentrations (μg/mL) of chemopreventive agent(s) (PBPs/EGCG) in 0.1 M phosphate-buffered saline, pH 7.4, were added, and the reaction mixture was incubated at 37 °C for 30 min (*19*). In another set of experiments rat liver microsomes were preincubated with different concentrations of chemopreventive agent(s) (PBPs/EGCG) at 37 °C for 30 min, and then [^{3}H]-B(a)P and other components of the assay mix were added as

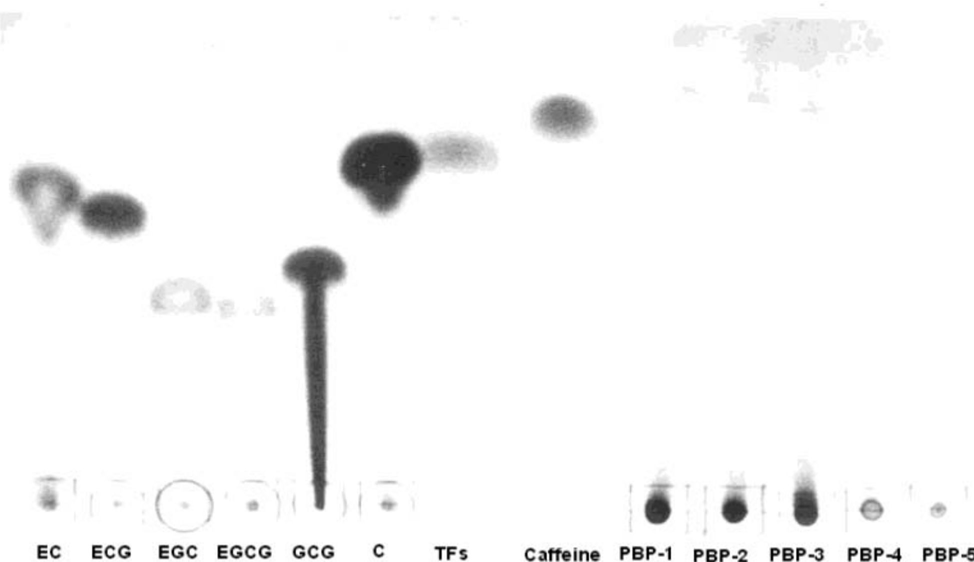


Figure 2. Thin-layer chromatogram showing absence of known biologically active and mobile monomeric free catechins (C, EC, ECG, EGC, EGCG, GCG) theaflavins (TFs) and caffeine in polymeric black tea polyphenol fractions PBP-1–PBP-5 [(+)-catechin (C), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), (+)-gallocatechin gallate (GCG)].

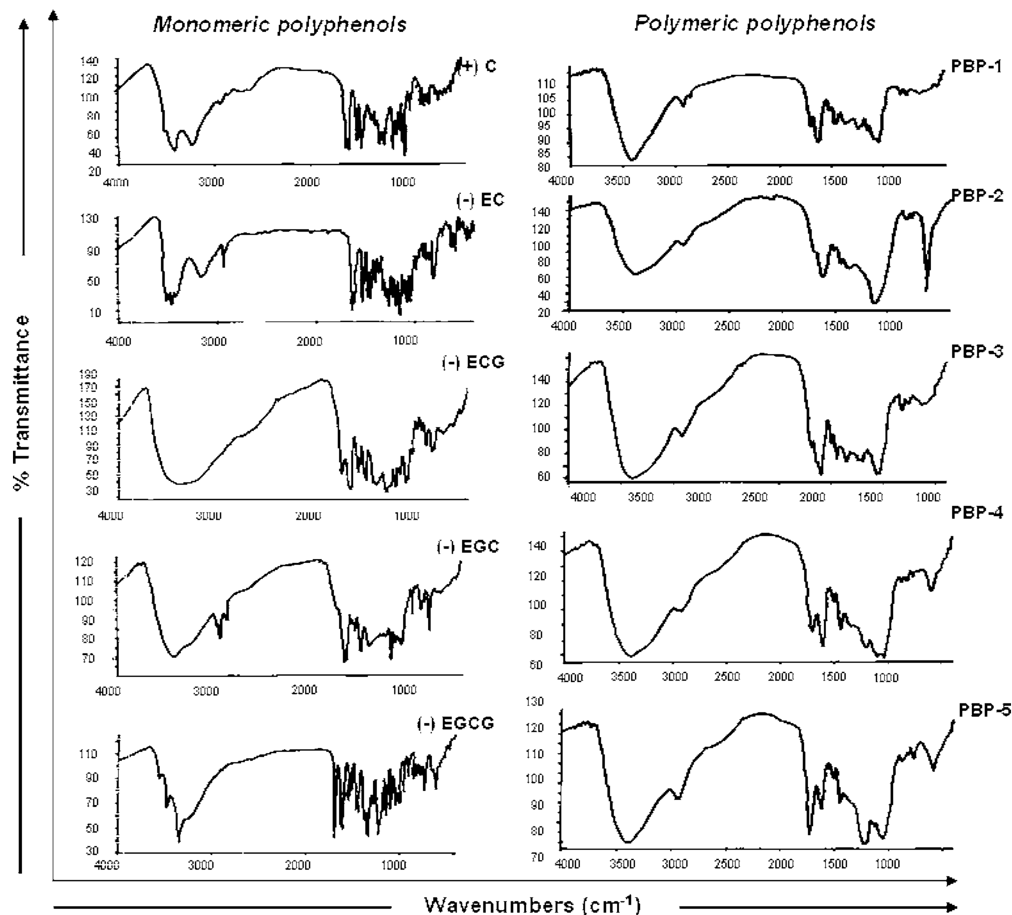


Figure 3. FT-IR spectra of monomeric free catechins (C, EC, ECG, EGC, EGCG) and polymeric black tea polyphenol fractions PBP-1–PBP-5 [(+)-catechin (C), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), (+)-gallocatechin gallate (GCG)].

mentioned earlier. In both cases the reaction was initiated by the addition of NADPH (0.6 μM) and the volume made up to 1 mL.

The reaction mixture was incubated at 37 °C for 30 min on a metabolic shaker water bath, in the dark and in the presence of air. The reaction was terminated by the addition of 0.1 mL of 10% SDS and double the volume of water-saturated phenol. DNA was isolated according to a standard phenol extraction procedure (three times). Excess, unmetabolized [3H]-B(a)P/B(a)P was removed by ether extraction (three times). The purity of the DNA samples recovered at the end of incubation was checked by measuring $A_{260/280}$ ratios (1.75−1.80), which were comparable to the ratios observed with the pure DNA employed in the assay. Contribution of non-covalently bound B(a)P

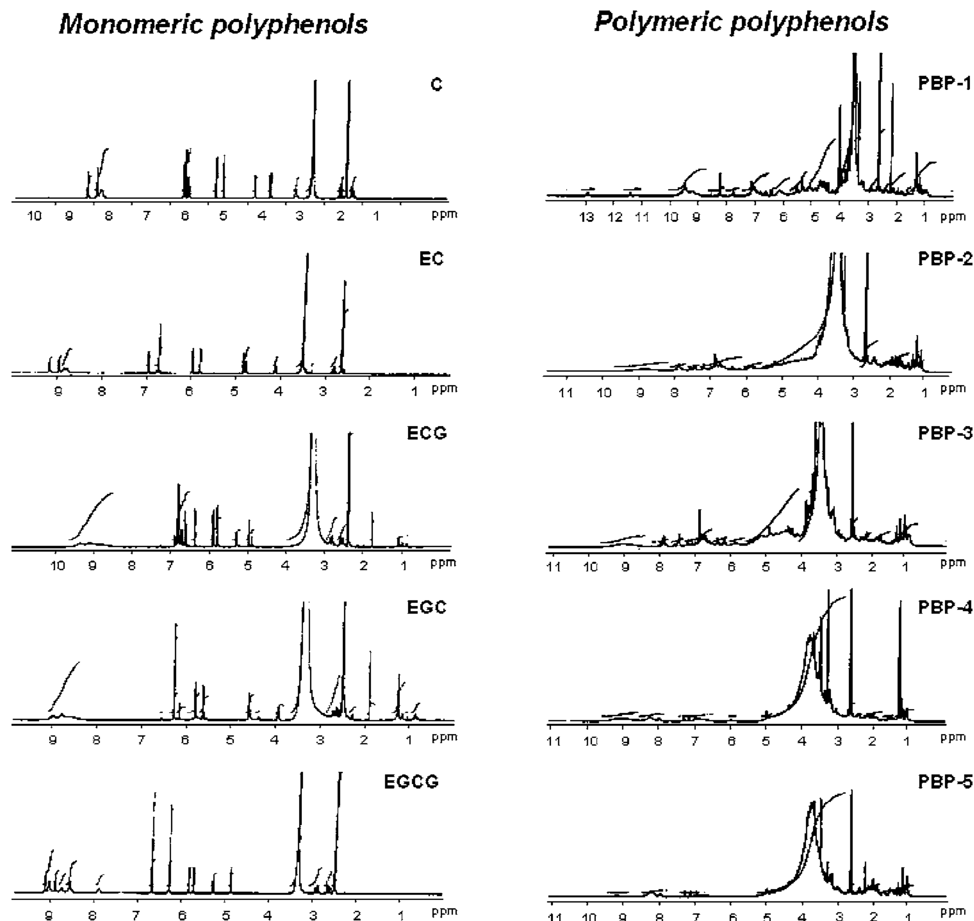


Figure 4. ^{1}H NMR spectra of monomeric free catechins (C, EC, ECG, EGC, EGCG) and polymeric black tea polyphenol fractions PBP-1–PBP-5 [(+)-catechin (C), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epigallocatechin gallate (EGCG), (+)-gallocatechin gallate (GCG)].

(under the experimental conditions) was ruled out by determining the CPM per microgram of DNA after reprecipitation/dialysis. A very small proportion of microsome-independent radioactivity, which may be due to light-catalyzed activation, etc., was subtracted from all microsome-mediated radioactivity values. Radioactivity measurements of DNA isolated from each PBPs/EGCG-treated sample were compared with DNA from untreated sample for determining the dose-dependent effect of PBPs/EGCG. Results, expressed as picomoles of [^{3}H]-B(a)P bound per milligram of DNA, are the mean ± standard error (SE) of at least six observations.

Determination of Carbon Monoxide (CO) Binding to CYP450. Total CYP450 activity as judged by CO binding was carried out according to the method of Omura and Sato (*20*) as described earlier (*19*).

Determination of Activities of CYP1A1, CYP1A2, and CYP2B1. The activities of the isozymes of CYP450, namely, CYP1A1, CYP1A2, and CYP2B1, were determined by monitoring the formation of resorufin from either ER, MR, or PR (*21*, *22*). Briefly, the assay mix contained 0.1 M sodium phosphate buffer, pH 7.4, 6.25 mM $MgSO_4$, 60 μM EDTA, 5 μM ER, MR, or PR, 100 μg of microsomal protein, and 100–250 μM NADPH in 1 mL total volume. Chemopreventive agents dissolved in 0.1 M sodium phosphate buffer, pH 7.4, were incorporated in 10 μL volumes to the assay mix. The concentration of PBPs/EGCG ranged from 0 to 800 μg/mL. The reaction time was 5 min with a preincubation of 5 min without the addition of NADPH. The reaction was stopped by the addition of 2 mL of chilled methanol. The precipitated protein was centrifuged down and the fluorescence of the supernatant measured at λ_{ex} 550 nm and λ_{em} 585 nm. Blanks contained no NADPH. Results expressed as nanomoles of resorufin formed per minute per milligram of protein are mean ± SE of at least five observations. IC_{50} values were determined from the graph.

To confirm that the observed inhibition or lack of inhibition was not due to interference in resorufin measurements, additional assays were conducted with (a) the parent compound (various concentrations of PBP-1–5) were added to known concentrations of resorufin) or (b) metabolic product (microsomes were incubated with or without PBP-1–5 at 37 °C for 30 min and then the resorufin was added).

Effect of Other Substrates [B(a)P and/or PBP-5] on Conversion of Ethoxyresorufin to Resorufin. Because PBP-5 inhibited the formation of resorufin from ethoxyresorufin, but did not inhibit microsome-catalyzed B(a)P-derived DNA adduct formation, it was necessary to study the formation of R from ER in the presence of various concentrations of B(a)P and/or PBP-5. The following experiments were carried out: (a) effect of various concentrations of PBP-4 and PBP-5 on the formation of R from ER; (b) effect of various concentrations of PBP-4 and PBP-5 in the presence of B(a)P (10 nmol) on the formation of R from ER; (c) effect of various concentrations of B(a)P in the presence of PBP-4 or PBP-5 (400 μg) on the formation of R from ER; and (d) effect of various concentrations of B(a)P on the formation of R from ER.

Fluorometric Evaluation of Microsomes Treated with B(a)P and/or PBP-4/PBP-5. To check for the interaction between microsomes, B(a)P, and PBP-4/PBP-5, another experiment was set up wherein microsomes were incubated with B(a)P and/or PBP-4/PBP-5 at 37 °C for 30 min, following which the tubes were kept on ice and centrifuged at 100000*g* for 1 h at 4 °C to recover the microsomes. The recovered microsomes were washed by resuspension and recovered after centrifugation. Microsomes were solubilized in 0.1 M NaOH, and the fluorescence spectrum was taken for each sample.

RESULTS

The physicochemical properties of PBP-1–PBP-5 isolated from black tea and EGCG isolated from green tea extract are presented in **Table 1**. It is seen from the table that these compounds differed from each other in color, pH of aqueous

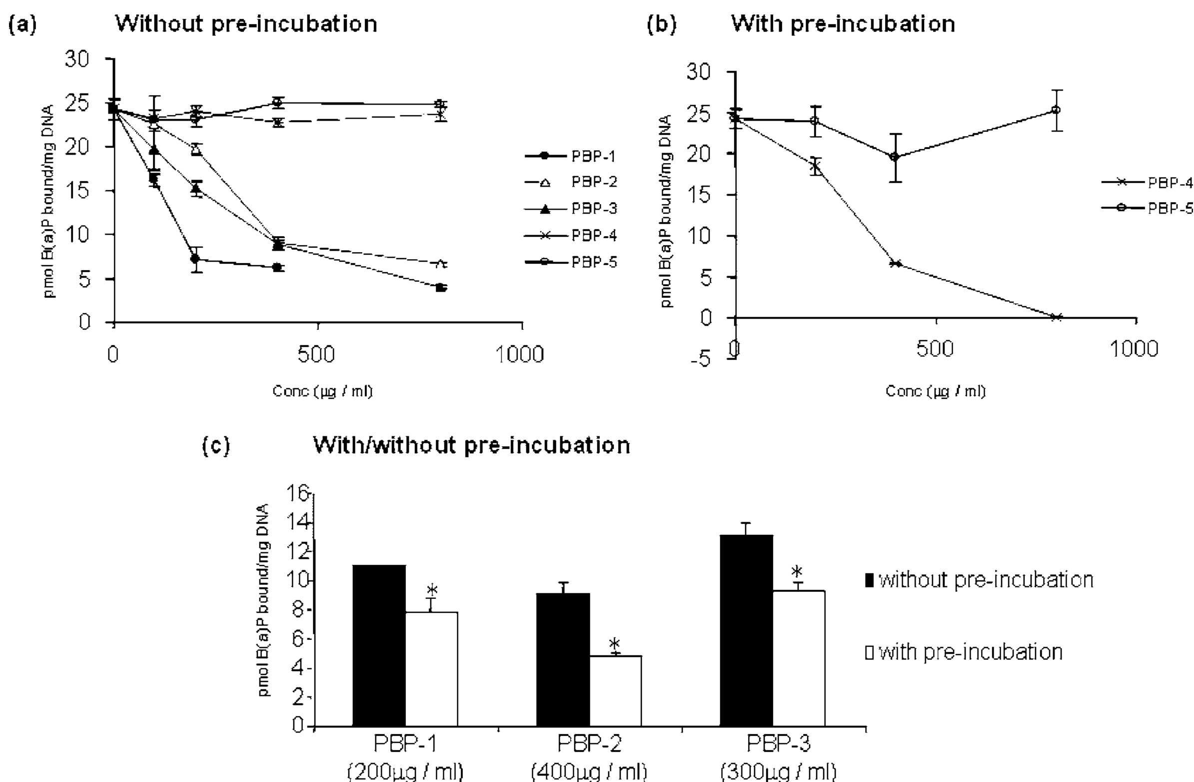


Figure 5. Effect of PBPs on [³H]-B(a)P-derived DNA adduct formation in vitro, employing rat liver microsomes: (**a**) without preincubation (individual PBP-1–PBP-5 was co-incubated with assay mix containing microsomes); (**b**) with preincubation (individual PBP-1–PBP-5 was preincubated with microsomes for 30 min and then added to the assay mix); (**c**) comparison of effect(s) of PBP-1–PBP-3 with and without preincubation at a dose near IC_{50}. For further details see text. Data expressed as pmol of B(a)P bound per mg of DNA are mean ± SE of six (**a**, **b**) or three observations (**c**). *, significantly different when compared with respective "without preincubation" group, $P \leq 0.05$.

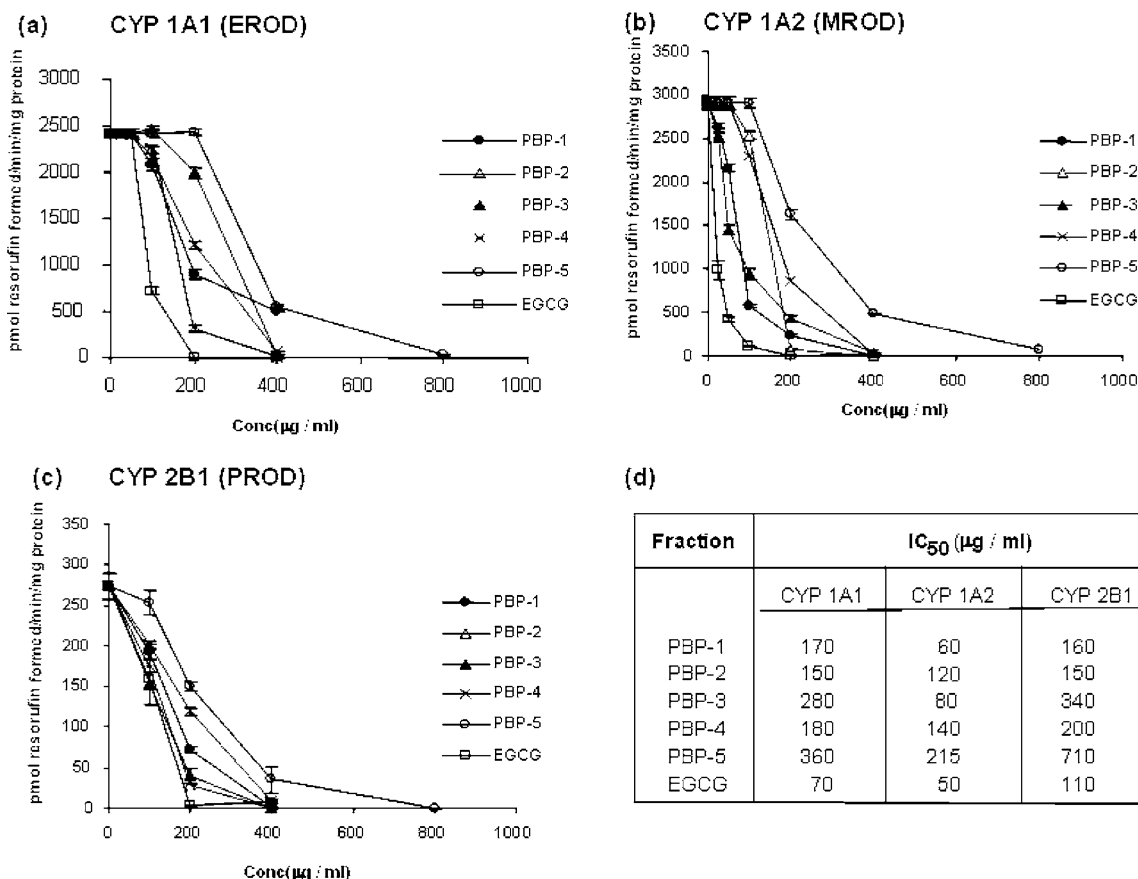
solution, λ_{max1}, λ_{max2}, ratios of $\lambda_{max1}/\lambda_{max2}$, yields,, etc. It is also noteworthy that all of the PBPs isolated from black tea reacted weakly with ferric chloride, whereas EGCG, a free catechin from green tea, gave a strong characteristic reaction (*23*). Similarly, all PBPs were retained at the origin in TLC separation carried out employing silica gel as the stationary phase and chloroform/ethyl acetate/formic acid (6:4:1) as the mobile phase, whereas EGCG migrated as expected.

The TLC evaluation of PBPs to ascertain that they were free from known biologically active components such as caffeine, C, EC, ECG, EGC, EGCG, GCG, and TFs presented in **Figure 2** suggested that all PBPs isolated according to the procedure of Brown et al. (*15*) were free from contamination by these compounds. FT-IR spectra show poor resolution for PBPs in the fingerprint region (800−1600 cm^{-1}) as compared to monomeric free catechins (**Figure 3**). Comparison of NMR spectra between PBPs and monomeric free catechins also reveals poor peak resolution for the former, suggesting PBPs to be polymeric in nature (**Figure 4**). These results show that PBPs isolated from black tea and used for the evaluation of their biological activity were free from known biologically active components of black tea.

Experiments carried out in vitro to study the effect of PBPs on the formation of microsome-catalyzed B(a)P-derived DNA adducts (**Figure 5a**) show that PBP-1, PBP-2, and PBP-3 (100−800 μg/mL) brought about a dose-dependent decrease in the levels of [³H]-B(a)P-derived DNA adducts. The extent of decrease for PBP-1, PBP-2, and PBP-3 ranged from 13 to 75%, from 7 to 73%, and from 19 to 85% of control values (24.23 ± 0.74 pmol/mg of DNA, 100%), respectively. However, inclusion of PBP-4 or PBP-5 (100−800 μg/mL) in the assay did not show significant effect under the experimental conditions used (**Figure 5a**). When rat liver microsomes were preincubated with individual PBP-1−5 and then incubation mix was employed for the assay, a dose-dependent inhibition of B(a)P-derived DNA adducts was observed not only with PBP-1, PBP-2, and PBP-3 but with PBP-4 also. The extent of inhibition ranged from 24 to 100% of control values (24.23 ± 0.74 pmol/mg of DNA, 100%) (**Figure 5b**).

Comparison of the extent of inhibition in assays between simultaneous addition (co-incubation with assay mix) of PBP versus those preincubated with microsomes at the same dose (closer to IC_{50}) of PBP-1, PBP-2, and PBP-3 showed a relatively higher degree or percent inhibition of DNA adduct formation with preincubation of PBP-1, PBP-2, or PBP-3 with microsomes (**Figure 5c**). Thus, PBP-4 showed inhibitory activity only after preincubation with microsomes, whereas PBP-5 failed to show any effect under both of the experimental conditions employed (**Figure 5b**).

One of the possible reasons for the decrease of [³H]-B(a)P-derived DNA adduct formation in the presence of PBPs is inhibition of activity of cytochrome P450s. Hence, the effect of each PBP was studied on the activity of cytochrome P450 as judged by CO-binding activity of microsomes. All PBPs showed significant reduction in CO-binding activity of microsomes, although this was due to PBP-derived color interference rather than true inhibition of CYP450 activity (data not shown). Because the effect of PBPs on the total CYP450 activity could not be assessed, their effects on the activities of different isozymes of CYP450, namely, CYP1A1 (EROD), CYP1A2 (MROD), and CYP2B1 (PROD), were studied by employing biochemical probes wherein the effect of PBPs on the formation of product, that is, resorufin, was measured (**Figure 6a−c**). The observations illustrate the potent inhibitory effect of PBPs on



Fraction	IC50 (µg / ml)		
	CYP 1A1	CYP 1A2	CYP 2B1
PBP-1	170	60	160
PBP-2	150	120	150
PBP-3	280	80	340
PBP-4	180	140	200
PBP-5	360	215	710
EGCG	70	50	110

Figure 6. Effect(s) of PBPs and EGCG on the activity of CYP1A1, CYP1A2, and CYP2B1 in Aroclor 1254-treated rat liver microsomes in vitro. Data expressed as (i) pmol of resorufin formed/min/mg of protein are mean ± SE of five observations (**a**–**c**) and (ii) inhibitory concentration 50% (IC_{50}, μg/mL) have been derived from graphs **a**–**c**.

the activities of specific CYP450 isozymes in Aroclor 1254-treated rat liver microsomes. All PBPs as well as EGCG inhibited the formation of resorufin catalyzed by CYP1A1 or CYP1A2 or CYP2B1 isozymes from respective substrates in a dose-dependent manner.

To compare the efficacy of various PBPs within themselves as well as with that of EGCG and also to check whether preferential inhibition of any specific isozymes of cytochrome P450 was observed, IC_{50} values (μg/mL) are presented in **Figure 6d**.

It is seen from the data in **Figure 6d** that relatively lower IC_{50} values were observed for all PBPs and EGCG in inhibiting CYP1A2 (MROD) than CYP1A1 (EROD) and/or CYP2B1 (PROD) activity. Inhibitory effects of all PBPs and EGCG were in the order MROD > EROD > PROD. These results suggest that all PBPs continue to possess CYP450 inhibitory activity as seen with free catechins/EGCG/green tea polyphenols.

Among PBPs, inhibition for MROD was in the order PBP-1 > PBP-3 > PBP-2 > PBP-4 > PBP-5, whereas inhibition for EROD/PROD followed essentially the same order, that is, PBP-2 > PBP-1 > PBP-4 > PBP-3 > PBP-5. PBP-5 appeared to be least effective among various PBPs for inhibiting all three isozymes of CYP450 studied (**Figure 6d**). It may be noted that the hierarchy of IC_{50} based on the DNA adduct assay was in agreement with that of the MROD assay, rather than the EROD assay.

The disagreement between [^{3}H]-B(a)P-derived DNA adduct formation and the formation of resorufin from ethoxyresorufin in the case of PBP-4 and/or PBP-5 is probably due to the absence of benzo[*a*]pyrene in assays evaluating the conversion of ER to R; hence, the formation of R from ER was checked in the presence of (a) various concentrations of PBP-4 and/or PBP-5 alone, (b) various concentrations of PBP-4 and/or PBP-5 in the presence of a fixed concentration of B(a)P, (c) various concentrations of B(a)P in the presence of a fixed concentration of PBP-4 and/or PBP-5, and (d) various concentrations of B(a)P alone.

Observations presented in **Figure 7a** show that both PBP-4 and PBP-5 inhibited the formation of R from ER in a dose-dependent manner and that PBP-4 was relatively more potent than PBP-5. When the effects of the same doses of PBP-4 and PBP-5 on the formation of R from ER were studied in the presence of 10 nmol of B(a)P, there was significant decrease in the levels of resorufin formed in the presence of 10 nmol of B(a)P alone (~87%), and this was further decreased (~9 and 6%) on inclusion of various concentrations of PBP-4 and PBP-5, respectively (**Figure 7b**), although this was not dose-dependent. These results suggest that B(a)P as well as PBP-4 and/or PBP-5 independently inhibit the formation of R from ER and that the combination is relatively more potent. Similarly, in the presence of 400 μg/mL PBP-4 or PBP-5, the addition of various concentrations, that is, 5, 10, or 20 nmol of B(a)P, resulted in further decreases of 65% for PBP-4 and 16% for PBP-5, in the levels of R formed, although no dose-related effects were observed with different concentrations of B(a)P (**Figure 7c**).

Results from experiments carried out to study the effect of various concentrations of B(a)P alone on the formation of R from ER showed a significant decrease (~87%) in the levels of R formed (**Figure 7d**).

Critical evaluation of results with PBP-5 (**Figure 7c**) suggest that B(a)P appeared to be a relatively more effective inhibitor than PBP-5 in inhibiting the formation of R from ER probably

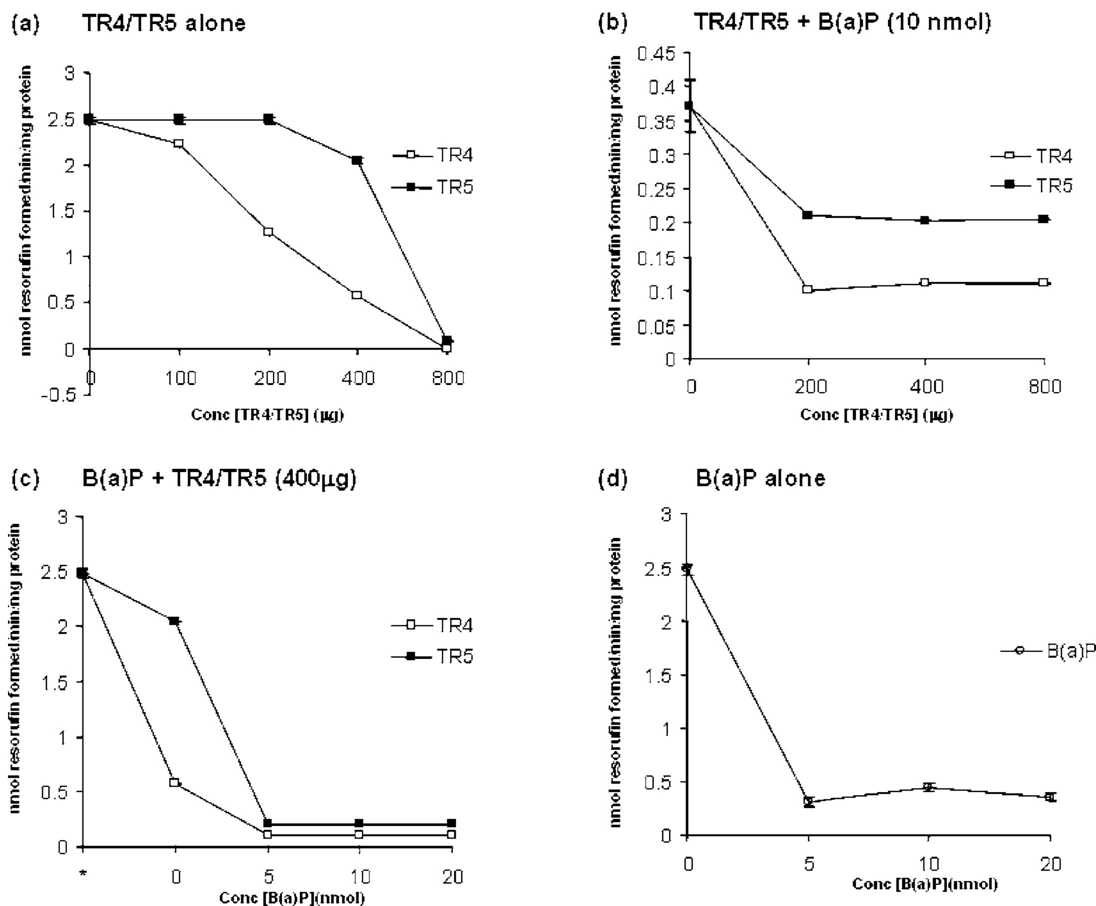


Figure 7. Effect of PBP-4/PBP-5 or B(a)P alone and in combination of PBP-4/PBP-5 with B(a)P on microsome-catalyzed resorufin formation from ethoxyresorufin (ER) in vitro. Assay mix containing microsomes and substrate (ER) were incubated with (**a**) various concentrations of PBP-4/PBP-5 (μg/mL), (**b**) various concentrations of PBP-4/PBP-5 and a fixed concentration of B(a)P (10 nmol), (**c**) various concentrations of B(a)P and a fixed concentration of PBP-4/PBP-5 (400 μg/mL), and (**d**) various concentrations of B(a)P alone. For details see text.

because of the relatively higher affinity of B(a)P than that of PBP-5 for microsomes. In the absence of exact molecular weights of PBP-1−PBP-5, it is difficult to compare the inhibitory activity at equimolar concentrations.

Comparison of fluorescence ratios (410/480) between control microsomes and those treated with (i) PBP-4/PBP-5 alone and (ii) B(a)P alone as well as (iii) PBP-4/PBP-5 + B(a)P showed that PBP-4/PBP-5 + B(a)P was relatively closer to the ratio shown by B(a)P alone, suggesting a relatively higher affinity for B(a)P when compared to PBP-4/PBP-5 (data not shown).

DISCUSSION

The formation of carcinogen−DNA adducts is one of the early events in chemically induced carcinogenesis and considered to be essential for tumor initiation (*24*). Formation of carcinogen−DNA adducts in most carcinogens is dependent on the formation of reactive species catalyzed by CYP450 isozymes. Inhibition of the formation of reactive species or scavenging of reactive species (before it reacts with DNA) by chemopreventive agents is likely to prevent the biological consequences and may be used as a prescreen to identify potential chemopreventive environmental agents (*25*). Earlier observations have demonstrated that a number of plant-derived antioxidants inhibit the formation of carcinogen−DNA adducts in vitro (*19*) and in vivo (*26*, *27*).

In the present study black tea-derived newly formed major polymeric polyphenols (PBP-1−PBP-4) and a green tea-derived most potent polyphenol, that is, EGCG (used as positive control) have shown a dose-dependent inhibition of microsome-catalyzed [3H]-B(a)P-derived DNA adduct formation (with and/or without preincubation of PBPs with microsomes), although the degrees of inhibition were different. The higher degree of inhibition with PBP-1−PBP-4 preincubated microsomes suggests products of PBP-1−PBP-4 to be relatively more inhibitory than the parent compounds. These results show that polymeric polyphenols formed newly in black tea from free catechins [green tea polyphenols (GTPs)] in the leaves do continue to possess inhibitory activity exhibited by EGCG. Because exact molecular weights of different PBPs are not known, comparison of inhibitory activity of PBPs versus EGCG could not be conducted at equimolar concentrations in this experiment. However, significant inhibitory activity shown by PBPs—known to be of high molecular weights (∼700−40000) (*28*)—at equal weight levels used for EGCG (458) suggests that PBPs may prove to be equivalent to or even better than EGCG. The absence of inhibitory activity of PBP-5, even after preincubation with microsomes, is surprising and suggests that it possesses a very high molecular weight and/or that it may even differ from PBP-1−PBP-4 in other aspects. Subsequent studies have shown that PBP-1−PBP-4-induced inhibition of microsome-catalyzed [3H−B(a)P]-derived DNA adduct formation is due to inhibition of isozymes of CYP450. Earlier observations have shown that the addition of EC, EGC, ECG, EGCG,and GTPs to rat liver microsomes resulted in a dose-dependent inhibition of CYP450-dependent aryl hydrocarbon hydroxylose (AHH), 7-ethoxycoumarin-*O*-deethylase (ECOD), and EROD activities (*29*). The

results of the present study suggest that PBPs continue to possess CYP450 inhibitory property as in the case of EGCG.

Several standard chromatographic techniques such as Sephadex LH-20 chromatography (*12*) and reverse phase HPLC (*13*, *30*) as well as modern analytical techniques such as electrospray ionization ion trap multiple mass spectrometry (ESI-MS) as well as HPLC-ESI-MS, equipped with diode array (*31*), have not been successful in the preparative fractionation of PBPs. Hence, solvent fractionation of PBPs was employed. PBPs thus isolated have been shown to be free of other known biologically active components such as caffeine, free catechins, and theaflavins, present in black tea, and evidence suggesting the polymeric nature of PBPs has been presented (**Figures 2−4**).

It must be noted that the reported concentration of PBPs in black tea is 35.9% (wt %/wt of dry tea powder) (*32*). In 1.5% black tea-derived brew, concentration of PBPs is likely to be 5400μg/mL, whereas in our study the concentrations of PBPs employed are 200−800 μg/mL (6.5−27-fold less) and hence are likely to be biologically relevant.

B(a)P is predominantly metabolized by CYP1A1/CYP1A2; however, the hierarchy of IC_{50} of TRs for inhibition of DNA adduct formation (PBP-1 > PBP-3 > PBP-2 > PBP-4 > PBP-5) was not in line with the hierarchy based on the inhibition of EROD (PBP-2 > PBP-1 > PBP-4 > PBP-3 > PBP-5); instead, it was in line with the inhibition of MROD (PBP-1 > PBP-3 > PBP-2 > PBP-4 > PBP-5). This is probably due to the relatively higher activity of CYP1A2 (2940 ± 71 pmol of resorufin formed/min/mg of protein) as compared to that of CYP1A1 (2470 ± 45 pmol of resorufin formed/min/mg of protein) in the Aroclor-treated rat liver microsomes employed in the assays. In addition, the role of relative affinity and substrate specificity of isozyme protein(s) for B(a)P versus PBPs (DNA adduct assay) and ER versus PBPs (EROD assay) is likely and probably responsible for the observed differences in hierarchy of various PBPs in both assays.

The major detoxification pathway for most of the polycyclic aromatic hydrocarbons (PAHs) and nitrosamines (major classes of carcinogens present in tobacco smoke) is mediated by the 1A1/1A2 and 2B classes of CYP450 isozymes. Hence, the present in vitro study suggests that PBPs are likely to inhibit conversion of B(a)P and NNK to their ultimate carcinogenic forms and may be relatively more effective against PAHs than nitrosamines as 1A2 is inhibited more readily than 2B1. Because of overlapping substrate specificity of CYP450 isoforms in the metabolism of a compound, for example, B(a)P, inhibition of one isoform predominantly involved in the metabolism of B(a)P does not necessarily suggest a protective mechanism of action. In our study because the inhibition of adduct formation observed takes into consideration the contribution or lack of it of all isoforms (studied by us, i.e., CYP1A1, CYP1A2, and CYP 2B1 and those not studied by us) as well as potential scavenging ability of PBPs (not studied or demonstrated by us), a protective mechanism(s) of action of PBPs is demonstrated or is evident under the experimental conditions employed.

Experiments conducted with PBP-4/PBP-5 alone or B(a)P alone and in combination with each other suggest that microsomes have higher affinity for B(a)P than PBP-4/PBP-5 or ER. It should also be noted that the activity of PBPs observed cannot be extrapolated to the in vivo situation because of differences in solubility, absorption, distribution, and metabolism in the two systems. EGCG used as positive control in the present in vitro experiment has been reported to inhibit the activity of CYP450 isozymes in vivo that metabolize carcinogens such as NNK and B(a)P (*33*, *34*) and also to inhibit tumorigenesis by environmental carcinogens including nitrosamines and polycyclic aromatic hydrocarbons (PAHs) (*35*, *36*). A similar activity by PBPs needs to be established in vivo before they can be predicted to be chemopreventive agents like EGCG. Studies along these lines are in progress.

ACKNOWLEDGMENT

We thank Dr. I. N. N. Namboothiri, Department of Chemistry, IIT, Powai, Mumbai, and Dr. L. Mombassawala, Dr. (Mrs) M. Gandhi, and other staff at RSIC, IIT, Powai, Mumbai, for FT-IR and NMR analysis/interpretation, Dr. S. S. Pakhale, Dr. R. Thapliyal, and S. S. Dolas for useful discussions, and Prasad Phase for excellent technical help.

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Received for review January 1, 2004. Revised manuscript received April 17, 2004. Accepted April 21, 2004. We thank the National Tea Research Foundation, Kolkata, for partial financial support to the project and the Council for Scientific and Industrial Research for the award of a Senior Research Fellowship to R.K. (2002−2003),

JF049979O