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Preclinical factors influencing the relative contributions of Phase I and II enzymes to the metabolism of the experimental anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid

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Abstract

It is important to determine the relative contribution of each metabolic pathway (f_p) and of enzymes to the net metabolism of a drug. The aim of this study was to investigate, using a human liver bank, the f_p of the anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and the effects of various inhibitors and inducers on f_p. The mean apparent K_m and V_{max} values (N = 14) were 21 ± 5 μM and 0.04 ± 0.02 nmol/min/mg, respectively, for 6-methylhydroxylation, and 143 ± 79 μM and 0.71 ± 0.52 nmol/min/mg, respectively, for acyl glucuronidation in human liver microsomes. 6-Methylhydroxylation and acyl glucuronidation contributed 26 and 74%, respectively, to DMXAA metabolism at 5 μM; values were 7 and 93% at 350 μM DMXAA. There was a significant relationship between the ratio of metabolic activity by Phase II and I reactions ($R_{II/I}$) and uridine diphosphate glucuronosyltransferase (UGT2B7) protein level ($r = 0.605$, $P = 0.022$), whereas a reverse correlation between $R_{II/I}$ and cytochrome P450 (CYP1A) protein level was observed ($r = -0.540$, $P = 0.046$). Various compounds inhibited either DMXAA glucuronidation or 6-methylhydroxylation, or both pathways. Pretreatment of rats with β-naphthoflavone, but not phenobarbitone and cimetidine, increased the percentage of the contribution by 6-methylhydroxylation to 17% from 4% of control at 5 μM DMXAA. Our results indicate that the f_p of DMXAA is subject to substrate concentration, inhibition, induction, and the protein levels of enzymes that biotransform DMXAA. However, clinical studies are important to verify the conclusions drawn from *in vitro* data.

Keywords: DMXAA; Induction; Inhibition; Metabolism

1. Introduction

Metabolism including Phase I and II reactions has been regarded as one of the most important and complex processes in the body, leading to the excretion of most drugs [1]. CYPs and UGTs are the most important Phase I and II drug-metabolizing enzyme families, responsible for the metabolism of more than 95% of therapeutic drugs [2–6]. The relative abundance of the hepatic CYPs in humans has been determined as: CYP1A2 (13%), 2A6 (4%), 2B6 (<1%), 2C (20%), 2D6 (2%), 2E1 (7%), and 3A4 (30%) [4,7]. The significance of the individual CYP enzymes in human drug metabolism varies, with CYP3A, CYP2D, and CYP2C being responsible for the metabolism of 50, 25, and 20%, respectively, of the currently known drugs [4,8]. Both CYPs and UGTs have broad and overlapping substrate specificity. Therefore, a single drug can be metabolized by two or more reactions/enzymes. The majority of the human CYPs and UGTs may be inhibited and/or induced after exposure of the individual to drugs or other exogenous compounds [2,4]. For example, both UGT1A9 and UGT2B7 are inducible by various compounds such as

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Abbreviations: AIC, Akaike's information criterion; BNF, β-naphthoflavone; CMT, cimetidine; CYP, cytochrome P450; UGT, uridine diphosphate glucuronosyltransferase; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; DMXAA-G, DMXAA acyl glucuronide; f_p, relative contribution of each metabolic pathway; K_m, Michaelis–Menten constant; 6-OH-MXAA, 6-hydroxymethyl-5-methylxanthenone-4-acetic acid; PB, phenobarbitone; RAF, relative activity factor; $R_{II/I}$, ratio of metabolic activity by Phase II and I reactions; V_{max}, maximum metabolic velocity.

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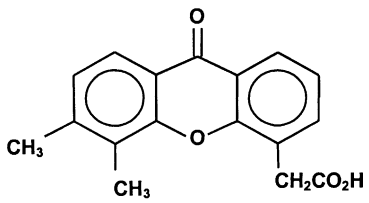
BNF [3], *t*-butylhydroquinone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [9], whereas CYP1A2 is inducible by smoking [10]. The induction and inhibition of enzymes responsible for drug metabolism have been considered as major factors leading to significant changes in drug effects [11–13]. Enzyme induction and inhibition may be of particular importance with anti-cancer drugs as these drugs with narrow therapeutic ranges are often administered in multi-drug combinations at maximum tolerated doses.

It is important to determine the identity, the f_p, and the enzyme for a drug, as this is associated with the *in vitro–in vivo* extrapolation with respect to metabolic clearance, toxicity, and drug–drug interactions. Detailed and quantitative metabolite profiling using *in vivo* animal models can provide information on the f_p, but this approach is costly and time-consuming. Species differences may cause difficulty in extrapolating animal data to humans. The relative contribution of each enzyme (mainly CYP) to the metabolism of a drug can be determined by several approaches, including correction for specific enzyme contents determined by western blot analysis in human liver [7], kinetic and inhibition studies using selective inhibitors (chemicals and antibodies), and the RAF approach [14,15]. However, the resultant data will vary, depending on the method used, which may only give an approximation of the real situation. Moreover, this may be difficult for drugs that are mainly metabolized by UGTs, due to the lack of selective inhibitors (chemicals and antibodies), the latency issue, and the technical problem with the determination of the contents of UGT enzymes.

The investigational anti-cancer drug DMXAA (Fig. 1), developed as an analogue of flavone-8-acetic acid by the Auckland Cancer Society Research Center, has completed Phase I clinical trials recently in New Zealand and the United Kingdom under the direction of the Cancer Research Campaign's Phase I/II Clinical Trials Committee [16]. As a new biological response modifier, the mode of action, the toxicology, and the pharmacological properties of DMXAA are remarkably different from most classical chemotherapeutic agents. The induction of cytokines (particularly tumour necrosis factor-α) [16–18], serotonin [19,20] and nitric oxide [21,22], and the anti-vascular [23], anti-angiogenetic [24], and immuno-modulating effects [25,26] are considered to be major mechanisms of action of DMXAA. *In vitro* and *in vivo* studies have indicated that acyl glucuronidation is the dominant metabolic pathway for DMXAA in animals and humans, with 6-methylhydroxylation the less important pathway, giving rise to DMXAA-G and 6-OH-MXAA [27–29]. Phenotyping studies have indicated that DMXAA glucuronidation is catalyzed by UGT1A9 and UGT2B7 [30], and that of 6-methylhydroxylation by CYP1A2 [31].



5,6-dimethylxanthenone-4-acetic acid

Fig. 1. Chemical structure of DMXAA.

The aim of this study was to determine the relative contribution of the Phase I (6-methylhydroxylation) and II (glucuronidation) pathways to the metabolism of DMXAA, using an established human liver bank. The effects of various CYP and UGT substrates/inhibitors and enzyme protein levels (UGT2B7 and CYP1A), determined by western blot analysis, on the metabolism of DMXAA were also investigated in human liver microsomes. In addition, the effects of several known drug-metabolizing enzyme inducers, including BNF, PB, and CMT [11,32–34], on the metabolism of DMXAA were examined, using a rat model.

2. Materials and methods

2.1. Chemicals and reagents

DMXAA and 2,5-dimethylxanthenone-4-acetic acid (SN24350, as the internal standard) were synthesized in the Auckland Cancer Society Research Center [35]. To avoid degradation, DMXAA was protected from light exposure by using amber glass [36]. Authentic DMXAA-G and 6-OH-MXAA were isolated and purified from the bile and urine of rats treated with DMXAA, and their structures were confirmed by mass spectrometry and [^{1}H]NMR [29]. Diclofenac, BNF, cyproheptadine, α-naphthoflavone, bicinchoninic acid reagent, Brij 58, chlorzoxazone, diethyldithiocarbamate, erythromycin, fenclofenac, fenoprofen, indomethacin, quinidine, D-saccharic acid 1,4-lactone, testosterone, tolbutamide, troleandomycin, PB, and CMT were obtained from the Sigma-Aldrich Co. Sulphaphenazole was obtained from Novartis. Ketoconazole was purchased from ICN Biomedicals. The polyclonal antiserum raised in goats recognizing human CYP1A1/1A2 and UGT2B7 was obtained from the Gentest Corp. Anti-goat or anti-rabbit immunoglobulin G (IgG) raised in donkeys was purchased from ICN Biomedicals. Electrophoresis products were obtained from Bio-Rad, and NADPH and UDPGA from Roche Diagnostics NZ Ltd. All other reagents were of analytical or HPLC grade as appropriate.

2.2. Human liver bank

Human liver samples (N = 14) were obtained from individuals who underwent liver resection for metastasis of colon cancer or hydatid disease, or from a transplant donor. Histological examination of the resected livers ensured the use of healthy liver tissue. The details of

the donors have been described elsewhere [31]. Ethical approval from the Northern New Zealand Research Ethics Committee and written informed consent for use of liver tissues for research were obtained.

2.3. Animal treatment

Healthy male Wistar Kyoto rats (180–230 g) were housed under constant temperature, lighting, and humidity according to institutional guidelines. Sterile food and water was available *ad lib*. All animal procedures were approved by the Animals Ethics Committee of the University of Auckland. The rats (N = 5 for each group) were treated with BNF, PB, CMT, or vehicle. BNF or CMT (80 mg/kg) was administered by intraperitoneal injection for 3 days, and PB was given orally in drinking water (0.1%, w/w) for 5 days. The animals were killed, and livers were collected.

2.4. Preparation of liver microsomes

The microsomes from human and rat livers were prepared by differential centrifugation, as described previously [37]. Livers and microsomes were stored at −80° until used. Microsomal protein concentration was determined by the Bicinchoninic Acid Protein Assay (Pierce), and bovine serum albumin was used as a standard [38]. The CYP content was determined as described [39].

2.5. Microsomal incubation and inhibition studies

The *in vitro* kinetic and inhibition studies of DMXAA glucuronidation and 6-methylhydroxylation with rat (untreated and pretreated) and human liver microsomes were performed using optimized incubation conditions with respect to microsomal protein concentration, incubation time, and substrate concentration for each species [31,40]. For glucuronidation, Brij 58 (ratio of Brij 58 to microsomal protein was 0.1−0.25:1, w/w) was incubated with microsomes at pH 6.8, along with D-saccharic acid 1,4-lactone to inhibit the activity of β-glucuronidase. All incubations were performed in triplicate, were initiated by the addition of UDPGA or NADPH, and conducted at 37° in a shaking water bath. The inhibition of liver DMXAA acyl glucuronidation and 6-methylhydroxylation *in vitro* by various UGT substrates and CYP inhibitors/substrates with various sources of human liver microsomes was investigated. Pooled human liver microsomes (from HL6, HL7, and HL8) were used for DMXAA glucuronidation as this pathway is catalyzed by multiple UGT enzymes (UGT1A9/UGT2B7) [30], and the glucuronidation activity for DMXAA was similar in these human livers. CYP1A2 catalyzes the 6-methylhydroxylation of DMXAA [31], and significant interindividual variations in the inhibition of this metabolic pathway may occur; thus, three sources of human livers (HL12, HL13, and HL14) were used. The concentrations of DMXAA were 140 μM for glucuronidation, and 20 μM for 6-methylhydroxylation, the apparent K_m values for each metabolic pathway in humans. The inhibitors and/or substrates for CYPs and UGTs were tested initially at 0.5 to 500 μM. If significant inhibition was observed, the latter were then used to determine the inhibitor concentration range for K_i. The mechanism-based CYP inhibitors diethyldithiocarbamate and troleandomycin [41] were preincubated in the presence of NADPH at 37° for 15 min prior to the addition of DMXAA. Incubations were stopped by cooling on ice, adding 2 vol. of an ice-cold acetonitrile:methanol mixture (3:1, v/v) containing 2 μM internal standard, and vortexing vigorously. Mixtures were centrifuged (3000 *g* for 10 min at 37°) to remove the precipitated microsomal protein. The supernatant was removed and evaporated under nitrogen gas, and the residue was reconstituted with mobile phase and injected into the HPLC. Substrate consumption did not exceed 10% at any substrate concentration. Formation rates (v) were expressed in nanomoles per milligram of protein per minute.

2.6. HPLC

The determination of DMXAA-G and 6-OH-MXAA in liver microsomal preparations by HPLC, with fluorescence detection, has been described previously [31,40]. Briefly, the HPLC system consisted of a solvent delivery system, a model SF250 fluorescence detector (excitation and emission wavelengths of 345 and 409 nm, respectively), a model 460 autosampler, and a model D450 data processing system (all from the Kontron Instrument Co.). A Luna C18 guard column and a 5 μm Spherex analytical column (150 × 4.6 mm; Phenomenex Co.) were used. The mobile phase (flow rate 2.5 mL/min) was acetonitrile:10 mM ammonium acetate buffer (24:76, v/v, pH 5.0). All HPLC methods had acceptable accuracy (85−115% of true values) and precision (intra- and inter-assay coefficient variations < 15%). Assay specificity was indicated by the absence of interfering chromatographic peaks in microsomal samples and in incubations with potential inhibitors.

2.7. Immunoblot analysis

SDS–PAGE was performed according to Laemmli [42]. After electrophoresis, the separated microsomal proteins were transferred onto Hybond-P membranes (Amersham). Detection of CYP1A or UGT2B7 was carried out using a primary antibody recognizing human CYP1A1/1A2 raised in goat with rat CYP1A1 as immunogen or human UGT2B7 raised in rabbit. Biotinylated donkey anti-goat or -rabbit IgG was used as the secondary antibody. Streptavidin-horseradish peroxidase was bound to the secondary antibody, and the light signal was developed using the ECL system (Amersham). The signal intensities on the blots were quantified using an MD30 image analysis system (Leading Edge) equipped with a video camera mounted on

a Leitz Diaplan microscope. Recombinant CYP1A2 and UGT2B7 from human B-lymphoblastoid cellular microsomes were used as standards.

2.8. Data analysis

Data are presented as means ± SD. Several models to describe the *in vitro* kinetics of DMXAA glucuronidation and 6-methylhydroxylation (one and two binding sites, substrate–activator and substrate–inhibitor complex formation, and the sigmoid models, Eqs. (1)–(4)) in rat and human liver microsomes were fitted and compared using the Prism 3.0 program (Graphpad Software Co.) as described [31].

$$v = \frac{V_{\max}[S]}{K_m + [S]} \tag{1}$$

$$v = \frac{V_{\max 1}[S]}{K_{m1} + [S]} + \frac{V_{\max 2}[S]}{K_{m2} + [S]} \tag{2}$$

$$v = \frac{V_{\max}[S]}{K + [S] + [S]^2/K_{is}} \tag{3}$$

$$v = \frac{V_{\max}[S]^{h'}}{K^{h'} + [S]^{h'}} \tag{4}$$

where v is the rate of metabolism; $V_{\max}$, the maximum velocity; K_m, the Michaelis–Menten constant; [S], the substrate concentration; K_{is}, the substrate inhibition constant; h', the Hill coefficient for cooperative substrate binding; and subscripts 1 and 2, the first and the second type of enzyme binding sites. The choice of model was confirmed by the F-test, comparison of the relative residuals and the standard error of the parameter estimates from the non-linear regression analysis, and comparison of the AIC [43]. Statistical significance was assessed, using a Student's t-test, at $P < 0.05$. The initial statistical analysis to evaluate the differences in the mean kinetic parameters among the different groups was performed by a two-way ANOVA with a Tukey−Kramer test.

The apparent K_i values and the nature of inhibition were initially estimated by Dixon plots, and then were confirmed by fitting and comparing several inhibition models (competitive, uncompetitive, and mixed inhibition) (Eqs. (5)–(7)) using the Prism 3.0 program. The appropriate model was chosen by F-test and AIC comparison:

$$v = \frac{V_{\max}[S]}{K_m(1 + [I]/K_{ic}) + [S]} \tag{5}$$

$$v = \frac{V_{\max}[S]}{K_m + [S](1 + [I]/K_{iu})} \tag{6}$$

$$v = \frac{V_{\max}[S]}{K_m(1 + [I]/K_{ic}) + [S](1 + [I]/K_{iu})} \tag{7}$$

where [I] is the inhibitor concentration, and subscripts c and u represent competitive and uncompetitive inhibition.

The f_p of each metabolic pathway [p = glucuronidation (II) and 6-methylhydroxylation (I)] to net DMXAA metabolism in human liver microsomes was predicted as a function of substrate concentration [S], using the reaction velocity ($v_{p(S)}$) based on the enzyme kinetic parameters (K_m and $V_{\max}$) determined for each pathway:

$$f_p\,(\%) = \frac{v_{p(S)}}{v_{\mathrm{I}(S)} + v_{\mathrm{II}(S)}} \times 100 \tag{8}$$

The ratio of metabolic activity by Phase II and I reactions ($R_{\mathrm{II/I}}$) was given by:

$$R_{\mathrm{II/I}} = \frac{v_{\mathrm{II}(S)}}{v_{\mathrm{I}(S)}} \tag{9}$$

3. Results

3.1. Phase I and Phase II metabolism of DMXAA in human liver microsomes

Both glucuronidation and 6-methylhydroxylation of DMXAA in human liver microsomes followed Michaelis–Menten kinetics (typical data and Eadie−Hofstee plots are shown in Fig. 2), with mean K_m and $V_{\max}$ values of 143 ± 79 μM and 0.71 ± 0.52 nmol/min/mg for glucuronidation, and 21 ± 5 μM and 0.04 ± 0.02 nmol/min/mg for 6-methylhydroxylation. The relative contribution of the different metabolic pathways (f_p) to net metabolism of DMXAA over 5-350 μM is shown in Fig. 3A. At 5 μM DMXAA, 6-methylhydroxylation and glucuronidation contributed 26 and 74%, respectively, to overall biotransformation in human liver microsomes; values were 7 and 93%, respectively, at 350 μM DMXAA. Consistently, the ratio of metabolic activity by Phase II and I reactions ($R_{\mathrm{II/I}}$) increased from 3 at 5 μM DMXAA to 12 at 350 μM DMXAA (Fig. 3B).

3.2. Relationship of $R_{II/I}$ with drug-metabolizing enzyme levels

There was a 5-, 6-, and 5-fold interindividual variation in the contents of CYP, CYP1A, and UGT2B7, respectively, determined by western blot analysis in the 14 human livers (Table 1). This is consistent with the 3- to 7-fold interindividual variation in the K_m and $V_{\max}$ for 6-methylhydroxylation and glucuronidation of DMXAA. In addition, there was a significant relationship between $R_{\mathrm{II/I}}$ and UGT2B7 protein levels ($r = 0.605$, $P = 0.022$), whereas a reverse correlation between $R_{\mathrm{II/I}}$ and CYP1A protein levels was observed ($r = -0.540$, $P = 0.046$) (Fig. 4A and B). However, there was no significant correlation between $R_{\mathrm{II/I}}$ and total CYP contents ($r = 0.444$, $P = 0.1122$).

3.3. Inhibition studies

In human liver microsomes, fenclofenac, cyproheptadine, and diclofenac, and the anti-cancer drugs amsacrine, vinblastine, and vincristine significantly inhibited DMXAA

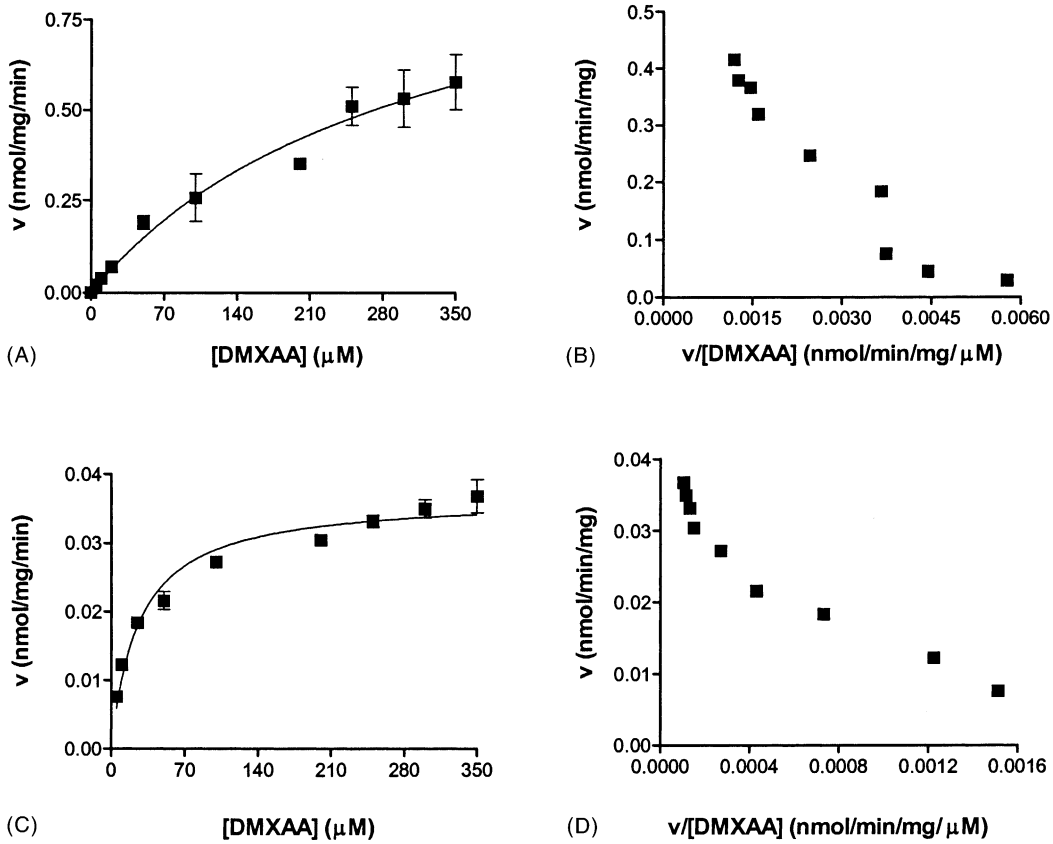


Fig. 2. Typical Michaelis–Menten (A and C) and Eadie–Hofstee (B and D) plots for DMXAA glucuronidation (A and B) and 6-methylhydroxylation (C and D) in human liver microsomes from HL4. Each data point is the mean ± SD of triplicate determinations in all the Michaelis–Menten plots. The curves in Michaelis–Menten plots represent the fit of one enzyme binding-site model.

glucuronidation to 10–50% of control with K_i values of 10–365 μM (for Dixon plots, see Fig. 5). α-Naphthoflavone at 0.5 and 5 μM reduced DMXAA 6-methylhydroxylation to 20 and 14% of control, respectively (K_i = 0.036 μM). In addition, cyproheptadine, diclofenac, phenacetin, α-naphthoflavone, SKF 525A, and the anticancer drugs daunorubicin and *N*-[2-(dimethylamino)-ethyl]acridine-4-carboxamide (DACA) inhibited DMXAA

Table 1
Enzyme levels and kinetic parameters for DMXAA 6-methylhydroxylation and glucuronidation by human liver microsomes[a]

Human livers	Total CYP (nmol/mg)	CYP1A level	UGT2B7 level	6-Methylhydroxylation			Glucuronidation	
				Ratio of metabolic activity	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)
HL1	0.93 ± 0.11	1.2 ± 0.2	2.4 ± 0.2	26.2	11 ± 1	0.03 ± 0.00	62 ± 11	1.12 ± 0.05
HL2	0.68 ± 0.10	1.3 ± 0.3	2.5 ± 0.3	40.7	23 ± 3	0.01 ± 0.00	258 ± 113	1.77 ± 0.32
HL3	0.46 ± 0.06	1.5 ± 0.3	2.1 ± 0.2	9.6	23 ± 2	0.03 ± 0.00	109 ± 13	0.58 ± 0.03
HL4	0.63 ± 0.07	1.3 ± 0.2	2.2 ± 0.3	9.5	26 ± 4	0.03 ± 0.00	234 ± 76	1.73 ± 0.14
HL5	0.35 ± 0.03	0.5 ± 0.1	2.3 ± 0.3	18.7	31 ± 5	0.02 ± 0.00	166 ± 28	1.13 ± 0.08
HL6	0.22 ± 0.02	0.7 ± 0.1	1.7 ± 0.2	25.6	20 ± 3	0.03 ± 0.00	108 ± 15	0.30 ± 0.05
HL7	0.244 ± 0.02	2.5 ± 0.2	1.5 ± 0.1	13.3	15 ± 2	0.05 ± 0.00	80 ± 11	0.38 ± 0.02
HL8	0.27 ± 0.02	2.1 ± 0.3	1.5 ± 0.2	14.3	17 ± 1	0.04 ± 0.00	72 ± 11	0.23 ± 0.01
HL9	0.24 ± 0.03	2.3 ± 0.2	0.5 ± 0.1	8.7	16 ± 1	0.05 ± 0.00	86 ± 6	0.23 ± 0.01
HL10	0.40 ± 0.06	2.1 ± 0.2	1.4 ± 0.1	5.2	16 ± 1	0.09 ± 0.00	86 ± 7	0.42 ± 0.01
HL11	0.35 ± 0.05	2.9 ± 0.2	1.5 ± 0.2	10.3	17 ± 1	0.04 ± 0.00	317 ± 111	0.58 ± 0.11
HL12	0.41 ± 0.04	1.9 ± 0.2	1.4 ± 0.2	9.8	26 ± 2	0.06 ± 0.00	133 ± 23	0.74 ± 0.05
HL13	0.38 ± 0.05	2.0 ± 0.2	1.6 ± 0.2	12.1	24 ± 2	0.05 ± 0.00	131 ± 9	0.57 ± 0.01
HL14	0.26 ± 0.01	1.9 ± 0.1	1.4 ± 0.1	12.8	22 ± 2	0.05 ± 0.00	83 ± 5	0.60 ± 0.01
Mean ± SD (range)	0.43 ± 0.23 (0.22–0.93)	1.8 ± 0.6 (0.5–29)	1.7 ± 0.5 (0.5–2.5)		21 ± 5 (11–31)	0.04 ± 0.02 (0.01–0.09)	143 ± 79 (62–317)	0.71 ± 0.52 (0.23–1.77)

[a] Parameters are reported as means ± SD from three determinations in microsomal preparations from 14 human livers. Glucuronidation activity was determined in Brij 58-activated microsomes. The units for CYP1A and UGT2B7 are arbitrary.

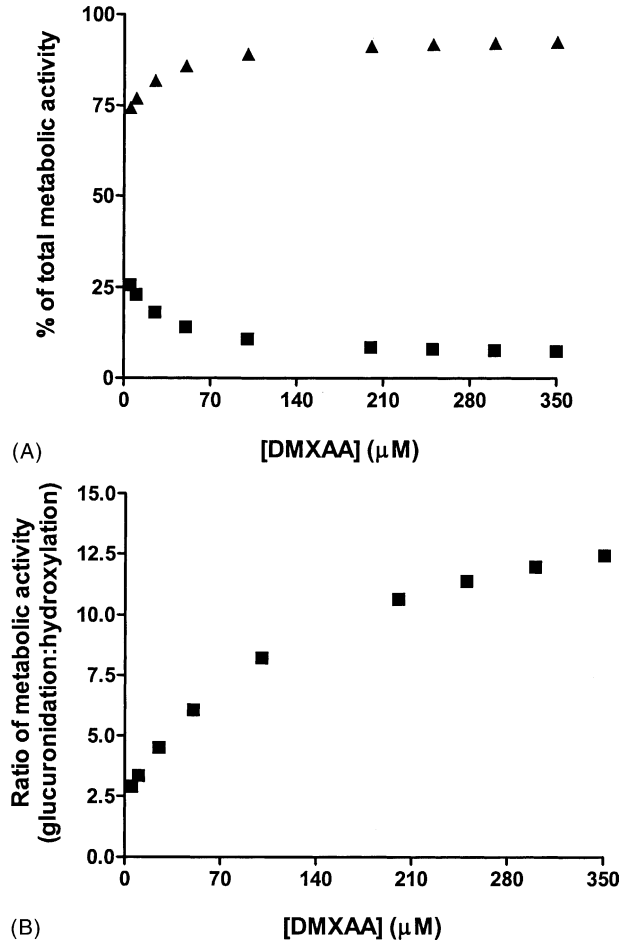


Fig. 3. (A) Relative contribution of Phase I (6-methylhydroxylation, ■) and Phase II (glucuronidation, ▲) metabolic pathways to overall DMXAA metabolism in human liver microsomes at 5–350 μM DMXAA. (B) The ratio of metabolic activity (glucuronidation:6-methylhydroxylation) over 5–350 μM DMXAA.

6-methylhydroxylation with K_i values of 0.59 to 350 μM (for Dixon plots, see Fig. 6). Other CYP inhibitors, including troleandomycin, ketoconazole, diethyldithiocarbamate, tobutamide, erythromycin, chlorzoxazone, sulphaphenazole, quinidine, and testosterone had little or negligible inhibitory effects on DMXAA 6-methylhydroxylation (data not shown). Diclofenac and cyprohepatadine inhibited both

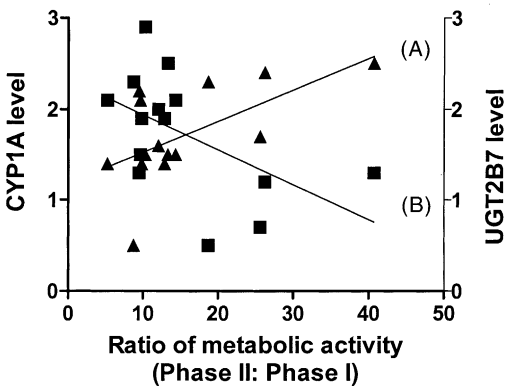


Fig. 4. Relationship between the ratio of metabolic activity (glucuronidation:6-methylhydroxylation) at 100 μM DMXAA and CYP1A levels (A, ■) and UGT2B7 levels (B, ▲) determined by western blot analysis.

glucuronidation (K_i = 10 and 53 μM, respectively) and 6-methylhydroxylation (K_i = 93 and 265 μM, respectively).

3.4. Induction studies

Pretreatment of BNF, PB, and CMT resulted in a 120, 95, and 42% increase in the total CYP contents, respectively, compared with the control (564 ± 23 pmol/mg microsomal protein, N = 5). The kinetic parameters for liver microsomal DMXAA 6-methylhydroxylation and glucuronidation in inducer-treated and untreated rats are shown in Table 2. DMXAA 6-methylhydroxylation and glucuronidation followed monophasic Michelis–Menten kinetics in either inducer-treated or untreated rats except for the BNF-pretreated rats where high- and low-affinity enzyme components for 6-methylhydroxylation were demonstrated by a best-fit two enzyme-site model (typical Michelis–Menten plots for microsomal DMXAA 6-methylhydroxylation are shown in Fig. 7, left column). The Eadie–Hofstee plots (Fig. 7, right column) suggested a biphasic kinetic for DMXAA 6-methylhydroxylation in BNF-treated rats. All inducers had little or negligible effects on the apparent K_m values for DMXAA 6-methylhydroxylation and glucuronidation except for BNF-treated rats where a 23-fold lower K_m and a 180-fold higher V_{max}/

Table 2
Kinetic parameters of liver microsomal DMXAA 6-methylhydroxylation and glucuronidation in inducer-treated and untreated rats[a]

Treatment	6-Methylhydroxylation			Glucuronidation in detergent-activated microsomes		
	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (mL/min/g)	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (mL/min/g)
Untreated	158 ± 21	0.04 ± 0.00	0.23 ± 0.04	118 ± 11	0.75 ± 0.03	6.36 ± 0.65
BNF	7 ± 1 (K_{m1})	0.03 ± 0.00 (V_{max1})	41.4 ± 591[b]	101 ± 12	1.37 ± 0.13[b]	10.1 ± 1.20[b]
	292 ± 115 (K_{m2})	0.06 ± 0.01[b] (V_{max2})	0.21 ± 0.08			
PB	135 ± 26	0.04 ± 0.00	0.30 ± 006[b]	134 ± 25	0.85 ± 0.02	6.34 ± 1.21
CMT	129 ± 10	0.04 ± 0.00	0.27 ± 0.02	144 ± 18	0.77 ± 0.03	5.35 ± 0.67

[a] Kinetic parameters are means ± SD from three determinations in pooled liver microsomes of rats (N = 5) receiving various inducers. BNF = β-naphthoflavone; PB = phenobarbitone; CMT = cimetidine.

[b] $P < 0.05$, compared with control rats.

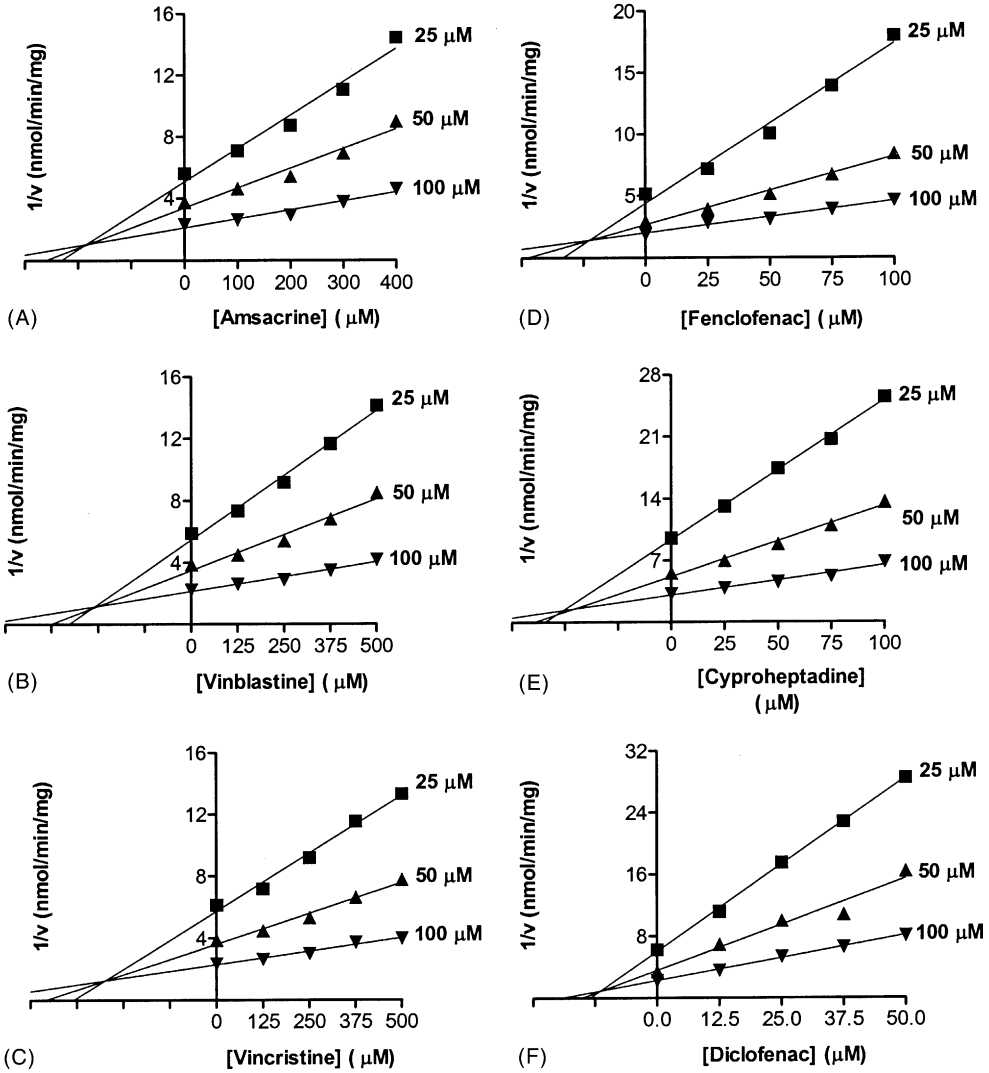


Fig. 5. Dixon plots for the inhibition of DMXAA glucuronidation by amsacrine (A), vinblastine (B), vincristine (C), fenclofenac (D), cyproheptadine (E), and diclofenac (F) in human liver microsomes. Each data point is the mean of at least three determinations.

K_m (CL_{int}) value was observed compared with control. BNF pretreatment significantly increased the percentage of metabolic activity by 6-methylhydroxylation to 17% from 4% of control at 5 μM DMXAA, but this was reduced to the level of control when the DMXAA concentration increased to 350 μM (Fig. 8). However, PB or CMT pretreatment had little effect on the change of the percentage of metabolic activity by 6-methylhydroxylation.

4. Discussion

Our results based on this study, together with previous investigations, have indicated that acyl glucuronidation and 6-methylhydroxylation are the two major metabolic pathways for DMXAA, with the former being the predominant one and the latter a lesser important one for its metabolic clearance. Previous studies have indicated that: DMXAA-G was the predominant urinary metabolite (up to 60% of the total dose of DMXAA) in patients receiving DMXAA [31]; up to 50% of the DMXAA dose was recovered as DMXAA-G in an isolated rat liver perfusion model [27]; and the major biliary metabolite was DMXAA-G in the mouse [44]. Like other drugs that are glucuronidated extensively, the clearance of DMXAA *in vivo* may be influenced by a variety of factors, including the rate of production, hydrolysis, and renal clearance of the acyl glucuronide [3,45]. Factors affecting one or more of these processes may lead to alterations in the clearance of DMXAA. However, caution should be taken with extrapolation from *in vitro* data to the *in vivo* situation for DMXAA, as this is complicated by conjugation–deconjugation recycling via the hydrolysis of the reactive acyl glucuronide during circulation in the blood (systematic recycling), or during passage through the gut after biliary excretion (entero-hepatic recycling) [2,46].

However, the relative contribution of glucuronidation and 6-methylhydroxylation to net DMXAA metabolism may be

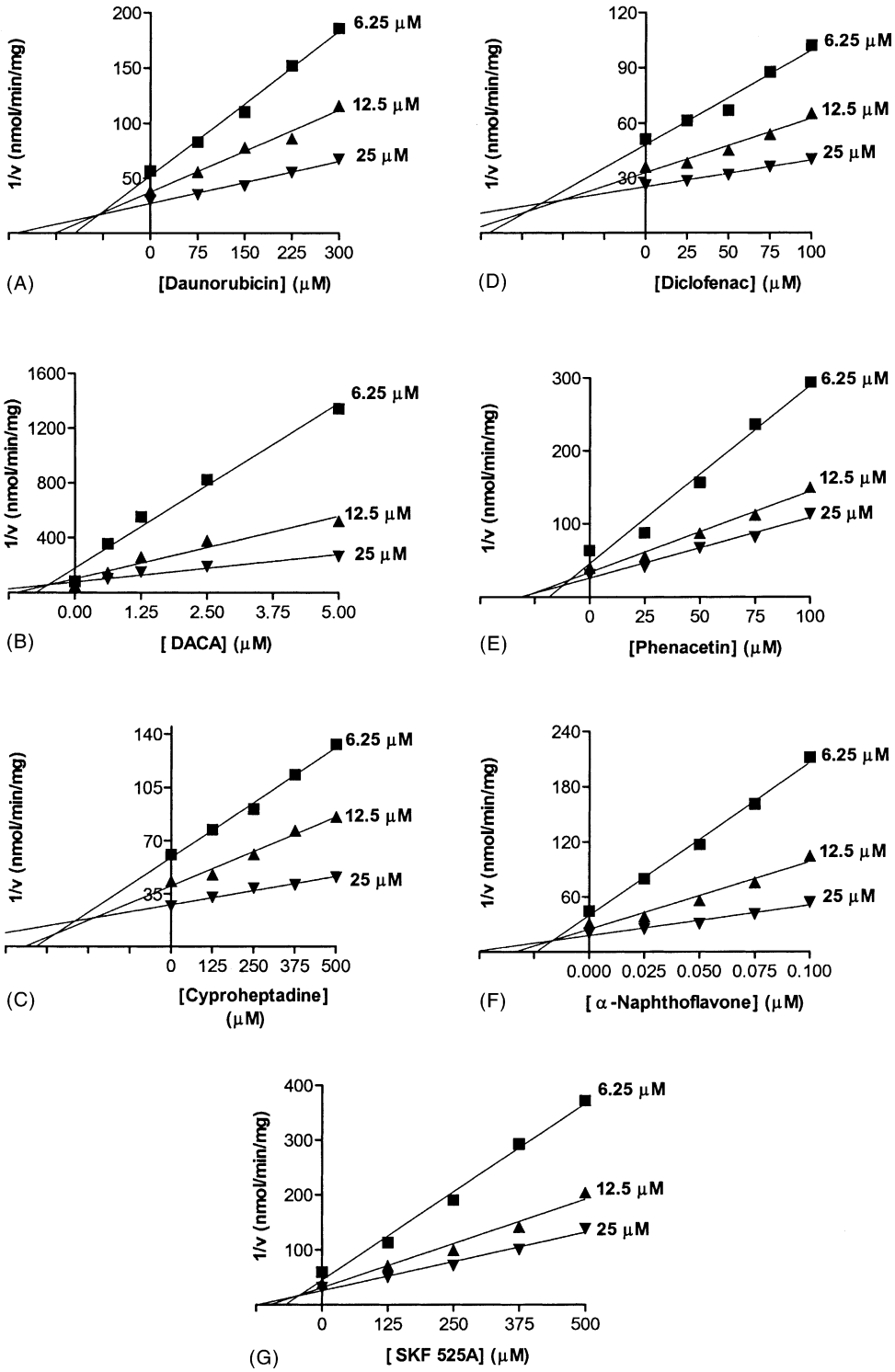


Fig. 6. Dixon plots for the inhibition of DMXAA 6-methylhydroxylation by daunorubicin (A), DACA (B), cyproheptadine (C), diclofenac (D), phenacetin (E), α-naphthoflavone (F), and SKF 525A (G) in human liver microsomes. Each data point is the mean of at least three determinations.

subject to substrate concentration. This may be due to the 6- and 1.5-fold higher K_m and intrinsic clearance values for glucuronidation, compared with those for 6-methylhydroxylation, suggesting that the UGTs (UGT1A9 and UGT2B7) were the high-capacity component, whereas the enzyme for 6-methylhydroxylation (CYP1A2) was low-capacity. This is demonstrated, for example, in the reduction of the percent contribution of 6-methylhydroxylation from 26% at 5 μM DMXAA to 7% at 350 μM DMXAA. Data from the Phase I clinical trial of DMXAA indicated that the concentration ratio of DMXAA-G to 6-OH-MXAA increased with increasing doses of DMXAA, and that the systemic clear-

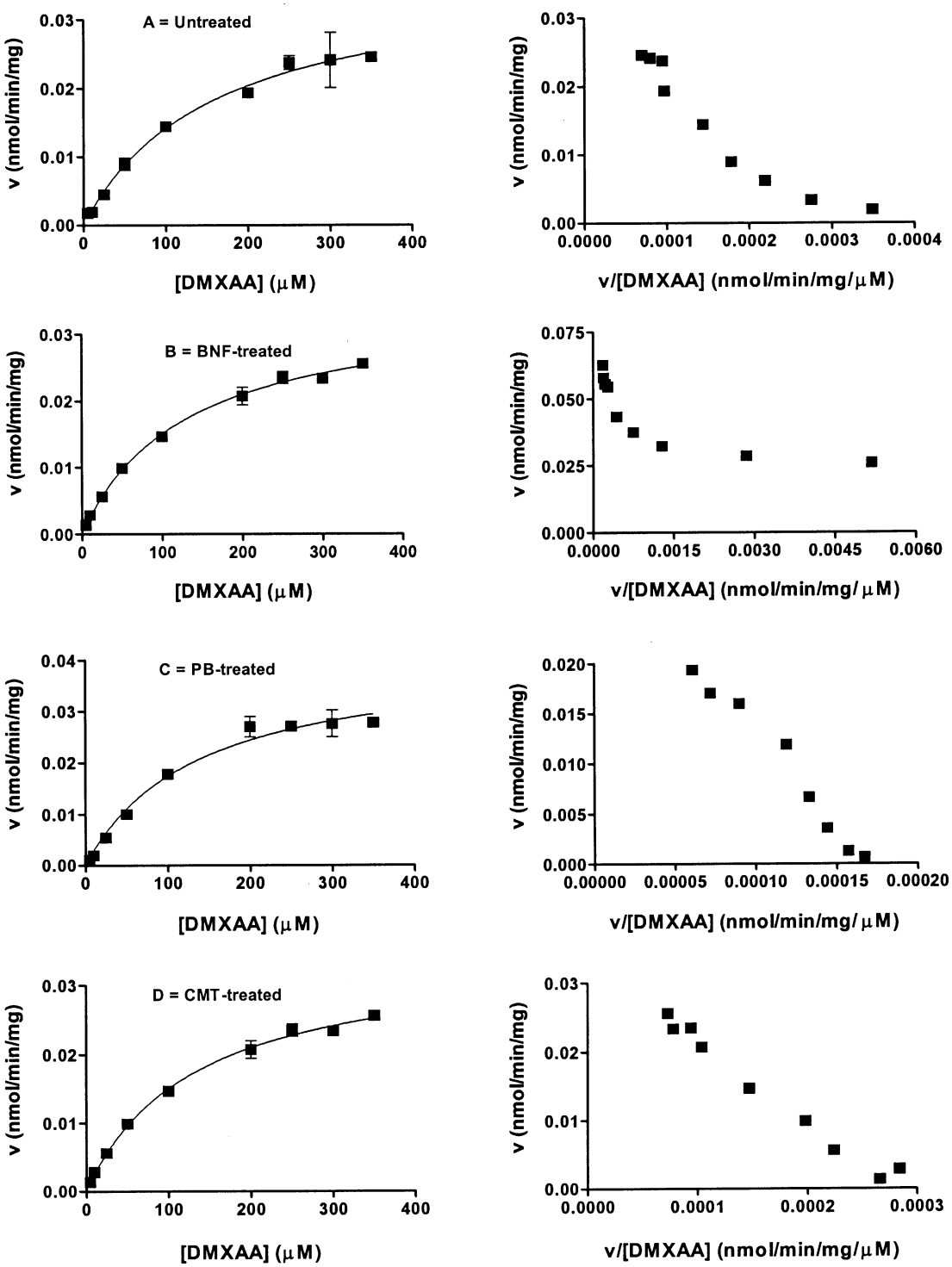


Fig. 7. Michaelis–Menten plots (left column) and Eadie–Hofstee plots (right column) for the metabolism of DMXAA in control rats (A) and rats pretreated with BNF (B), PB (C), and CMT (D). Values are means ± SD, N = 5 for points in all the Michaelis–Menten plots.

ance values in cancer patients at low doses of DMXAA were more variable than those found at high doses (unpublished results). This may be due to CYP1A2 contributing more to the overall clearance of DMXAA at low doses, whereas at higher doses CYP1A2 activity levels may be more subject to inhibition, induction, individual smoking habits, and gender, resulting in significant interindividual variation [10].

For drugs metabolized by two such enzyme systems, the remarkable change of the f_p values may have important pharmacokinetic and toxicological implications. For example, the shifting of a major metabolic pathway may lead to an altered metabolite profile in the bile and urine, resulting in pharmacological consequences, especially if the metabolites are toxic and/or pharmacologically active

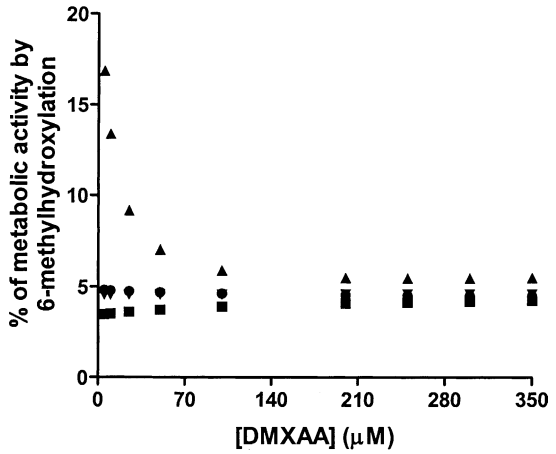


Fig. 8. Percent contribution of 6-methylhydroxylation to net metabolism of DMXAA over 5–350 μM in the control rats (■) and rats pretreated with BNF (▲), PB (▼), and CMT (●). Values are means from triplicate determinations.

[1,47]. For instance, tamoxifen resistance has been attributed, in part, to its altered metabolite profile, leading to reduced tamoxifen in the tumour and more accumulation of estrogenic metabolites [48]. As DMXAA-G undergoes covalent binding to albumin *in vitro* and *in vivo* in humans, adduct formation with albumin is proportional to the concentration of DMXAA-G and the DMXAA dosage [28]. Therefore, it could be predicted that shifting of the major DMXAA metabolic pathway from Phase I (6-methylhydroxylation) to Phase II (glucuronidation) would lead to increased DMXAA–albumin adduct formation, possibly resulting in clinically significant toxicity. A shift in the importance of one major metabolic pathway to another with changing concentration may also have implications for drug–drug interactions [49]. The contribution to overall clearance of a particular metabolic pathway subject to inhibition is an important determining factor in the prediction of *in vivo* drug interactions [50]. Our *in vitro* and *in vivo* studies have indicated that glucuronidation is the major metabolic pathway for DMXAA, whereas 6-methylhydroxylation is a minor metabolic pathway accounting for up to 30% of total clearance [27–29]. Thus, alterations in the latter pathway of DMXAA metabolism would not be expected to have a clinically significant effect on DMXAA disposition, and it can be predicted that only those compounds inducing DMXAA glucuronidation or both metabolic pathways are likely to cause drug interaction with increased *in vivo* clearance of DMXAA. However, there remains the possibility that at low DMXAA concentrations, the major metabolic pathway may be CYP1A2-catalyzed 6-methylhydroxylation. Under these circumstances, factors that induce (e.g. smoking) or inhibit (e.g. fluvoxamine) CYP1A2 could have important effects on the total elimination rate of DMXAA.

Our results have also indicated that the relative contributions of glucuronidation and 6-methylhydroxylation were subject to the protein levels of enzymes involved in the biotransformation of DMXAA, as indicated by the significant interindividual variation of the kinetic parameters (K_m and V_{max}) for glucuronidation and 6-methylhydroxylation of DMXAA in 14 human livers. In addition, genetic polymorphisms in UGT2B7 [51] and CYP1A2 [4] have been reported, which may contribute to the change of f_p values. Furthermore, since UGT1A9, UGT2B7, and CYP1A2 are all present in extrahepatic tissues such as the intestines and the kidneys [4,5], their contributions to the net clearance of DMXAA should be taken into account.

This study has indicated that various inducers have differential effects on Phase I and II metabolism of DMXAA in the rat. Although all inducers examined increased the total CYP content, BNF and PB, but not CMT, significantly increased the 6-methylhydroxylation rate of DMXAA. On the other hand, BNF but not PB and CMT induced DMXAA glucuronidation in the rat. BNF significantly induced both DMXAA 6-methylhydroxylation and glucuronidation, but it has a more complicated effect on the former metabolic pathway, as indicated by the biphasic kinetics of 6-methylhydroxylation. The reason for the transformation from monophasic to biphasic kinetics is unknown. Possible explanations include the change in the liver CYP1A1/CYP1A2 ratio and the involvement of multiple enzymes with very different affinity to substrate after pretreatment of BNF that has been shown to be a potent inducer of CYP1A [33,34]. CYP1A has been shown to be involved in DMXAA 6-methylhydroxylation in the rat, as indicated by the significant inhibition by the known CYP1A inhibitors furafylline and α-naphthoflavone (unpublished data). Since the V_{max} (0.04 ± 0.00 nmol/min/mg) of DMXAA 6-methylhydroxylation in rat liver microsomes was comparable to that in human liver microsomes (0.04 ± 0.02 nmol/min/mg), and negligible CYP1A2 protein level has been observed in laboratory rats [52], this reaction may be catalyzed in the rat primarily by CYP1A1 and/or other CYP isoforms rather than CYP1A2. It appears that the inducing effects of BNF and PB on CYP content and CYP1A activity determined by DMXAA 6-methylhydroxylation were comparable with those results reported by others [33,34].

In conclusion, the relative contributions of Phase I and II metabolism to the clearance of DMXAA are subject to substrate concentration, inhibition, induction, and protein levels of the enzymes that biotransform DMXAA. The involvement of multiple enzymes for DMXAA metabolism indicates a low potential of drug–DMXAA interaction. However, clinical trials are important to verify the conclusions drawn from the *in vitro* data.

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