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Protection against Trp-P-2 DNA Adduct Formation in C57bl6 Mice by Purpurin Is Accompanied by Induction of Cytochrome P450

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Purpurin, an anthraquinone constituent from madder root, has previously been reported as antimutagenic in the Ames *Salmonella* bacterial mutagenicity assay and as antigenotoxic in *Drosophila melanogaster*, against a range of environmental carcinogens. Short-term dietary supplementation with purpurin inhibits the formation of hepatic DNA adducts in male C57bl6 mice after a single dose of the heterocyclic amine dietary carcinogen Trp-P-2 (30 mg/kg). Inhibition of adduct formation was dose-dependent. No DNA adducts were observed in animals treated only with purpurin. The decrease in adduct formation was accompanied by significant, dose-dependent inductions of hepatic cytochrome P450-dependent dealkylations of methoxy- (CYP1A2), ethoxy- (CYP1A1), and pentoxy- (CYP2B) resorufins, total cytochrome P450, and NADPH cytochrome P450 reductase. It is hypothesized that purpurin exhibits chemopreventive potential by inhibiting the cytochrome P450-dependent metabolism of heterocyclic amines to their genotoxic *N*-hydroxylamines.

KEYWORDS: Purpurin; cytochrome P450; chemoprevention; heterocyclic amines; DNA adducts; Trp-P-2; inhibition

INTRODUCTION

Heterocyclic amines are formed in the cooking of proteinaceous foods (reviewed in refs *1*−*3*). These compounds have been identified as potent bacterial mutagens (*4*) and rodent carcinogens (*5*) and are now suspected as human carcinogens (*2*, *6*). Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) has been isolated from preparations of cooked red meats and tobacco smoke (*7*, *8*) and has been identified as a rodent carcinogen (*9*). Consequently, humans are frequently exposed to this carcinogen. Several anthraquinone pigments have been isolated from extracts of madder root (*Rubia tinctorum*) (*10*), which has uses as a herbal treatment for kidney and bladder stones (*11*) and as a food coloring. We have investigated previously the antimutagenic and antigenotoxic properties of purpurin (1,2,4-trihydroxy-9,10-anthraquinone) (**Figure 1**), one of several hydroxyanthraquinones found in madder, using the Ames *Salmonella* mutagenicity test (*12*) and a *Drosophila melanogaster* genotoxicity test (*13*). In each procedure, purpurin showed marked protection against heterocyclic amines (*12*, *13*) and against 1-aminopyrene and 1-nitropyrene (unpublished observations).

The mechanism of in vitro purpurin antimutagenesis has been investigated and has been observed to have two components: inhibition of cytochrome P450-dependent bioactivation and

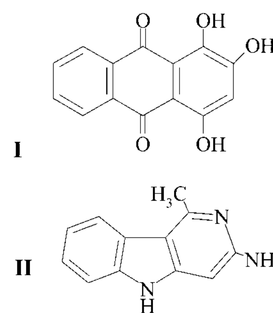

Figure 1. Structures of purpurin (**I**) and Trp-P-2 (**II**).

chemical reduction of the mutagenic *N*-hydroxylamine metabolite to a nondirectly mutagenic amine (*14*). Further investigation on the inhibition of cytochromes P450 using recombinant human cytochromes P450 expressed in *Escherichia coli* has demonstrated that inhibition by purpurin is specific for the CYP1 enzymes, particularly CYP1A2 and CYP1B1 (*15*). Previous studies with the structurally related compound anthraflavic acid (2,6-dihydroxyanthraquinone) report that although this compound inhibits CYP1A in vitro, it is an inducer of rat CYP1A1 and CYP1A2 in vivo (*16*, *17*). Furthermore, microsomal fractions from anthraflavic acid-induced animals were responsible for increased bioactivation of heterocyclic amines in the Ames test (*16*). Here we report preliminary experiments concerning the effects of short-term purpurin pretreatment upon DNA adduct formation by Trp-P-2 in vivo using the *Ah*-receptor-responsive mouse strain C57bl6. Furthermore, the

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Table 1. Cytochrome P450 Concentrations and NADPH Cytochrome P450 Reductase Activities in Hepatic Microsomes from C57bl6 Mice after Pretreatment with Trp-P-2 and/or Purpurin[a]

	control	Trp-P-2	purpurin (5%)	purpurin (0.25%) + Trp-P-2	purpurin (0.5%) + Trp-P-2	purpurin (5%) + Trp-P-2
cytochrome P450 (nmol mg^{-1} of protein)	0.514 ± 0.216	0.418 ± 0.101	1.899 ± 0.143 c	0.779 ± 0.194 a	1.352 ± 0.245 c	2.137 ± 0.133 c
NADPH cytochrome P450 reductase (nmol min^{-1} mg^{-1} of protein)	50.4 ± 4.0	43.1 ± 5.0 b	77.2 ± 6.8 b	49.1 ± 14.7	92.8 ± 34.4 a	100.8 ± 6.3 c

[a] Assays were performed in duplicate for three samples from different individuals, and the results are expressed as mean ± standard deviation. Single-tailed Student's *t* tests were used to determine significant differences from control values: a ($P < 0.10$); b ($P < 0.05$); c ($P < 0.01$).

effects of this pretreatment on hepatic expressions of cytochromes P450 and NADPH cytochrome P450 reductase were quantified to determine the relevance of purpurin-dependent modulation of cytochrome P450 in chemoprevention.

MATERIALS AND METHODS

Resorufin, alkoxyresorufins, cytochrome *c*, RNase A, and nuclease P1 (Sigma, St. Louis, MO), RNase T1, micrococcal endonuclease, phosphodiesterase I, and phosphodiesterase II (Worthington Biochemical Corp., Lakewood, NJ), Trp-P-2 and purpurin (Wako, Osaka, Japan), proteinase K (Merck, Darmstadt, Germany), apyrase (Yamasa Co., Noda, Japan), and T4 polynucleotide kinase (Takara, Ohtsu, Japan) were all purchased.

Male C57bl6 mice (15−20 g) were obtained from Charles River, Japan. Treatment groups consisted of three mice housed together with access to diet and water ad libitum. Six groups of mice received control diet, control diet and Trp-P-2 (30 mg/kg), purpurin diet (5%), or Trp-P-2 (30 mg/kg) and one of three purpurin diets (0.25, 0.5, or 5% w/w). Animals were weighed daily. Trp-P-2 was given by gastric intubation in a single dose dissolved in sterile water on day 3. For controls, animals not receiving Trp-P-2, water only was given. On day 4 animals were sacrificed by cervical dislocation, and liver, kidneys, colon, and lungs were removed, washed with ice-cold KCl (1.15% w/v), and frozen in liquid nitrogen. Samples were stored at −80 °C until use.

Frozen livers were halved and half used to prepare microsomes for mixed-function oxidation assays as described previously (*14*). DNA was isolated from the remaining half of the livers and from the other tissues by phenol−chloroform extraction and ethanol precipitation.

Total hepatic cytochrome P450 content was determined by the reduced carbon monoxide difference spectral method of Omura and Sato (*18*). NADPH cytochrome P450 reductase activity was quantified by monitoring the production of reduced cytochrome *c* at 550 nm (*19*). Generation of resorufin from ethoxy-, methoxy-, and pentoxyresorufins, indicative of CYP1A1, CYP1A2, and CYP2B activities, respectively, was followed fluorometrically as described previously (*20*, *21*).

Hepatic DNA adducts were determined using the nuclease P1 ^{32}P-postlabeling method in combination with thin-layer chromatography as described by Ochiai et al. (*22*).

All of the results obtained were evaluated using single-tailed Student's *t* tests.

RESULTS AND DISCUSSION

Throughout the trial, the average weight and average gain in weight of the animals were not significantly different between groups and control (data not shown). The liver weights of the purpurin-treated animals were significantly ($P < 0.01$) increased (1.32 ± 0.08 g compared to 0.88 ± 0.13 g in the controls). Evidence that purpurin was absorbed across the gastrointestinal wall was apparent because of the marked red-orange coloration of the urine and, upon dissection, the deep purple discoloration of the gastrointestinal tract and liver.

Purpurin treatment was responsible for dose-dependent induction of both total cytochrome P450 and NADPH cytochrome P450 reductase (**Table 1**). Trp-P-2 treatment had a small, but significant ($P < 0.05$), inhibitory effect upon the reductase activity but no effect upon total cytochrome P450 (**Table 1**). In contrast, Trp-P-2 given in addition to purpurin (5%) demonstrated a significant ($P < 0.01$) synergistic effect upon reductase activity (**Table 1**). Further investigation is required to elucidate the underlying mechanism of this synergism.

Marker enzyme activities demonstrated clearly that induction by purpurin was nonspecific. CYP1A1, CYP1A2, and CYP2B activities were each very markedly induced (**Figure 2**). This is in contrast to observations after anthraflavic acid treatment, when only CYP1A isoforms were induced (*16*). CYP-dependent activities were induced dose-dependently by the different purpurin diets. Induction by low doses of purpurin (0.25 and 0.5%) of the CYP1A2-dependent dealkylation of methoxyresorufin, however, was greater than either that of ethoxyresorufin (CYP1A1) or pentoxyresorufin (CYP2B). Preferential induction of methoxyresorufin *O*-dealkylase is in agreement with observations made after pretreatment with 1-hydroxyanthraquinone and 1,4-dihydroanthraquinone (*23*). Pretreatment with Trp-P-2 alone was responsible for an induction of CYP1A2 as expected (*24*), but no induction of CYP1A1 or CYP2B activities was observed. In contrast to NADPH cytochrome P450 reductase, the high level of induction of CYP1A2 did not demonstrate any synergistic effects of combinatorial treatment with purpurin and Trp-P-2 (**Figure 1**). These in vivo results contradict those obtained with purpurin and other polyphenolics in vitro, where dose-dependent inhibition of cytochrome P450 was observed (*12*, *17*, *25*, *26*). In vitro inhibition studies demonstrated, however, that this inhibition is competitive that is, purpurin competes with substrate for access to the active site (*14*, *15*). Consequently, as a probable substrate for these enzymes, it is not inconceivable that purpurin, and other anthraquinone, treatments induce significant levels of expression in vivo. Induction of CYP1A2, the isoform responsible for bioactivation of aromatic amine carcinogens (*27* and references cited therein), is expected to increase the metabolism of Trp-P-2 to genotoxic intermediates.

Considering the very marked induction of CYP1A2, an increase in Trp-P-2 hepatic DNA adduct formation is expected. However, a dose-dependent (trend value 5.35) and significant ($P < 0.10$) decrease in adducts was observed after purpurin treatment (**Figure 3**). These results, obtained after short-term supplementation with purpurin, suggest further investigations are warranted. A previous long-term feeding study using 1% (w/w) purpurin diet for 520 days was responsible for the formation of bladder and kidney tumors (*28*). However, the trend observed here suggests that lower doses of purpurin (0.25%) given over a longer dose period may prevent adduct formation without the observed toxicity. Further research is required to determine the safe optimum dose.

In contrast to in vitro studies (*12*), induction and not inhibition of cytochrome P450 was observed but DNA adduct formation

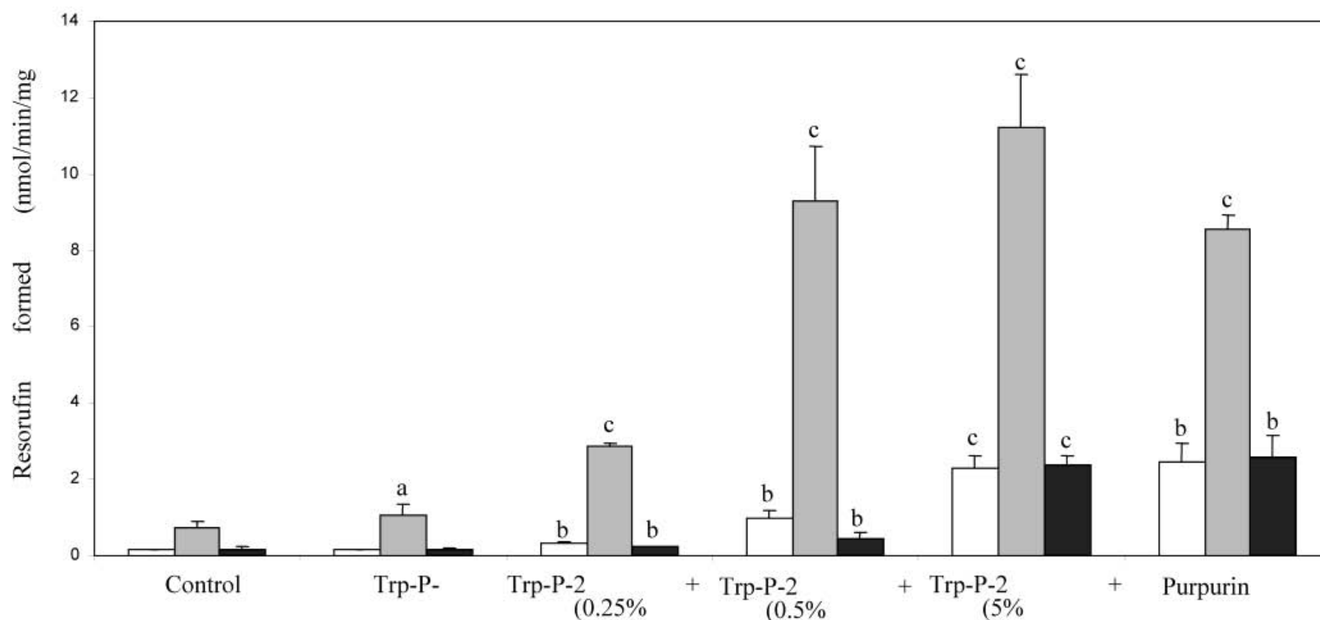

Figure 2. Induction of cytochrome P450-dependent dealkylations by pretreatment with purpurin. Investigations of ethoxy- (white bars), methoxy- (gray bars), and pentoxy- (black bars) resorufin *O*-dealkylations were performed in duplicate for groups consisting of three individuals. Results are presented as mean and standard deviation. Single-tailed Student's *t* analyses of the data were employed to determine significant increases compared with control: a ($P < 0.10$); b ($P < 0.05$); c ($P < 0.01$).

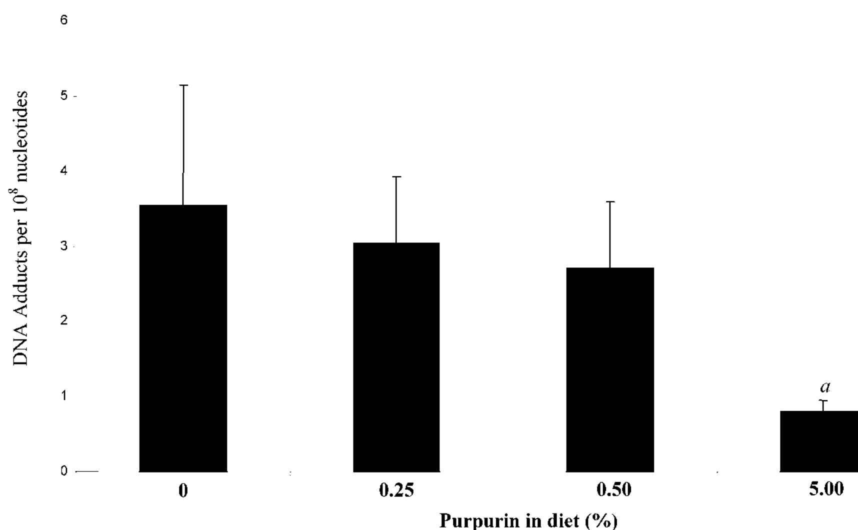

Figure 3. Effects of purpurin pretreatments on Trp-P-2 hepatic DNA adduct formation. Results are expressed as mean and standard deviation of adduct formation in three mice performed in duplicate: "a" represents values significantly ($P < 0.01$) different from animals receiving control diet.

was still decreased. Previously, anthraflavic acid-induced microsomes have demonstrated an increase in bioactivation of heterocyclic amines in the Ames test (*16*). Some further thought has to be applied to determine the mechanism of purpurin-dependent inhibition of DNA adduct formation. One possibility is that in mammals it is the chemical reduction of the proximate carcinogen, previously observed in vitro, that is the more important mechanism (*14*). This may explain why only the highest dose of purpurin decreased adduct formation as high doses may be required to obtain a plasma concentration sufficient to reduce the *N*-hydroxylamine. Another hypothesis is that purpurin induces both phase I metabolism and phase II metabolism, leading to an increased rate of clearance. It has already been demonstrated that some anthraquinones induce UDP-glucuronyltransferase and DT-diaphorase (*23*) but that anthraflavic acid does not induce glutathione *S*-transferase (*16*). It is possible that increased glucuronidation of Trp-P-2 contributes to the decrease in observed DNA adducts after purpurin treatment. However, studies on phase II conjugation enzymes and pharmacokinetics are required to help evaluate the true mechanism of purpurin anticarcinogenesis.

Kidney, colon, and lung tissues were investigated for the presence of DNA adducts, in addition to the liver. Under the dosing regimen employed here, no Trp-P-2 DNA adducts were observed in any of these tissues (data not shown). This is in agreement with observations in rats receiving long-term dietary Trp-P-2, in which tumors were observed only in the liver, urinary bladder, and mammary gland (*29*).

Our preliminary results have demonstrated the potential of purpurin as a chemopreventive agent. Future studies to determine the mechanism of in vivo inhibition of adduct formation employing longer treatment periods and concentrations of purpurin well below toxic levels are required to ascertain the potential of purpurin in chemoprevention.

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