Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 1895–1904

BIOORGANIC & MEDICINAL CHEMISTRY

Mechanism of Biochemical Action of Substituted 4-Methylbenzopyran-2-ones. Part II: Mechanism-based Inhibition of Rat Liver Microsome-mediated Aflatoxin B_1–DNA Binding by the Candidate Antimutagen 7,8-Diacetoxy-4-methylcoumarin

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Received 25 March 1998; accepted 3 June 1998

Abstract—7,8-Diacetoxy-4-methylcoumarin (DAMC), with no prerequisite for oxidative biotransformation has been reported to produce suicide inactivation of microsomal cytochrome P-450-catalysed formation of aflatoxin B_1-8,9-oxide that binds to DNA. Parenteral administration of DAMC to rats caused significant inhibition of AFB_1 binding to hepatic DNA in vivo as well as AFB_1-induced micronuclei formation in bone marrow cells. These results highlight the antimutagenic potential of DAMC.

Introduction

Aflatoxin B_1 (AFB_1) is considered to be an important aetiological factor for causing liver cancer in human beings.[1] AFB_1-8,9 oxide (AFB_1 epoxide), the ultimate carcinogen avidly interacts with cellular DNA (Fig. 1) resulting in the formation of AFB_1-N^7-guanine adduct.[2,3] AFB_1 epoxidation is catalysed by specific isoform of cytochrome P-450 (P-450) in hepatic microsomes.[4] Chemoprevention of carcinogenesis due to AFB_1 is being vigorously followed[5,6], P-450 enzymes are often thought as targets for chemoprevention against cancer induced by chemicals.[7] Minor dietary constituents, such as indoles, isothiocyanates, allium organosulphides, polyphenols, etc. have attracted considerable attention as prospective cancer preventive agents whose effects on phase I and/or phase II enzymes of detoxification have been intensely pursued.[8–12] Coumarins form an important class of naturally occurring dietary polyphenols and their role as anticarcinogens has received little attention. Substituted 4-methylcoumarins are an important class of natural products, some of which possess choleretic,[13] analgesic,[14] antispermatogenic[15] and diuretic properties.[16] As a part of our study on the chemistry and biological effects of 4-methylcoumarin derivatives (benzopyran-2-ones), we had earlier demonstrated the inhibitory effect of several diacetoxy 4-methylcoumarins on rat liver microsome-mediated AFB_1–DNA binding in vitro.[17] In this report, we have investigated the mechanism of inhibition of liver microsome-catalysed AFB_1–DNA binding by 7,8-diacetoxy-4-methylcoumarin (DAMC) and its potential as an antimutagen.

Materials and Methods

Chemicals

[3*H*] AFB_1-(G) was obtained from Moravek Biochemicals (Brea, CA, USA). Calf thymus DNA, AFB_1,

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0968-0896/98/$—see front matter
PII: S0968-0896(98)00111-4

1-chloro-2,4-dinitrobenzene (CDNB), ethoxyresorufin, pentoxyresorufin and NADPH were the products of Sigma Chemical Co. (St. Louis, MO, USA). *p*-Hydroxymercuri benzoate (PHMB) was purchased from E. Merck (Germany). 7,8-Dihydroxy-4-methylcoumarin (DHMC) was synthesised in our laboratory by the well-known Pechmann condensation of resorcinol with ethyl acetoacetate, its diacetoxy derivative (DAMC) was prepared by the acetylation of DHMC with acetic anhydride/ pyridine. DHMC was characterised from its mp 234 °C (lit.[18] mp 235 °C). The structure of DAMC was confirmed from various spectral data reported by us earlier.[19]

Animals

Male albino rats of wistar strain weighing around 190–200 g, fed on rat chow supplied by Hindustan Lever Ltd, Mumbai (India) were used.

Preparation of liver microsomes

Male rats were fasted overnight and sacrificed by exsanguination, liver excised and microsomes prepared as described earlier.[20]

Preparation of phenobarbital (PB) treated liver microsomes

Male rats were administered PB sodium salt ip (100 mg/kg) for three days, after which the animals were sacrificed and microsomes prepared as mentioned above.

Liver microsome-catalysed AFB_1-DNA binding assay

The incubation mixture for the assay of microsome-mediated AFB_1-DNA binding consisted of 100 mM phosphate buffer (pH 7.4), 2 mM NADPH, [3*H*] AFB_1 (250 μCi/μM) added in 0.02 mL DMSO, liver microsomes equivalent to 1 mg protein, 0.1 mg calf thymus DNA and with or without 25–100 μM inhibitor DAMC added in 0.02 mL DMSO and water to a total volume of 1 mL. Duplicate samples were incubated at 37 °C for 30 min. After incubation, 2 mL of extraction mixture (phenol/chloroform/isoamyl alcohol, 50:50:1, v/v/v) was added along with 0.9 mg of calf thymus DNA as a carrier to each tube. DNA was isolated by the procedure of Wang and Cerutti[21] the recovery of DNA was 50–70%. Isolated DNA was dissolved in 0.1 N NaCl, one aliquot was added to a vial containing 8 mL of Bray's Scintillation fluid for determination of radioactivity using a Beckman Liquid Scintillation Counter (Model LS 6000) and 0.2 mL was used for measurement of DNA.[22] Results of [3*H*] AFB_1 binding to DNA were corrected for DNA recovery and expressed as pmol of [3*H*] AFB_1 bound/mg DNA/30 min.

Kinetics of time dependent inhibition of liver microsome-catalysed AFB_1–DNA binding by DAMC

Rat liver microsomes (1 mg protein) were mixed with a fixed concentration of DAMC (30 to 90 μM added in 0.02 mL of DMSO) in 100 mM phosphate buffer (pH 7.4), with or without 2 mM NADPH and water to make total volume 0.8 mL. The contents (scaled up as per requirement) were preincubated at 37 °C in a shaking water bath. The aliquots (0.5 mL portion) were removed periodically into another set of tubes containing [3*H*] AFB_1 (250 μCi/μM) in 0.02 mL DMSO, 0.1 mg calf thymus DNA, 2 mM NADPH to a final volume of 1 mL and incubated for 30 min at 37 °C. At the end of incubation, the extraction mixture and carrier DNA were added as described above. Isolation of DNA and quantitation of [3*H*] AFB_1 binding was carried out as described earlier. In experiments where PHMB was used, the mercurial compound was included in the preincubation with microsomes at a concentration of 10^{-2} M. Rat liver microsome (1 mg protein) preincubated without DAMC and assayed for AFB_1–DNA binding as described above served as the control (taken as 100%). The effect of DAMC on preincubation with microsomes mediating AFB_1–DNA binding as percent of control was plotted against the time of preincubation.

Kinetics of time dependent inhibition of liver microsome catalysed ethoxyresorufin *O*-deethylase (EROD)/pentoxyresorufin *O*-dealkylase (PROD) by DAMC

(a) Assay of EROD/PROD was carried out by the method of Lubet et al.[23] The reaction mixture consisted of 0.05 M Tris–HCl buffer (pH 7.5), 0.025 M $MgCl_2$, 10 μM ethoxyresorufin/pentoxyresorufin and 1 mg of enzyme. The reaction was initiated by the addition of 125 μM NADPH and the rate of dealkylation of resorufin was followed by measuring the flourescence using excitation $\lambda = 522$ nm and emission $\lambda = 586$ nm.

(b) Time dependent inhibition. Liver microsomes were preincubated with DAMC as described earlier. The reaction mixture for the assay of EROD/PROD was added at the end of preincubation and the rate of dealkylation of resorufin was followed as described above.

Preparation of DAMC treated liver microsomes for assay of [3*H*] AFB_1–DNA binding in vitro

Rats were injected DAMC ip (300 mg/kg body weight) in 0.2 mL DMSO followed 1 h later by administration of another dose of DAMC. The animals were sacrificed 2 h after the second injection, livers excised, homogenised and microsomes prepared.[21] Microsomes thus prepared

were used to assay [3*H*]AFB_1 binding to calf thymus DNA as described earlier.

Effect of DAMC administration on rat hepatic AFB_1–DNA binding in vivo

The first group of rats was injected ip 0.2 mL DMSO, followed 1 h later by the administration of 0.2 mL DMSO and [3*H*] AFB_1 (40 μg AFB_1 containing 15 μCi [3*H*] AFB_1/100 g body weight). The second group of rats was administered ip 300 mg/kg [b.w.] of DAMC in 0.2 mL DMSO. One hour later, the animals received another dose of DAMC ip and [3*H*] AFB_1, as mentioned earlier. A third group of rats was injected ip 150 mg/kg/b.w. of DAMC in 0.2 mL DMSO followed 1 h later by the administration of another dose of DAMC and [3*H*] AFB_1 as mentioned above. The animals were sacrificed 2 h after AFB_1 injection by exsanguination. Livers were excised and placed in ice-cold 0.25 M sucrose containing 2 mM $CaCl_2$ and 10 mM Tris–HCl buffer (pH 7.5). Liver was homogenized in the same medium and crude nuclear pellet was obtained by centrifugation of the homogenate at 600 *g* in a Sorval Refrigerated Centrifuge (Model RC-5). Isolation of DNA from the nuclear pellet and quantitation of AFB_1–DNA binding were as described under microsome-mediated AFB_1–DNA binding assay.

Hepatic microsome-mediated transformation of DAMC

(a) *Spectral studies*: the reaction mixture consisted of 100 mM phosphate buffer (pH 7.4), liver microsomes (1 mg protein) and 100 μM DAMC/DHMC in 0.02 mL DMSO and with or without 2 mM NADPH incubated for 30 min at 37 °C. After incubation, the contents of the tubes were transferred to a spectrophotometer cuvette and the spectrum was scanned against appropriate reagent blank using Beckman spectrophotometer (Model DU64).

(b) *Reaction product isolation and characterization*: the reaction mixture contained 1 g DAMC in 15 mL DMSO, 100 mM phosphate buffer (pH 7.4) and microsomes in a total volume of 50 mL, incubated at 37 °C for 30 min. The contents of the reaction mixture were centrifuged, the clear supernatent was lyophilised. The residue was dissolved in methanol and concentrated on a boiling water bath yielding yellow coloured solid mass. This was dissolved in minimum volume of methanol, made into a slurry with silica gel (3 g) and loaded on to a column of silica gel. The methanolic eluate (1 L) was concentrated on a water bath yielding a white coloured compound (yield 70%), which was dried in a desiccator containing $CaC1_2$ and P_2O_5. The compound was identified as 7,8-dihydroxy-4-methylcoumarin (DHMC), mp 234 °C (lit.[18] mp 235 °C). The UV, IR, ^1H NMR and mass spectra of the compound were in agreement with the structure and those reported for this compound available in our laboratories.

Effect of DAMC on AFB_1-induced micronuclei in bone marrow cells

The first group of rats received 0.4 mL of DMSO alone in two equal doses with an interval of 1 h, the second group of rats was injected ip 0.2 mL DMSO followed 1 h later by 0.2 mL DMSO and AFB_1 (8 mg/kg/b.w. in 0.1 mL of DMSO), the third set of rats was injected DAMC ip (300 mg/kg b.w.) in 0.2 mL DMSO followed by 1 h later by another dose of DAMC and AFB_1 as mentioned earlier, fourth group of rats received DAMC (300 mg/kg/b.w.) followed 1 h later by another dose of DAMC (as mentioned earlier). All the animals were sacrificed 26 h after the last injection, the femur bone isolated and flushed with 2 mL of Hank's balanced salt solution (HBSS). The method of Schmid[24] was followed for preparation of smear and staining. Accordingly, cells in 0.2 mL HBSS were treated with acetic acid/methanol (1:3, v/v) and centrifuged. Cell pellet was suspended in 0.2 mL of acetic acid/methanol (1:3, v/v) and a drop of this suspension was placed on a chilled wet slide to obtain a uniform smear. The smears were air-dried and stained with hematoxylin and eosin (H and E).

The slides were viewed under oil immersion lens of the light microscope (Nikon).

Long-term feeding of DAMC supplemented diet

Rat chow was powdered finely and intimately mixed with DAMC (1% w/w). Requisite quantity of water was added to the mixture, small pellets were made and fed to the rats. The animals were sacrificed 15 days after the feeding of DAMC supplemented diet and liver cytosol prepared. Hepatic cytosol was also prepared out of rats not receiving DAMC.

Glutathione (GSH) *S*-transferase

The method of Habig et al.[25] using CDNB as the substrate was used for the enzyme assay.

Protein assay

Protein was determined by the method of Lowry et al.[26]

Results

Time dependent inhibition of liver microsome-catalysed AFB_1–DNA binding in vitro

The effect of preincubation of rat liver microsomes with DAMC leading to inactivation of P-450 mediated AFB_1 binding to exogenous DNA is illustrated in Figure 2. Microsomes were separately incubated with different concentrations of the inhibitor to varying intervals from 0 to 60 min, followed by the addition of cofactor (NADPH) and the substrate [3*H*] AFB_1 to initiate the epoxidation of AFB_1 (Fig. 1), the product (AFB_1 epoxide) being trapped as the adduct of DNA. It is evident from the kinetic plot (Fig. 2) that the time needed for enzyme inactivation (leading to DNA adduction) is inversely proportional to the concentration of the inhibitor. AFB_1–DNA binding was inhibited up to 90% by 90 μM concentration of DAMC at 45 min of preincubation and nearing total inhibition at 60 min (Fig. 2).

Effect of PHMB on the inhibition of AFB_1–DNA binding by DAMC

PHMB was included in the preincubation of rat liver microsomes with DAMC. It is clear from the data (Fig. 2) that the addition of PHMB results in the rapid reversal of the drastic inhibition of AFB_1–DNA binding caused by DAMC.

Role of the oxidative metabolism of DAMC in the inhibition of AFB_1–DNA binding catalysed by liver microsomes

Rat liver microsomes were preincubated with DAMC in the presence or absence of NADPH. The results

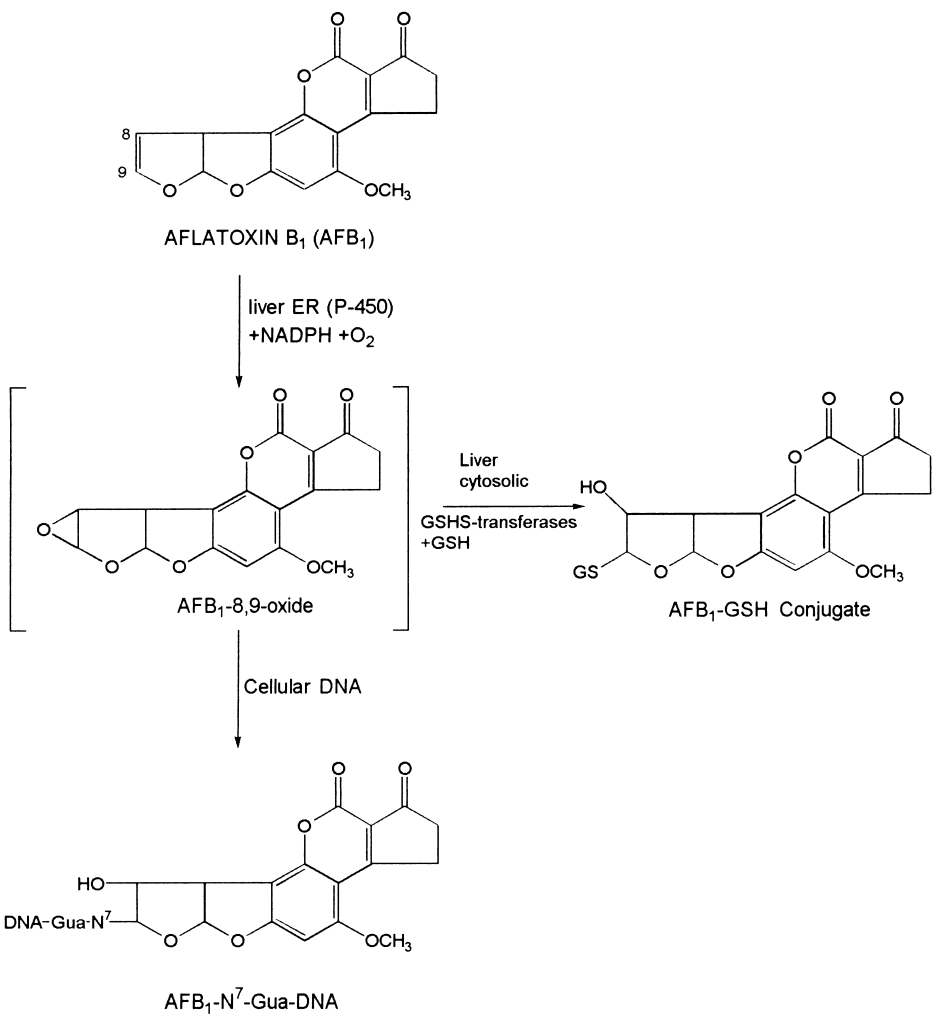

Figure 1. Metabolic activation of aflatoxin B_1.

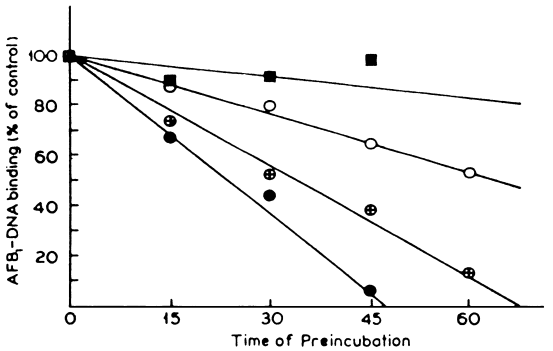

Figure 2. Kinetics of inhibition of AFB_1-DNA binding upon preincubation of rat liver microsomes with DAMC. Details are described in the text. Concentration during preincubation of DAMC: ●, 90 μM; ⊗, 60 μM; ○, 30 μM; PHMB: ■, 10^{-2} M.

tabulated in Table 1 indicate that addition of NADPH to the preincubation mixture makes no significant difference in the extent of inhibition of liver microsome catalysed AFB_1–DNA binding. PB treated liver microsomes were preincubated with microsomes in order to compare with normal liver microsomes in effecting the inhibition of AFB_1–DNA binding. PB-treated rat liver microsomes upon preincubation with DAMC produced no significant change in causing inhibition of AFB_1–DNA binding over the normal liver microsomes (Table 1).

Role of liver microsomal deacetylase in the biotransformation of DAMC

Change in UV spectra of DAMC upon incubation with rat liver microsomes was compared with that of DHMC. DAMC and DHMC have distinct λ_{max} values. But upon incubation with rat liver microsomes, both DAMC and DHMC exhibit identical λ_{max} values (Table 2). It is clear from the data (Fig. 3) that DAMC is mostly converted to DHMC (prominent peak at 324 nm). When NADPH is included in the preincubation mixture, both DAMC and DHMC are converted to a common single metabolite, exhibiting λ_{max} around 380 nm.

Table 2. Incubation Of DAMC/DHMC with rat liver microsomes: UV spectral details

Compd incubated	I Compd in buffer λ_{max} (nm)	II Compd + microsomes λ_{max}(nm)	III Compd microsomes + NADPH λ_{max}(nm)
DAMC (A)	280	324, 342	380
DHMC (B)	324	324, 342	380

DAMC/DHMC was added in 0.1 mL DMSO to 3 mL of 100 mM phosphate buffer (pH 7.4) and incubated at 37 °C for 30 min. The contents were transferred to a spectrophotometer cuvette and UV spectra recorded. Similarly DAMC/DHMC was added (in 0.1 mL DMSO) to microsomes in the same buffer containing NADPH (where indicated) and UV spectra recorded as described earlier, λ_{max} values of major peaks are tabulated (Fig. 3).

The product of hydrolysis of DAMC catalysed by rat liver microsomes was fully characterized as 7,8-dihydroxy-4-methylcoumarin (DHMC), mp 234 °C (lit.[18] mp 235 °C). The spectral data (IR, UV, ^{1}H NMR and mass) of the product DHMC were identical to those of an authentic synthetic sample of DHMC available in our laboratories.

Effect of DAMC/DHMC administration on rat liver microsome mediated AFB_1–DNA binding

Rats were administrated separately ip DAMC/DHMC (2 mmoles/kg) and sacrificed 2 h later. Liver microsomes were assayed for AFB_1–DNA binding. Results shown in Table 3 illustrate that the DAMC-treated liver microsomes possessed remarkably reduced activity to catalyse binding of AFB_1 to calf thymus DNA as compared to control rat liver microsomes ($p < 0.001$). DHMC administration to rats had no effect on the activity of hepatic microsomes to modulate AFB_1–DNA binding (Table 3).

Modulation of hepatic AFB_1–DNA binding in vivo by DAMC

Rats were separately administrated two doses of DAMC in order to study the effect on [3*H*] AFB_1 binding to

Table 1. Role of oxidative metabolism of DAMC in the inhibition of rat liver microsome mediated AFB_1-DNA binding

Experiment no.	Preincubation of DAMC with	%AFB_1–DNA binding remaining after:		
		15 min	30 min	45 min
1	Normal liver microsomes with NADPH	66.70 ± 2.88	46.90 ± 4.00	15.80 ± 1.5
2	Normal liver microsomes without NADPH	72.30 ± 1.92*	58.80 ± 4.90*	16.20 ± 0.8*
3	PB-treated liver microsomes + NADPH	74.40 ± 6.12*	51.10 ± 6.88*	15.60 ± 1.3*

DAMC was preincubated with normal/PB-treated rat liver microsomes in the presence or absence of NADPH followed by the assay of AFB_1–DNA binding as described in the text. Values are mean ± SEM of three animals.
*Not significant compared to experiment no. 1.

liver DNA in vivo (Table 4). The results tabulated in Table 4 clearly demonstrate the inhibitory effect of DAMC. Accordingly, injection of low dose (300 mg/kg/b.w.) as well as high dose (600 mg/kg/b.w.) of DAMC resulted in approximately 19 and 34% inhibition of hepatic AFB_1-DNA binding in vivo, respectively (Table 4). Histopathalogical examination revealed no hepatotoxicity due to administration of DAMC (unpublished observations) described above.

The time-dependent DAMC-mediated inactivation of rat liver microsomal CYP1A and CYP2B families as demonstrated by EROD and PROD, respectively, is illustrated in Figure 4. It is clear from the kinetic plot (Fig. 4) that DAMC caused irreversible inhibition of

Table 3. Influence of DAMC/DHMC pretreatment on rat liver microsome mediated AFB_1–DNA binding in vitro

Treatment	AFB_1–DNA binding
	p mol/mg DNA/30 min
Control	193.2 ± 8.8
DAMC	107.8 ± 3.1*
DHMC	186.2 ± 5.0 N.S.

Male rats separately injected ip DAMC/DHMC (2 mmole/kg) and sacrificed after 2 h. Liver microsomes were assayed for AFB_1-DNA binding. Values are mean ± SEM of eight observations.

*$p < 0.001$ compared to control.

N.S: Not significant compared to control.

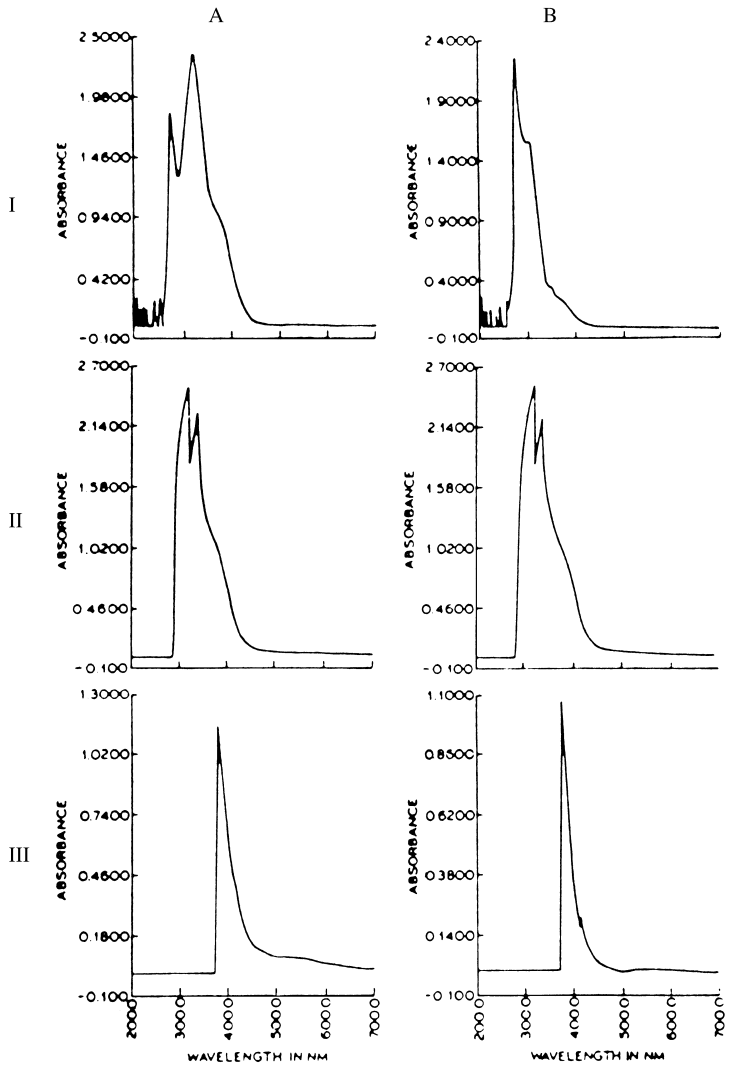

Figure 3. Rat liver microsomes in 100 mM phosphate buffer (pH 7.4), separately incubated with DAMC/DHMC (100 μM) and UV spectra recorded. The details are described in Table 2.

Table 4. Inhibitory action of DAMC pretreatment on rat hepatic AFB_1–DNA binding in vivo

Group	Treatment	Hepatic AFB_1–DNA binding p mol/mg DNA	Percent of control
1	Control	33.00 ± 1.40	—
2	DAMC (HD)	$22.00 \pm 1.05^{*}$	66.6 (33.4)
3	DAMC(LD)	$27.00 \pm 0.60^{**}$	81.8 (18.2)

The details of AFB_1–DNA binding assay is described under Materials and Methods. Values are mean ± SEM of four observations. HD: High dose (300 mg/kg/b.w. in two doses). LD: Low dose (150 mg/kg b.w. in two doses).
$^{*}p < 0.01$.
$^{**}p < 0.05$ as compared to control.
The numbers in parentheses denote the percent inhibition of AFB_1–DNA binding.

EROD and PROD in a dose-dependent manner. As observed in the case of inhibition of AFB_1–DNA binding, the inclusion of PHMB in the reaction mixture reversed the inhibition of EROD and PROD due to DAMC.

Modulation of AFB_1-induced micronuclei in bone marrow cells of rats administered DAMC

Experiments with intact rats (Table 5) demonstrated the inhibition of AFB_1-induced micronuclei in bone marrow cells by the administration of DAMC.

Table 5. Inhibition of AFB_1-induced micronuclei in bone marrow cells of rats administered DAMC

Group	Treatment	Micronuclei/1000 cells
1	Control	2.38 ± 0.18
2	AFB_1	$7.00 \pm 0.27^{*}$
3	AFB_1 + DAMC	$4.38 \pm 0.32^{**}$
4	DAMC	2.5 ± 0.28

The details of micronucleus assay is described under Materials and Methods. Values are mean ± SEM of eight observations.
$^{*}p < 0.001$ as compared to control.
$^{**}p < 0.01$ as compared to AFB_1.

Effect of feeding of rats with DAMC supplemented diet

The results (Table 6) demonstrate that GSH *S*-transferase activity of rat liver cytosol is significantly enhanced by long-term feeding of DAMC. The influence of addition of DAMC-fed rat liver cytosol to the microsome-mediated AFB_1–DNA binding was compared to that of control liver cytosol (Table 7). It is evident from the data that DAMC-fed rat liver cytosol caused significant reduction in AFB_1–DNA binding over the control cytosol.

Discussion

The P-450 linked mixed function oxidases play a key role in the oxidative metabolism of a wide variety of xenobiotics.[27] AFB_1, a potent hepatocarcinogen

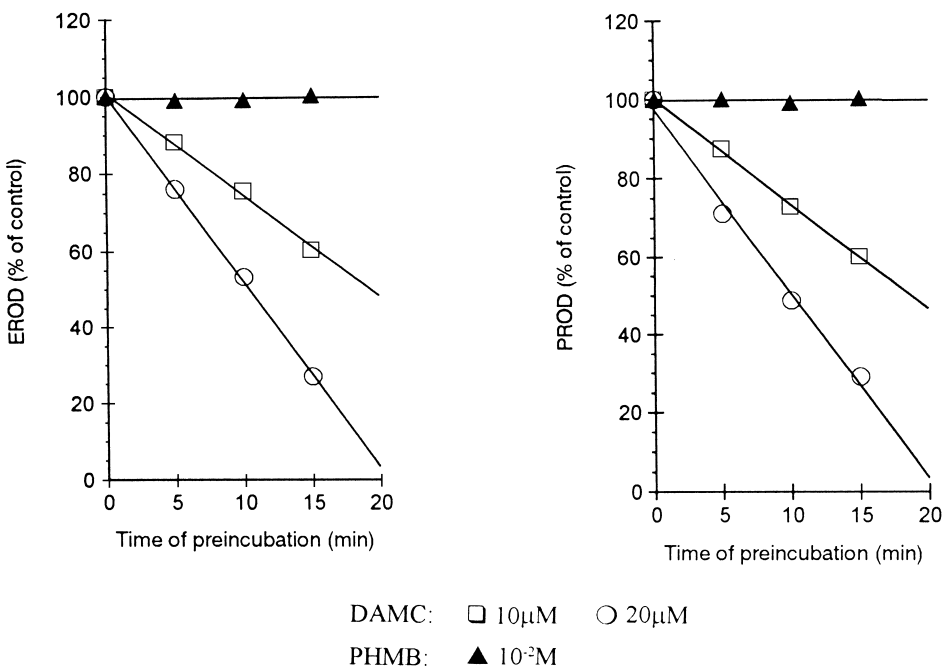

Figure 4. Kinetics of inhibition of EROD/PROD upon preincubation of rat liver microsomes with DAMC.

Table 6. Stimulation of liver cytosolic GSH *S*-transferase in DAMC-fed rats

Treatment	Liver weight / Body weight	GSH S-transferase / μmol CDNB conjugated/min/mg protein
Control	0.060 ± 0.008	2.85 ± 0.05
DAMC-fed	0.056 ± 0.001	4.30 ± 0.02*

Animals were fed 1% DAMC in diet for 15 days, sacrificed and liver cytosol prepared. Control animals did not receive DAMC. Cytosolic GSH *S*-transferase was assayed by the GSH conjugation of CDNB.
*$p < 0.01$ as compared to control. Values are mean ± SEM of four observations.

Table 7. Modulation of rat liver microsome mediated AFB_1–DNA binding in vitro by DAMC-fed rat liver cytosol

Group	Microsomes	AFB_1–DNA binding	
		Cytosol	pmol/mg DNA/30 min
1	+	—	186.6 ± 5.2
2	+	C	137.8 ± 2.3
3	+	DAMC	109.8 ± 2.0**

Male rats were fed 1% DAMC in the diet for 15 days, sacrificed and liver cytosol prepared (DAMC). Liver microsomes and cytosol (C) were also prepared from normal rats (receiving no DAMC). Values represent mean ± SEM of eight observations.
*$p < 0.001$ compared to Group 1.
**$p < 0.001$ compared to Group 2.

produced by *Aspergillus flavus*, is known to be a substrate for hepatic P-450 enzyme system resulting in the formation of several products (Fig. 1). AFB_1-8,9-epoxide upon interaction with cellular DNA yields 8,9-dihydro-8-(guan-7-yl)-9-hydroxy AFB_1.[2] Blocking of metabolic activation of AFB_1 (formation of AFB_1-8,9-epoxide) is thought to be helpful in the inhibition of AFB_1–DNA binding and thereby helpful in prevention of AFB_1-induced carcinogenesis.[27,28] Hence, the search for effective inhibitor(s) of AFB_1–DNA binding has been an attractive pursuit. In this context, our laboratory has focussed attention on the derivatives of 4-methylcoumarins. We had earlier demonstrated[17] the effectiveness of diacetoxy-4-methylcoumarins in forging the inhibition of liver microsome catalysed AFB_1–DNA binding in vitro and also hypothesised the possibility of mechanism-based inhibition of P-450. In this paper, the experimental results are reported to substantiate such a viewpoint. Time-dependant inactivation of liver microsomes catalysed-AFB_1–DNA binding was demonstrated by preincubating rat liver microsomes with DAMC, followed by the assay of AFB_1–DNA binding. The remaining microsomal activity (catalysis of AFB_1–DNA binding) was expressed as the function of time of preincubation (Fig. 2). The kinetic plot (Fig. 2) clearly highlights the time dependent inhibition of liver microsomal catalysed AFB_1–DNA binding characteristic of mechanism based inhibition.[29] The data clearly indicate that DAMC caused progressive loss of catalytic activity of rat liver microsomes with time, near total inactivation resulted in 60 min at 90 μM concentration of the inhibitor (Fig. 2). The action of DAMC described here is characteristic of suicide inhibitors, such as chloramphenicol,[30] secobarbital,[31] 21-chlorinated steroids,[32] dichloromethyl compounds[33] and *N*-alkylaminobenzotriazoles.[34] It is interesting to note that DAMC-caused inactivation of microsomal AFB_1–DNA binding is independent of NADPH (Table 1). Also, PB-treated rat liver microsomes upon preincubation with DAMC inhibited AFB_1–DNA binding (even in the presence or absence of NADPH) no higher than the untreated rat liver microsomes (Table 1). These findings clearly ruled out the role of oxidative metabolism of DAMC in effecting the mechanism-based inhibition of rat liver microsome catalysed AFB_1–DNA binding. Mechanism based inhibition of P-450-linked MFO by definition necessitates at least one catalytic P-450 cycle during or subsequent to the oxygen transfer step when the drug is activated to the inhibitory species.[35] Since DAMC without undergoing P-450 catalysed oxidation caused mechanism-based inhibition, we have described such an inhibition as pseudomechanism-based inhibition. Incubation of DAMC with rat liver microsomes resulted in the formation of DHMC (Fig. 3) as seen by the disappearance of DAMC peak at 280 nm with concomitant appearance of DHMC (Fig. 3) peak at 324 nm in the UV spectrum. The product of hydrolysis of DAMC by rat liver microsomes was confirmed to be DHMC from its mp and spectral data. These observations confirm that the hydrolysis of DAMC by rat liver microsomes is quite facile leading to the formation of DHMC and contributes to the rare incidence of the enzymatic deacetylation (Fig. 5) of a foreign compound (DAMC) catalysed by liver microsomes.[36] There exists the possibility of the action of liver microsomal transacetylase on DAMC leading to the transfer of acetyl groups to the active site lysine residue of apoprotein P-450 resulting in the inhibition of AFB_1 epoxidation (as measured by DNA adduct). Chloroamphenicol is known to inhibit P-450 linked MFO activities by such a mechanism.[30] Although we have not provided data for the action of DAMC similar to chloramphenicol, we believe that such a mechanism of action is quite possible. The role of liver microsomal transacetylase (involving thiol group at the active site) in the inhibitory action of DAMC (Fig. 5) is further substantiated by the action of the thiol blocking agent PHMB[37] whose addition to the preincubation system (Fig. 2) resulted in the reversal of inhibition of AFB_1–DNA binding caused by DAMC. Further,

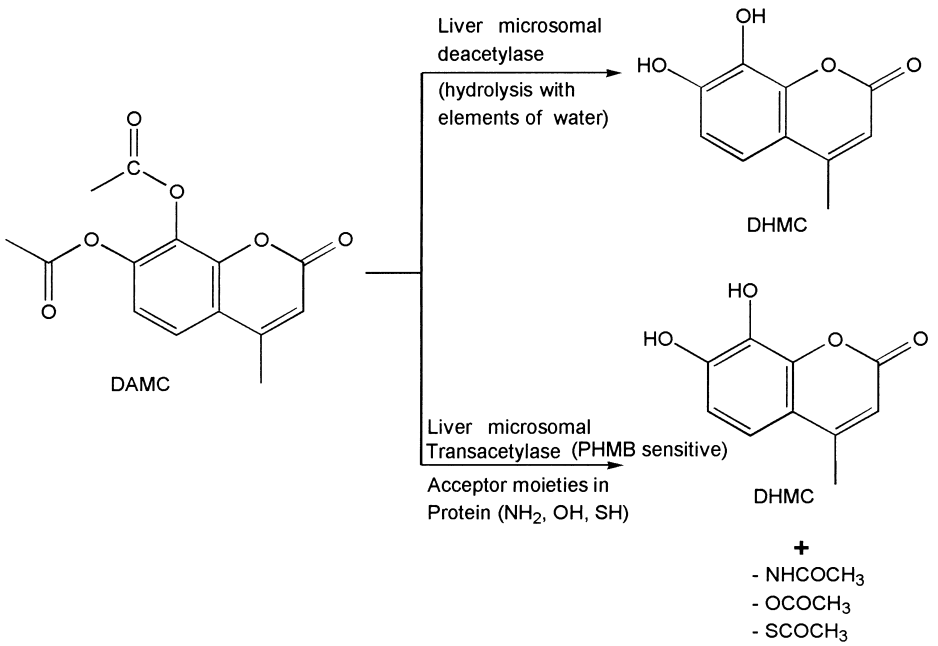

Figure 5. Liver microsome-catalysed deacetylation of DAMC.

DAMC injected rat liver microsomes significantly inhibited AFB_1–DNA binding in vitro (Table 3). These results clearly point out that DAMC causes irreversible inhibition. Once confirmed that DAMC is an effective suicide inhibitor of liver microsome catalysed AFB_1–DNA binding in vitro, an effort was made to examine the effect of DAMC on hepatic AFB_1–DNA binding in vivo. DAMC proved to be effective in significantly reducing the hepatic AFB_1–DNA binding in vivo (Table 4). Since DHMC is the product formed during microsomal preincubation with DAMC and the former also inhibits AFB_1-DNA binding in vitro,[17] it was thought interesting to examine whether DHMC had any ability to irreversibly inactivate microsomal P-450. Preincubation of rat liver microsomes with DHMC failed to inhibit irreversibly microsome mediated AFB_1-DNA binding (data not shown). This indicates that inhibition of AFB_1-DNA binding observed earlier is definitely due to irreversible inhibition caused by DAMC alone. Further demonstration that DHMC-treated rat liver microsomes can hardly inhibit (Table 3) the binding of AFB_1 to exogenous DNA (unlike the action of DAMC) leads to the conclusion that dihydroxycoumarin (DHMC) is not the suicide inhibitor of P-450 specific for the catalysis of AFB_1 activation to the epoxide. In addition, DAMC was also found to produce mechanism-based inhibition of EROD and PROD (Fig. 4), which are well known to be specific for P-4501A and P-4502B families, respectively.[7,38] These results clearly point out the efficacy of DAMC to cause mechanism-based inhibition of cytochrome P-450-linked mixed function oxidase. The aforementioned results indicate that DAMC qualifies to be considered as a potential antimutagen. Such a conclusion was substantiated by the marked inhibitory effect of DAMC on micronuclei induction in rat bone marrow cells by AFB_1 (Table 5). Several classes of compounds have been reported to cause the inhibition of hepatic P-450-linked AFB_1 epoxidation,[11,37,39–41] but a few such inhibitors merit similarity to DAMC causing very effective irreversible inhibition of AFB_1-epoxidation leading to the inhibition of AFB_1–DNA binding. Present studies have demonstrated for the first time DAMC as the specific irreversible inhibitor of AFB_1–DNA binding catalysed by microsomes as well as in the intact animal. Other xenobiotics[37,39–44] including the widely studied oltipraz (1,2-dithiole-3-thione) are also known to inhibit hepatic AFB_1–DNA binding. It is not clear whether oltipraz irreversibly inactivates human liver P450 3A4 responsible for the formation of AFB_1-exoepoxide from AFB_1.[45] Rats fed DAMC in the diet for two weeks were found to have significantly elevated levels of hepatic cytosolic GSH *S*-transferase activity (Table 6). The reduction of microsome-mediated AFB_1-DNA binding by the addition of GSH and cytosol is known to be due to the action of GSH *S*-transferase[46,47] which catalyses the GSH-conjugation of AFB_1-epoxide (Fig. 1) sparing it from binding to DNA. The observation that DAMC-fed rat liver cytosol surpasses the control liver cytosol in diminishing the AFB_1-DNA binding (Table 6) clearly indicates that DAMC feeding to rat can obviously result in the induction of isoforms of GSH *S*-transferase

specific for the GSH conjugation of AFB_1-epoxide.[48] The influence of DAMC to stimulate the cytosolic GSH *S*-transferase further strengthens the antimutagenic property of DAMC. Coupled with its superb antioxidant activity found earlier,[49] DAMC can merit as a promising cancer preventive agent.

Acknowledgement

The financial assistance of the Danish International Development Agency (DANIDA, Denmark) is gratefully acknowledged.

References and Notes

1. IARC Monographs on the evaluation of carcinogenic risks to humans, Vol 56, some naturally occurring substances: food items and constituents. Heterocyclic aromatic amines and Mycotoxins Lyon: IARC, 1993.

2. Swenson, D. H.; Miller, E. C.; Miller, J. A. *Biochem. Biophys. Res. Commun.* **1974**, *60*, 1036.

3. Essigman, J. M.; Croy, R. G.; Bennett, R. A.; Wogan, G. N. *Drug Metab. Rev.* **1982**, *13*, 581.

4. Gallagher, E. P.; Stapleton, P. L.; Wienkers, L. D.; Kunze, K.; Eaton, D. L. *Proc. Am. Assoc. Cancer Res.* **1993**, *34*, 164.

5. Roebuck, B. D.; Liu, Y. L.; Rogers, A. E.; Groopman, J. D.; Kensler, T. W. *Cancer Res.* **1991**, *51*, 5501.

6. Kensler J. W.; Primano T.; Groopman J.; Zhang Y. Talalay P. *Proc. Int. Natl. Soc. Xenobiotics* **1996**, *10*, 18.

7. Yang, C. S.; Smith, T. J.; Hong, J. Y. *Cancer Res. (Suppl)* **1994**, *54*, 1982S.

8. Wattenberg, L. W. *Cancer Res. (Suppl)* **1992**, *52*, 2085S.

9. Zhang, Y.; Talalay, P. *Cancer Res. (Suppl)* **1994**, *54*, 1976S.

10. Wargovich, M. J.; Imada, O.; Stephen, L. C. *Cancer Lett.* ***1992***, 64, *39*.

11. Guengerich, F. P.; Kim, D. H. *Carcinogenesis* **1991**, *11*, 2275.

12. Fong, A. T.; Swansen, H. I.; Dashword, R. H.; William, D. F.; Hendricks, J. D.; Bailey, G. S. *Biochem. Pharmacol.* **1990**, *39*, 19.

13. Takeda, S.; Aburada, M. *J. Pharmacobio-Dyn* **1981**, *4*, 724 (*Chem. Abstr.* **1981**, *95*, 215075).

14. Yang, C. H.; Chaing, C.; Liu, K.; Perg, S.; Wang, R. *Yao Huseh Tung Pao* **1980**, *15*, 48 (*Chem. Abstr.* **1981**, *95*, 161758).

15. Tyagi, A.; Dixit, V. P.; Joshi, B. C. *Naturwissenschaften* **1980**, *67*, 104.

16. Deana, A. A.; Stokker, G. E.; Schultz, E. M.; Smith, R. L.; Cragoe, E. J.; Russo, H. F.; Watson, L. S. *J. Med. Chem.* **1983**, *26*, 580.

17. Raj, H. G.; Gupta, S.; Singh, S.; Singh, A.; Jha, A.; Bisht, K. S.; Sharma, S. K.; Jain, S. C.; Parmar, V. S. *Bioorg. Med. Chem.* **1996**, *4*, 2225.

18. Chakravarti, D. *J. Indian Chem. Soc.* **1935**, *12*, 536.

19. Parmar, V. S.; Bisht, K. S.; Jain, R.; Singh, S.; Shanna, S. K.; Gupta, S.; Malhotra, S.; Tyagi, O. D.; Vardhan, A.; Pati, H. N. *Indian J. Chem.* **1996**, *35B*, 220.

20. Allameh, A.; Saxena, M.; Raj, H. G. *Cancer Lett.* **1988**, *40*, 49.

21. Wang T. V.; Cerutti, P. A. *Cancer Res.* **1979**, *39*, 5165.

22. Burton, K. *Biochem. J.* **1956**, *62*, 315.

23. Lubet, R. A.; Mayer, R. J.; Cameson, J. W.; Nims, R. W.; Burke, M. D.; Wolff, T.; Guengerich, P. *Arch. Biochem. Biophys.* **1985**, *238*, 43.

24. Schmid, W. *Mutation Res.* **1975**, *31*, 9.

25. Habig, W. H.; Pabst, M. J.; Jakoby, W. B. *J. Biol. Chem.* **1974**, *249*, 7130.

26. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.

27. Kensler, T. W.; Enger, P. A.; Dolan, P. H.; Groopman, J. D.; Roebuck, B. D. *Cancer Res.* **1987**, *47*, 4271.

28. Guengerich, F. P. *J. Biol. Chem.* **1991**, *266*, 10019.

29. Copland, R. A. In '*Enzymes*' VCH Inc., New York, N.Y. 1996; pp. 237–261.

30. Halpert, J. R. *Biochem. Pharmacol.* **1981**, *30*, 875.

31. Lunetta, J. M.; Sugiyama, K.; Correia, M. A. *Mol. Pharmacol.* **1989**, *35*, 10.

32. Halpert, J.; Jaw, J. Y.; Cornfield, L. J.; Balfour, C.; Mash, E. A. *Drug Metab. Dispos.* **1989**, *17*, 26.

33. Halpert, J. R.; Balfour, C.; Miller, N. E.; Kaminski, L. S. *Mol. Pharmacol.* **1986**, *30*, 19.

34. Mathews, J. M.; Bend, J. R. *Mol. Pharmacol.* **1986**, *30*, 25.

35. Williams, D. A. In *Principles of Medicinal Chemistry* Foye, W. O.; Lemke, T. L.; Williams, D. A., Eds. B. I. Waverly Pvt (Ltd.) 1995, pp. 83–140.

36. Suzuki-Kurasaki, M.; Yoshioka, T.; Uematsu, T. *Biochem. J.* **1997**, *325*, 155.

37. Mandel, H. G.; Manson, M. M.; Judah, D. J.; Simpson, J. L.; Green, J. A.; Forrester, L. M.; Wolf, C. R.; Neal, G. E. *Cancer Res.* **1987**, *47*, 5218.

38. Troxel, M. C.; Buhler, D. R.; Hendricks, J. D.; Bailey, G. S. *Toxicol Appl. Pharmacol.* **1997**, *146*, 69.

39. Mandal, S.; Ahuja, A.; Shivapurkar, N. M.; Cheng, S. J.; Groopman, J. D.; Stoner, G. D. *Carcinogenesis* **1987**, *8*, 1615.

40. Gurtoo, H. L.; Dahms, R. P. *Biochem. Pharmacol.* **1979**, *28*, 3441.

41. Brienholt, V. B.; Schimerlik, M.; Dashwood, R.; Bailey, G. S. *Chem. Res. Toxicol.* **1995**, *8*, 506.

42. Takahashi, N.; Harttig, U.; William, S. D. E.; Bailey, G. S. *Carcinogenesis* **1995**, *17*, 79.

43. Zanger, R. C.; Springer, D. L.; McCarry, J. A.; Novak, R. F.; Primiano, T.; Buhler, D. R. *Carcinogenesis* **1992**, *13*, 2375.

44. Eaton, D. L.; Bammler, D. E. *Proc. Int. Natl Soc. Study Xenobiotics* **1996**, *10*, 15.

45. Guengerich, F. P.; Ueng, Y. F.; Johnson, W. W.; Harris, T. M.; Langouet, S.; Guillouzo, A.; Ketterer, B.; Yamazak, H.; Shimada, T. *Proc. Int. Natl. Soc. Study Xenobiotics* **1996**, *9*, 9.

46. Raj, H. G.; Clearfield, M. S.; Lotlikar, P. D. *Carcinogenesis* **1984**, *5*, 879.

47. Lotlikar, P. D.; Clearfield, M. S.; Jhee, E. C. *Cancer Lett.* **1984**, *24*, 241.

48. Ketterer, B. *Proc. Int. Natl Soc. Study Xenobiotics* **1995**, *7*, 24.

49. Raj, H. G.; Parmar, V. S.; Jain, S. C.; Goel, S.; Poonam, Himanshu; Malhotra, S.; Singh, A.; Olsen, C. E.; Wengel, J. *Bioorg. Med. Chem.* **1998**, *6*, 833.