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Enhancement of phase II enzyme activity by purpurin resulting in the suppression of MeIQx–DNA-adduct formation in mice

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Abstract

We previously demonstrated using a bacterial system that the antigenotoxic activity of the anthraquinone compounds purpurin and alizarin was due to the suppression of microsomal enzyme activity involved in the activation of mutagens. In the present study we determined the effect of purpurin and alizarin on (i) MeIQx–DNA-adduct formation in mouse tissues and (ii) the activity of phases I and II enzymes in liver fractions, the liver being the target tissue of MeIQx. The amount of MeIQx–DNA adduct formed was determined using ^{32}P-postlabeling methods. Methoxyresorufin-*O*-demethylase (MROD) and ethoxyresorufin-*O*-deethylase (EROD) enzyme activities, which reflect CYP 1A activity, were measured as markers for phase I enzymes, and UDP–glucuronyltransferase (UGT) and glutathione *S*-transferase (GST) activities were determined as markers for phase II enzymes. Mice fed with a diet containing 0.5% purpurin for 3 days prior to MeIQx administration had 70% fewer MeIQx–DNA adducts in the lung and kidney, and fewer DNA adducts (insignificant, statistically) in the liver compared with mice fed a diet lacking purpurin. MROD and EROD activities in the liver of these mice increased six- and eight-fold, respectively, and were higher than those determined for the control mice within 1 day following commencement of purpurin treatment. These elevated activities were maintained during treatment and declined immediately following removal of purpurin from the diet. GST and UGT activities gradually increased 2.5- and 3-fold, respectively, following purpurin treatment, and were maintained at significantly high levels even after purpurin administration ceased. Alizarin did not significantly affect DNA-adduct formation and enzyme activity, except in the case of UGT. Taken together, our results show that purpurin reduced MeIQx–DNA-adduct formation by maintaining elevated phase II enzyme activities, thereby facilitating accelerated excretion of MeIQx.
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Keywords: MeIQx–DNA adduct; Purpurin; CYP 1A; GST; Phase I enzyme; Phase II enzyme

1. Introduction

The intake of antimutagens and anticarcinogens present in food components may lead to a decreased risk of cancer onset. We previously demonstrated the antigenotoxic properties of several food components including chlorophyll and its derivatives [1–4], green tea polyphenols [5,6], and glycine betaine and pseudouridine in beer [7–9]. Recently, we showed that the anthraquinone pigment purpurin, a red pigment isolated

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CYP, cytochrome P450; EROD, ethoxyresorufin-*O*-deethylase; GSH, glutathione reduced form; GST, glutathione *S*-transferase; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MROD, methoxyresorufin-*O*-demethylase; NAT, *N*-acetyltransferase; UDP, uridine 5′-diphosphate; UGT, UDP–glucuronyltransferase; UDPGA, UDP–glucuronic acid

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from madder root [10], was an effective inhibitor against heterocyclic amine-induced genotoxicity in both bacteria [11] and Drosophila [12]. Anthraquinone compounds are found widely in plants, and some of these are used as herbal medicines or food pigments. Investigations have demonstrated the preventive effect of anthraquinones against genotoxicity or cytotoxicity [13,14], and the modulation of metabolic enzyme activities in response to xenobiotics [15–18]. We demonstrated that purpurin and alizarin inhibit the activity of human recombinant cytochrome P450 (CYP) isozymes CYP 1A1, CYP 1A2 and CYP 1B1, resulting in the antimutagenic effect observed in recombinant Salmonella possessing these CYPs [19].

Many mutagens including MeIQx are activated by phase I enzymes such as CYPs, and their active metabolites, especially those are acetylated by NAT (*N*-acetyltransferase), bind to DNA during the initiation of mutagenesis. On the other hand, metabolites are excreted after forming conjugates catalyzed by phase II enzymes in the detoxification pathway. In this study we set out to determine whether anthraquinone compounds affected MeIQx–DNA-adduct formation and the activity of phases I and II enzymes in mice. DNA-adduct formation represents one mutational event that can initiate carcinogenesis. MeIQx–DNA adducts were detected in rats [20] and Drosophila [3] following the feeding of MeIQx. MeIQx is converted to active metabolites by the microsomal enzyme CYP 1A, and is known to conjugate with glucuronic or sulfuric acid in a process catalyzed by phase II enzymes such as UGT [21–23]. In an effort to investigate the effect of MeIQx on enzyme activity *in vivo*, we measured the activities of ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin-*O*-demethylase (MROD) as markers for phase I CYP 1A, and UDP–glucuronyltransferase (UGT) and glutathione *S*-transferase (GST) activities as markers for phase II enzymes in liver dissected from mice following administration of anthraquinone compounds. MeIQx–DNA-adduct formation was suppressed in tissues following feeding with purpurin, although the extent of suppression varied in different organs and was dependent on the treatment regimen. Treatment with purpurin enhanced the activities of both phase I enzymes in the liver, while the activities of both phase II enzymes gradually increased in liver fractions and were maintained even after removal of purpurin from the diet. It is expected that other tissues were similarly affected by purpurin. These results suggest that purpurin administration reduces genotoxicity by enhancing MeIQx metabolism.

2. Materials and methods

2.1. Reagents

Purpurin [81-54-9] was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). GSH [70-18-8], CDNB [97-00-7], alizarin [72-48-0] and MeIQx [77094-11-2] were from Wako Pure Chemicals (Osaka, Japan). Resorufin [34994-50-8], methoxyresorufin [5725-89-3], ethoxyresorufin [5725-91-7], UDPGA [78132-48-6] and RNase A were from Sigma Chemical (St. Louis, MO). Proteinase K was from Merck (Darmstadt, Germany), 4-nitrophenol [100-02-7] was from ICN Biomedicals (Costa Mesa, CA) and apyrase and nuclease P1 [54576-84-0] were from Yamasa Co. (Noda, Japan). T4 polynucleotide kinase was from Takara (Ohtsu, Japan), and RNase T1, micrococcal endonuclease and phosphodiesterases I and II were from Worthington Biochemical Corp (Lakewood, NJ). All chemicals used in this study were of commercially available pure grade.

2.2. Animal treatment

Male C57BL/6N mice (5 weeks old) were obtained from CLEA Japan Inc. (Tokyo, Japan). Animals were maintained according to guidelines proposed by the Animal and Care Committee at Okayama University. Anthraquinone-treated groups consisted of six mice, while non-treated groups consisted of two mice. One or two mice were housed in each cage with access to a daily diet (CE-2, CLEA Japan Inc., Tokyo, Japan, 6 g/(day mouse)) and water, *ad libitum*. MeIQx (0.3 mg/(day mouse)) and/or anthraquinones (30 mg/(day mouse)) were used for the treatment. The chemical structures of purpurin and alizarin are shown in Fig. 1. Mice were treated with MeIQx and anthraquinones (purpurin or alizarin) according to the regimen shown in Fig. 2. MeIQx was given to mice for 3 days from day 4 to day 6 in every group except the negative control. As shown in Fig. 2(A), mice of the pre-treated group (Group 3) were treated with anthraquinone from days 1 to 3, while the pre-treated and simultaneously treated group (Group 4) was treated with anthraquione from day 1 until the end of the feeding experiment (day 6). Mice were sacrificed by cervical dislocation at 24 h following the last feed. Organs (liver, lung and kidney) were dissected, immediately frozen, and subsequently stored at −80 °C until use. Whole kidney, lung and half of the liver were used for the detection of DNA adducts. The remaining half of the liver was used for measuring enzyme activities. In order to determine purpurine-induced changes in enzyme activities, mice were treated according to the shown in Fig. 2(B). Three mice were sacrificed at the day shown in Fig. 2(B) and liver enzyme activities were measured.

2.3. DNA isolation from tissue and quantification of DNA adducts

DNA was isolated from mouse lung, kidney and liver using phenol–chloroform extraction and ethanol precipitation. The

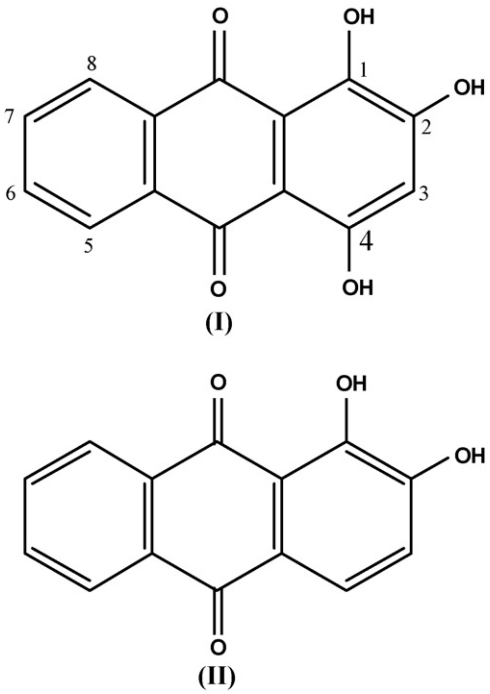

Fig. 1. Structures of pigments used in this study: purpurin (I) and alizarin (II).

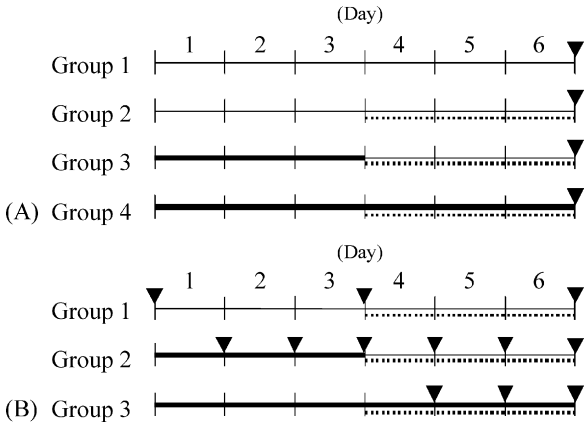

Fig. 2. Administration regimen of MeIQx and anthraquinones. Each mouse was fed a daily diet of 6 g. A fine line shows treatment with a normal diet and a bold line shows treatment with a diet containing purpurin or alizarin in experiment A and purpurin in experiment B at 30 mg/(day mice). A dotted line shows treatment with a diet containing MeIQx (0.3 mg/(day mice)). (A) Group 1 consisted of two mice and all other groups consisted of six mice. All mice were sacrificed at the point in the experiment shown by the closed triangle. (B) Group 1 and Group 3 consisted of nine mice and Group 2 consisted of 18 mice. Three mice were sacrificed at each point shown by the closed triangles.

amount of DNA adducts present in the tissue samples was determined using the nuclease P1 ^{32}P-postlabeling method as previously described [3,20]. Statistical analysis was performed using the Student's *t*-test.

2.4. Measurement of enzyme activity

Dissected liver was homogenized in 3 volumes of ice-cold 0.15 M KCl and centrifuged at 9000 × *g* for 20 min at 4 °C (Hitachi CR 20F, Hitachi, Japan). This S9 fraction was immediately frozen in liquid nitrogen and subsequently kept at −80 °C until use. Protein concentration was determined by the method of Bradford [24]. CYP 1A activity was determined fluorometrically by measuring the generation of resorufin from ethoxy- and methoxyresorufin as previously described [19,25] using a Hitachi F3010 Fluorescence Spectrophotometer (Hitachi, Japan). UGT activity was determined by measuring the reduction in absorbance of *p*-nitrophenol at 405 nm as described by Jakoby [26]. GST activity was determined by the method of Habig et al. [27], using CDNB as a substrate, with slight modification in that reaction mixtures were diluted 10-fold with 0.1 M potassium phosphate buffer (pH 6.5) prior to absorbance measurements. Statistical analysis was performed using the Student's *t*-test.

3. Results

3.1. Effect of pre-treatment or prolonged treatment with purpurin on MeIQx–DNA-adduct formation

Mice were fed with MeIQx and/or purpurin according to the regimen shown in Fig. 2(A). A comparison of the bodyweight of mice in each group indicated no marked differences between the groups, regardless of the treatment regimen employed. The amount of MeIQx–DNA adducts in DNA isolated from each tissue was determined using ^{32}P-postlabeling methods. MeIQx–DNA adducts were present in all tissues of mice fed with MeIQx, but not in untreated mouse tissues. Our results showed that the amount of MeIQx–DNA adducts formed in mice fed with purpurin was reduced, with levels ranging from marginally detectable to statistically significant compared with control mice (Table 1). In the case of pre-treatment with purpurin (Group 3), adduct formation was marginally suppressed in the liver, but significantly suppressed in the lung and kidney ($P = 0.045$ and 0.005, respectively). In contrast, prolonged treatment with purpurin had little effect on adduct formation. Pre-treatment with alizarin resulted in a marginal reduction in the amount of adducts present in the lung and kidney (Table 1).

Table 1
Effect of pre-treatment or prolonged treatment with anthraquinone pigments on MeIQx–DNA-adduct formation

Anthraquinone treatment	DNA-adduct formation levels (no. of adducts/10^8 nucleotides)		
	Liver	Lung	Kidney
Group 2	153 ± 65	2.43 ± 1.58	20.3 ± 8.8
Purpurin			
Group 3	100 ± 82	0.84 ± 0.63*	6.8 ± 2.9**
Group 4	144 ± 55	2.10 ± 1.83	13.6 ± 6.3
Alizarin			
Group 3	170 ± 83	1.81 ± 2.29	12.8 ± 9.3
Group 4	136 ± 54	2.62 ± 1.57	23.2 ± 11.4

Mice were treated according to the regimen shown in Fig. 2(A). Each group consisted of six mice and all groups were fed with a diet containing MeIQx (0.3 mg/(day mouse)) during days 4–6. Group 3 mice were administered with anthraquinones (30 mg/(day mouse)) during days 1–3, while Group 4 mice were administered during days 1–6. Results are shown as mean ± S.D. on six mice. No adducts were detected in control mice fed on a diet lacking MeIQx. *$P < 0.05$, **$P < 0.01$. The significance of the differences was evaluated using the Student's *t*-test.

3.2. Effect of anthraquinones on phases I and II enzyme activity

Xenobiotics such as carcinogens are converted to active forms by phase I metabolic enzymes in microsomes, and then form conjugates, through the action of phase II enzymes, prior to being excreted. We set out to determine whether the anthraquinone compounds used in this study could modulate the activity of phases I and II enzymes. Enzyme activity was determined in liver S9 fractions prepared from mice sacrificed at the end of the feeding experiment. MROD and EROD were used as markers for the phase I enzyme CYP 1A, while UGT and GST were used as markers for phase II enzymes. The results are shown in Table 2. When purpurin treatment ceased prior to the administration of MeIQx (Group 3 in Fig. 2(A)), MROD and EROD activities decreased. On the other hand, MROD and EROD activities increased 5.5- and 7.4-fold, respectively, in liver fractions prepared from mice continuously fed purpurin for 6 days (Group 4 in Fig. 2(A)). UGT activity in Group 4 mice was two-fold higher than that in untreated mice. However, in the case of pre-treatment with purpurin, UGT activity remained unchanged (Group 3). GST activity increased in both Groups 3 and 4. In the case of treatment with alizarin, liver UGT activity increased while MROD, EROD and GST activities remained unchanged. We confirmed that treatment with MeIQx alone was insufficient to modulate the aforementioned enzyme activities (data not shown).

3.3. Treatment-dependent change in enzyme activity induced by purpurin

In an effort to elucidate the change in enzyme activity following purpurin treatment, each enzyme activity was measured every day for 6 days according to the regimen shown in Fig. 2(B). All animals were fed a diet lacking MeIQx for 3 days prior to being administered

Table 2
Effect of pre-treatment or prolonged treatment with 0.5% anthraquinone pigments on induction of phases I and II enzyme activities in the liver

Anthraquinone treatment	Phase I enzyme activity		Phase II enzyme activity	
	MROD (pmol/(min mg protein))	EROD (pmol/(min mg protein))	UGT (μmol/(min mg protein))	GST (mmol/(min mg protein))
Group 2	46.5 ± 0.7	10.4 ± 0.5	5.41 ± 0.31	1.56 ± 0.11
Purpurin				
Group 3	31.1 ± 0.4**	8.7 ± 0.1**	5.43 ± 1.10	2.28 ± 0.09**
Group 4	254.1 ± 31.4**	77.4 ± 18.5**	10.21 ± 1.41**	2.44 ± 0.25**
Alizarin				
Group 3	55.8 ± 17.7	14.2 ± 3.8	6.88 ± 0.52*	1.38 ± 0.24
Group 4	37.1 ± 7.3	12.6 ± 2.9	10.9 ± 2.37*	1.83 ± 0.61

Mice were treated according to the regimen shown in Fig. 2(A), in the same way as described in the note of Table 1. Livers were obtained from three out of six mice in each group. Results are shown as mean ± S.D. of three mice. *$P < 0.05$, **$P < 0.01$. The significance of the differences was evaluated using the Student's *t*-test.

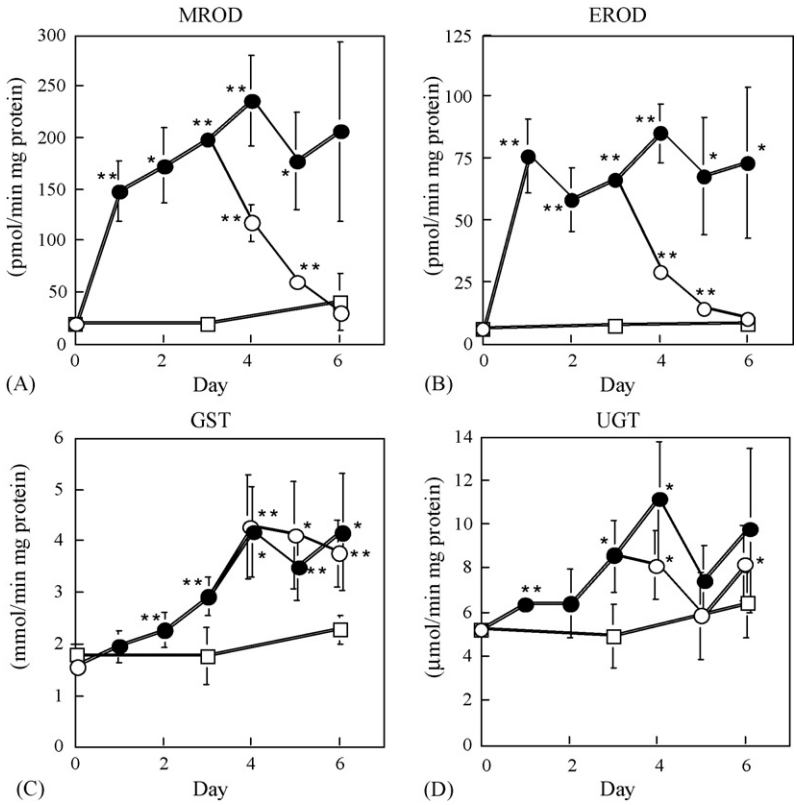

Fig. 3. The activity of (A) MROD, (B) EROD, (C) GST and (D) UGT in liver S9 fractions of mice treated with purpurin and MeIQx according to Fig. 2(B). The activity is presented as the average results derived from three mice with standard deviation. Open squares show the activity in tissue of mice fed a diet containing no purpurin, Group 1 in Fig. 2(B). Open circles indicate the activity in tissue of mice pre-treated with purpurin, Group 2, and closed circles show the activity in tissue of mice subjected to prolonged treatment with purpurin, Group 3. $^{*}P<0.05$, $^{**}P<0.01$. The significance of the differences compared with the activity at day 0 was evaluated using the Student's *t*-test.

MeIQx for 3 days. As shown in Fig. 3(A) and (B), MROD and EROD activities increased to significant levels after the first day in mice fed a diet containing purpurin. When mice were continuously fed a diet containing purpurin, MROD and EROD activities were maintained at a level 10-fold higher than basal levels. However, enzyme activity declined rapidly when purpurin administration ceased, and 3 days later at the end of the experiment, the requisite activity dropped to basal levels. On the other hand, the activity of phase II enzymes, and especially that of GST, gradually increased following purpurin administration, and this activity was maintained at a high level until the end of the experiment, even when purpurin was removed from the diet (Fig. 3(C) and (D)).

4. Discussion

We determined the effect of anthraquinone compounds on MeIQx–DNA-adduct formation and metabolic enzyme activity involved in MeIQx metabolism. Given that Ohgaki et al. demonstrated that the liver represents the main target of MeIQx in CDF1 mice [28], and that the liver is known to be the main organ where metabolism of xenobiotics occurs, we examined the effect of anthraquinones on (i) DNA-adduct formation in mouse liver, kidney and lung and (ii) metabolic enzyme activity in liver. Results showed that purpurin significantly suppressed the formation of MeIQx–DNA adducts in kidney and lung, and marginally in liver, and increased the activity of phase I CYP 1A and phase II enzymes in the liver. Pre-treatment with purpurin suppressed the formation of DNA adducts more effectively than prolonged treatment. In the case of pre-treatment with purpurin, the enhanced phase I enzyme activity decreased when MeIQx was incorporated. Here, the MeIQx active form might have been excreted following formation of the conjugate due to phase II enzyme activity being maintained at a high level. On the other hand, in the case of prolonged treatment with purpurin, as the activity of CYP 1A enzymes was maintained at

a higher level than the activity of phase II enzymes, the suppression of adduct formation appeared less effective, as active intermediates were constantly being produced by phase I enzymes. These results suggest that purpurin could accelerate the metabolism of MeIQx, thus resulting in the suppression of genotoxicity. In the *in vivo* study, alizarin displayed a reduced suppressive effect compared with purpurin in terms of the formation of DNA-adduct, due perhaps to decreased modulation of enzyme activity. *N*-Hydroxy MeIQx, formed by the action of CYP 1A, was shown to conjugate with glucuronic or sulfuric acid [21]. Although the DNA binding ability of *N*-acetoxy-PhIP was strongly inhibited in the presence of GST and GSH, the activity of *N*-acetoxy-MeIQx was not inhibited *in vitro* [29]. Boobis et al. reported that detoxification of *N*-hydroxy PhIP esters was catalyzed by GST [30]. Our results suggest that in mouse liver, GST is more effectively involved in detoxification of the MeIQx active intermediate than UGT. As suppression of adduct formation is more effective in mouse kidney and lung than in liver, a similar or more effective modulation of enzyme activity seems to be associated with the kidney and lung. The activation of phase II enzymes might be greater than the activation of phase I enzymes. We previously reported that purpurin inhibited the activity of CYP 1A and CYP 1B and was followed by suppression of mutation in a bacterial system [19]. There is the possibility that purpurin also directly inhibits the activity of CYPs *in vivo*. It is well known that NAT2 plays an important role in the metabolism of MeIQx [22,23]. Investigations concerning the effect of purpurin on NAT activity will need to be performed. Large amounts of purpurin increase the risk of neoplasm in bladder [31]. Recently, Mijatovic et al. reported that aloe-emodin, a plant anthraquinone, reduces the anticancer activity of cisplatin [32]. The inhibitory activity of anthraquinones against DNA damage or enzyme activity is dependent on anthraquinone structure, and in particular the site pertaining to hydroxy group substitution [13,17]. Compounds may be selected that enhance the activity of phase II detoxification enzymes, but have no effect on the activity of phase I enzymes. Further research concerning structure-activity relationships, including alternate strategies of administration, should be useful in ascertaining the potential use of purpurin in the area of chemoprevention.

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