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Preclinical factors affecting the interindividual variability in the clearance of the investigational anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid

Shufeng Zhou[a,*], Philip Kestell[b], Bruce C. Baguley[b], James W. Paxton[a]

[a]*Division of Pharmacology and Clinical Pharmacology, The University of Auckland, Private Bag 92019, Auckland, New Zealand*
[b]*Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand*

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Abstract

Cancer chemotherapy is characterized by significant interindividual variations in systemic clearance, therapeutic response, and toxicity. These variations are due mainly to genetic factors, leading to alterations in drug metabolism and/or target proteins. The aim of this study was to determine, using a human liver bank (N = 14), the interindividual variations in the expression and activity of liver enzymes that metabolize the investigational anticancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA), i.e cytochrome P450 (CYP1A2) and uridine diphosphate glucuronosyltransferase (UGT1A9/2B7). In addition, interindividual variations in enzyme inhibition, hydrolysis of DMXAA acyl glucuronide (DMXAA-G) by plasma and hepatic microsomes, and the binding of DMXAA by plasma proteins also were examined. The results indicated that there was approximately one order of magnitude of interindividual variation in the expression of CYP1A2 and UGT2B7, activity of the enzymes toward DMXAA, and inhibition potency (IC_{50}) by diclofenac, cyproheptadine, and α-naphthoflavone. The enzyme activities toward DMXAA and IC_{50} values were closely correlated with enzyme expression. There was a smaller (2- to 3-fold) variation in the enzyme-catalyzed hydrolysis of DMXAA acyl glucuronide in human plasma and liver microsomes (N = 6) and in the binding of DMXAA by plasma proteins in humans. In conclusion, the interindividual variability of DMXAA disposition observed *in vitro* might reflect the greater elimination variability (>one order of magnitude) in Phase I cancer patients. The variability in DMXAA clearance in these cancer patients would be due mainly to differences in its metabolism and its metabolic inhibition by co-administered drugs. To a lesser extent, variability in the clearance of DMXAA could be due to the hydrolysis of its acyl glucuronide and/or its binding to plasma proteins. Further study is needed to examine the genotype–phenotype relationship, and the result, together with therapeutic drug monitoring may provide a useful strategy for optimizing DMXAA treatment.

Keywords: DMXAA; CYP; Interindividual variation; Inhibition; UGT

1. Introduction

Cancer chemotherapy is characterized by significant interindividual variations in pharmacokinetics (i.e. clearance and half-life) and pharmacodynamics (i.e. therapeutic responses and drug toxicities) [1]. Such variability is due mainly to genetic factors, leading to altered drug metabolism and/or drug target proteins (e.g. receptors or signaling proteins) [1–5]. Many anticancer drugs have been reported to be metabolized by CYPs and UGTs, responsible for the metabolism of more than 95% of therapeutic drugs [6–10]. The significance of the individual CYP enzyme in human

* Corresponding author. Present address: Department of Pharmacy, Faculty of Science, National University of Singapore, Blk S4, Science Drive 4, S 117543 Singapore, Singapore. Tel.: +65-6874-2931; fax: +65-6779-1554.
E-mail address: phazsf@nus.edu.sg (S. Zhou).

Abbreviations: ANF, α-naphthoflavone; CYP, cytochrome P450; DMXAA, 5,6-dimethylxanthenone-4-acetic acid; DMXAA-G, DMXAA acyl glucuronide; IC_{50}, concentration inhibiting 50% of the reaction; k, the first order degradation constant; K_m, Michaelis–Menten constant; 6-OH-MXAA, 6-hydroxymethyl-5-methylxanthenone-4-acetic acid; TNF-α, tumor necrosis factor-α; V_{max}, the maximum metabolic velocity; UGT, uridine diphosphate glucuronosyltransferase.

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drug metabolism varies, with CYP3A, CYP2D, and CYP2C being responsible for the metabolism of 50, 25, and 20%, respectively of the drugs currently known [8,11]. 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%) [8,12]. In addition to differences in the levels among enzymes, there are large interindividual variations in the protein and/or mRNA levels for each enzyme [12,13], which is due to phenotypic differences or genetic polymorphisms. The interindividual differences in the enzyme levels are approximately 5-fold for CYP2C and CYP3A4, 12-fold for CYP2E1, 20-fold for CYP1A2, and >50-fold for CYP2D6, CYP2B6, and CYP2D6 [12]. The phenotypic differences in each enzyme are believed to be due largely to induction and inhibition by a number of exogenous factors, although genetic changes (e.g. gene mutation and single nucleotide polymorphisms) also may play a role [14–16]. Interindividual variability in the disposition of anticancer drugs with narrow therapeutic ranges may be of particular importance as they often are used at the maximum tolerated doses; and multiple drug administration is common. Many of them are prodrugs requiring biotransformation to form active species by enzymes that exhibit genetic polymorphisms; and these polymorphic enzymes can detoxify certain anticancer drugs.

The investigational anticancer drug DMXAA was developed as an analogue of flavone-8-acetic acid by the Auckland Cancer Society Research Center (ACSRC). DMXAA has been found to induce cytokines (particularly TNF-α [17–19], serotonin [20,21], and nitric oxide [22,23]). Its antivascular [24], anti-angiogenetic [25], and immunomodulating effects [26,27] are considered to be major components of its mechanisms of action. It recently has completed Phase I clinical trials in New Zealand and UK under the direction of the Cancer Research UK Phase I/II Clinical Trials Committee. In 63 cancer patients of the New Zealand arm, one partial response was observed in a patient with metastatic squamous cell carcinoma of the cervix who was administered a dose of 22 mg/kg DMXAA [17]. Dose-limiting toxicity was defined at 99 mg/kg and included cognitive impairment, acute reversible tremor, dyspnea, and visual disturbance. Non-linear pharmacokinetics was observed, with the plasma clearance decreasing with increasing dose. A plasma C_{max} of 2200 μM has been observed in 2 patients receiving high doses of DMXAA. A wide (20-fold) interindividual variation in the plasma clearance of DMXAA has been observed in cancer patients, with clearance values at low doses being more variable than those at the higher doses [17]. Data from Phase I patients also have suggested that glucuronidation may be the rate-limiting step for DMXAA elimination, whereas 6-methylhydroxylation is a minor pathway; up to 60% of the total dose was excreted as DMXAA-G, 5.5% as 6-OH-MXAA and 4.5% as the glucuronide of 6-OH-MXAA [28]. *In vitro* studies using human liver microsomes and perfused isolated rat liver as well as *in vivo* animal studies have indicated that acyl glucuronidation is the dominant metabolic pathway for DMXAA, with a lesser contribution from 6-methylhydroxylation [28–31]. Enzyme mapping studies have indicated that DMXAA glucuronidation is catalyzed by UGT1A9 and UGT2B7 [32], and 6-methylhydroxylation by CYP1A2 [33] (Fig. 1). Like other acyl glucuronides, DMXAA-G undergoes β-glucuronidase-catalyzed and non-enzymatic hydrolysis, intramolecular migration and covalent binding to plasma proteins (Fig. 1) [28]. Therefore, any factors affecting DMXAA metabolism (glucuronidation plus 6-methylhydroxylation) and the disposition of its acyl glucuronide would result in alterations in its clearance. DMXAA is highly bound by human plasma proteins (mainly albumin) in a concentration-dependent manner, and ligand competition studies have indicated that both sites I and II are involved in DMXAA binding [34].

The aim of this study was to determine, using a human liver bank, the interindividual variations in the expression and activity of liver enzymes that metabolize DMXAA and to analyze the frequency distribution of the resulting kinetic parameters (K_m and V_{max}). In addition, interindividual variations in enzyme inhibition, hydrolysis of DMXAA-G by human plasma and hepatic microsomes, and the binding of DMXAA by plasma proteins in humans (both healthy volunteers and cancer patients) were examined. Using preclinical and clinical models, examination of the interindividual variability in the disposition of DMXAA and its major metabolites would likely be predictive of the way individual cancer patients would clear DMXAA. This information would help the physician to optimize the therapeutic regimen for individual patients and to achieve the maximal therapeutic response with minimal toxicity.

2. Materials and methods

2.1. Chemicals and reagents

DMXAA and 2,5-dimethylxanthenone-4-acetic acid (SN24350, as internal standard) were synthesized in the ACSRC [35]. DMXAA was protected from light exposure to avoid degradation 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 structure confirmed by mass spectrometry and [^{1}H]-nuclear magnetic resonance [29]. Bromocresol green, cyproheptadine, diclofenac, ANF, bicinchoninic acid reagent, Brij 58, and D-saccharic acid 1,4-lactone were obtained from the Sigma-Aldrich Co. α-Nicotinamide adenine dinucleotide phosphate (reduced form, NADPH) and uridine diphosphate glucuronic acid (UDPGA) were from Roche Diagnostics NZ Ltd. The Centrisart micropartition device with a 20,000 molecular weight cut-off

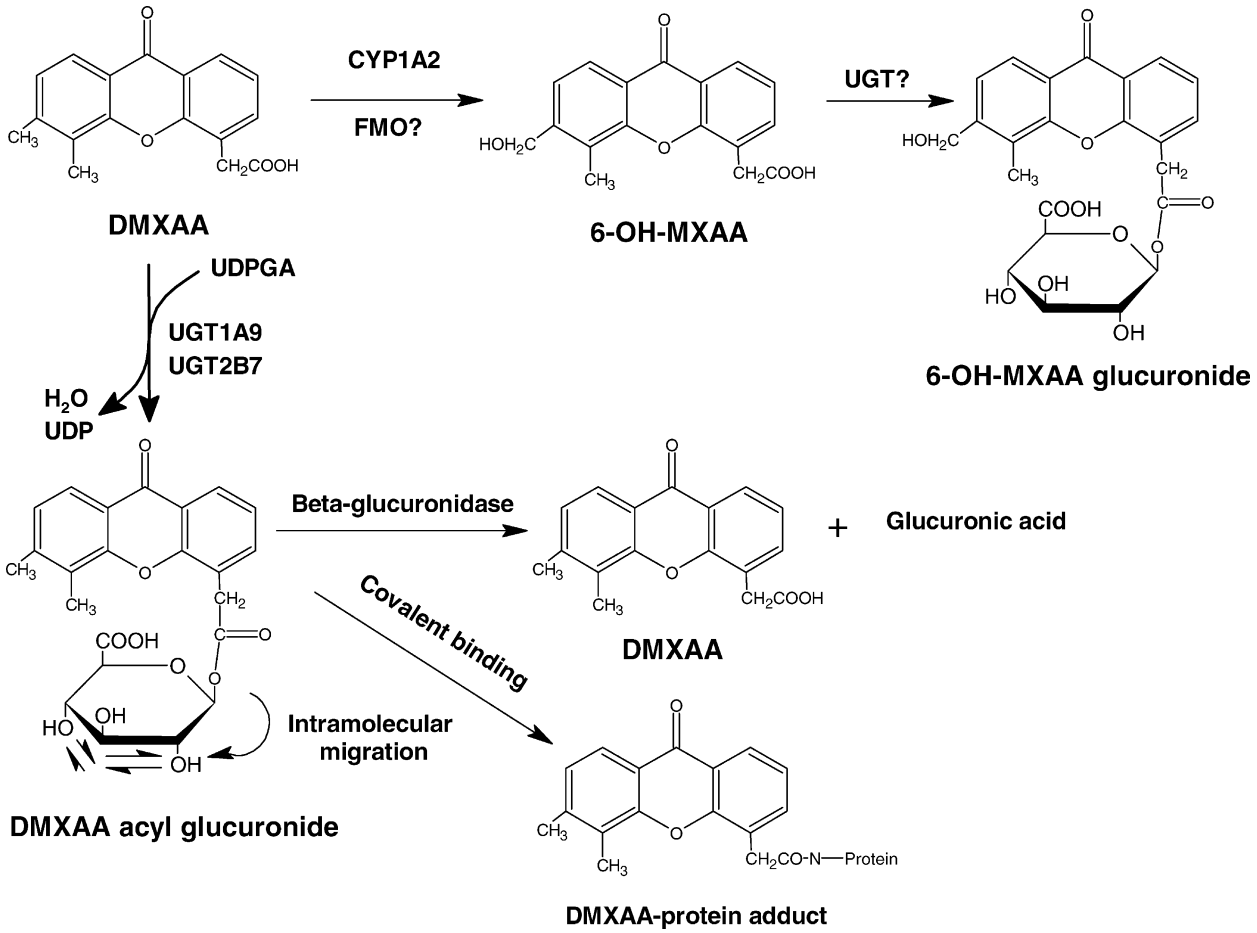


Fig. 1. Metabolism of DMXAA in humans.

was from Sartorious AG. The polyclonal antiserum raised in goat recognizing human CYP1A1/1A2 and UGT2B7 was obtained from Gentest Corp. Anti-goat or anti-rabbit immunoglobulin G (IgG) raised in donkey was purchased from ICN Biomedicals. Electrophoresis products were obtained from Bio-Rad. All other reagents were of analytical or HPLC grade.

2.2. Preparation of human plasma and liver microsomes

Fresh heparinized blood was obtained from healthy human volunteers (N = 17), and cancer patients (N = 5) in a Phase I DMXAA trial. Plasma from patients was sampled before the administration of DMXAA. Plasma was separated by centrifugation at 1000 *g* for 15 min at 37°. Human liver samples (N = 14) were donated by individuals who underwent liver resection for either metastasis of colon cancer or hydatid disease, and one liver sample was 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 [33]. Ethical approval from the Northern New Zealand Research Ethics Committee and written informed consent for liver tissues and blood used for research was obtained. The microsomes from human livers were prepared by differential centrifugation, as described previously [37]. Livers and microsomes were stored at −80° until used. Total protein concentrations in plasma and hepatic microsomes were determined using the bicinchoninic acid method [38]. Plasma albumin concentration was measured using the bromocresol green method [39]. The CYP content was determined as described [40].

2.3. In vitro kinetic and inhibition studies

The *in vitro* kinetic and inhibition studies of DMXAA glucuronidation and 6-methylhydroxylation with human liver microsomes were performed under optimized incubation conditions with respect to microsomal protein concentration, incubation time, and substrate concentration [33,41]. For glucuronidation, Brij 58 (0.1 to 0.25:1, ratio of Brij 58 to microsomal protein, w/w) was incubated with human liver 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 by diclofenac and cyproheptadine and 6-methylhydroxylation by ANF *in vitro* with various sources of human liver microsomes (N = 14) were

investigated. The concentrations of DMXAA were 140 μM for glucuronidation, and 20 μM for 6-methylhydroxylation (the apparent K_m values for each metabolism pathway in humans). Incubations were stopped by cooling on ice and adding 2 vol. of 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 22°) 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.

2.4. Plasma protein binding assay

Determination of the unbound fraction (f_u) of DMXAA in human plasma has been described previously [34,42]. DMXAA (500 μM) was incubated with human plasma for 30 min at 37° with shaking. This concentration was relevant to the *in vivo* plasma concentration. A 400 μL aliquot then was transferred to a Centrisart ultrafiltration device, centrifuged (2000 *g* for 30 min) at 37°, and the DMXAA concentration in the ultrafiltrate was determined by HPLC. A 100 μL sample of ultrafiltrate was mixed with 50 μL of 0.1 M phosphate buffer (pH 7.4) containing 10 μM internal standard, and 50 μL was injected into an HPLC column (see Section 2.6). A 100 μL aliquot of plasma was also taken to determine the total DMXAA concentration. The aliquot was mixed with 50 μL of methanol containing 20 μM internal standard, followed by 0.4 mL of ice-cold acetonitrile:methanol [3:1 (v/v)]. After centrifugation (2500 *g* for 15 min at 22°) to remove precipitated proteins, the supernatant was removed and evaporated to dryness under nitrogen. The residue was dissolved in 200 μL of mobile phase, and 50 μL was injected into the HPLC column.

2.5. Hydrolysis of DMXAA acyl glucuronide in human plasma and liver microsomes

In vitro studies of the degradation of DMXAA-G were performed by incubating samples containing 10 μM DMXAA-G and fresh human plasma (from 6 individuals) with either unheated or boiled (90° × 10 min) human liver microsomes (1 mg/mL; from 6 people, i.e. HL1–HL6) for up to 2 hr at 37°, in a water bath. Additional samples prepared for these experiments consisted of 10 μM DMXAA-G in 0.1 M phosphate buffer at pH 7.4, and 10 μM DMXAA-G in the same buffer but containing pooled human liver microsomes (1 mg/mL; from HL1–HL6) either with or without D-saccharic acid 1,4-lactone (20 mM). Aliquots (200 μL) of the incubation mixtures were taken at the following time points: 0, 10, 20, 30, 40, 60, 90, and 120 min. The reactions were stopped by the addition of 2 vol. of an ice-cold acetonitrile:methanol mixture [3:1 (v/v)] containing 2% acetic acid (v/v) and 2 μM IS and cooling on ice. All mixtures were centrifuged at 3500 *g* for 10 min at 22°; their supernatants were then collected and evaporated to dryness under nitrogen flow using a Speedvac. The residues were reconstituted with 200 μL of mobile phase, and injected into an HPLC column (see Section 2.6). All incubations were conducted in triplicate. DMXAA-G at 10 μM also was incubated at 37° in 0.1 M sodium phosphate buffer, pH 5.0, in a total volume of 300 μL for 30 min with either 2000 units/mL of β-glucuronidase or β-glucuronidase plus 20 mM D-saccharic acid 1,4-lactone. DMXAA-G was quantitated as described above.

2.6. HPLC and LC-MS

The determination of DMXAA-G and 6-OH-MXAA in liver microsomal samples by HPLC using fluorescence detection has been described previously [33,41]. Briefly, the HPLC system consisted of a solvent delivery system, a Model SF250 fluorescence detector (excitation and emission wavelength, 345 and 409 nm, respectively), a Model 460 autosampler, and a Model D450 data processing system (All from 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. The LC-MS identification of the metabolites formed in liver microsomes has been described previously [28,33]. The LC-MS system was fitted with either an atmospheric pressure chemical ionization or electrospray interface (Hewlett Packard). The mobile phase was the same as used for HPLC.

2.7. Immunoblot assay

SDS-PAGE was performed according to Laemmli [43]. After electrophoresis, the separated microsomal proteins were transferred to Hybond-P membranes (Amersham). Detection of CYP1A2 or UGT2B7 was carried out using either a primary antibody recognizing human CYP1A1/1A2 raised in goat, using rat CYP1A1 as an immunogen, or human UGT2B7 raised in rabbit. Either a biotinylated donkey anti-goat or anti-rabbit IgG was used as the secondary antibody. Streptavidin-horseradish peroxidase was then added to bind the secondary antibody and a light signal was developed using the ECL system (Amersham). The intensity of the signals 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-lymphoblast microsomes were used as standard.

2.8. Data analysis

Data are presented as means ± SD. Several models to describe the *in vitro* kinetics of DMXAA glucuronidation and 6-methylhydroxylation (single and two binding site, substrate–activator and substrate–inhibitor complex formation, and the sigmoid models) in human liver microsomes were fitted and compared using the Prism 3.0 program (Graphpad Software Co.) as described [33]. 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 nonlinear regression analysis, and by comparison of the Akaike's information criterion [44]. 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 analysis of variance with a Tukey–Kramer test. The IC_{50} (the concentration inhibiting 50% of enzyme activity) was determined by linear regression (% of control) against the logarithmic inhibitor concentration. The apparent K_i values and the nature of inhibition initially were estimated by Dixon plots, and then confirmed by fitting and comparing several inhibition models (competitive, uncompetitive, and mixed inhibition). The degradation half-life ($T_{1/2}$) was calculated as 0.693/k, where k is the degradation rate constant calculated by iterative nonlinear regression analysis of the measured data using the equation for first order reaction kinetics:

$$C_t = C_{(0)}\, e^{-kt}$$

where C_t is the concentration of DMXAA-G at time (t) after incubation; $C_{(0)}$ is the initial concentration of DMXAA-G; and k is the first order degradation constant.

3. Results

3.1. Interindividual variation in the protein levels of DMXAA-metabolizing enzymes

There was an approximately 5-fold interindividual variation in the total CYP content of 14 human liver samples (means ± SD: 0.430 ± 0.227 nmol/mg protein, range: 0.218–0.928 nmol/mg protein). As shown in Fig. 2, there was a 6- and 5-fold interindividual variation in the enzyme levels of CYP1A2 and UGT2B7, respectively as determined by immunoblot analysis in the 14 human livers. For CYP1A2, the highest level was observed in HL7 and HL1 and the lowest in samples HL2 and HL5; whereas the

Fig. 2. Protein levels of CYP1A2 and UGT2B7 as determined by immunoblot analysis in 14 liver samples. Human liver microsomes (15 μg) were loaded on a 10% SDS-polyacrylamide gel, electrophoresed, and transferred to a PVDF membrane. The blotted membrane was incubated with the antibody against human CYP1A1/1A2 or UGT2B7, and then incubated with a biotinylated secondary antibody. Light signals generated by the ECL method were captured on X-ray film and band signal intensity was determined. Note that recombinant CYP1A2 and UGT2B7 were used as standards (A, 1.5 μg; B, 15 μg).

Table 1
Interindividual variation in the expression and activity of DMXAA-metabolizing enzyme from 14 human livers, as determined by immunoblot analysis

Parameters	Mean	Range	Fold
Enzyme expression			
Total CYP content (nmol/mg protein)	0.430 ± 0.227	0.218–0.928	4.5
CYP1A2 (arbitrary unit)	1.8 ± 0.6	0.5–2.9	5.8
UGT2B7 (arbitrary unit)	1.7 ± 0.5	0.5–2.5	5.0
Enzyme activity			
Glucuronidation			
K_m (μM)	143 ± 79	62–317	5.1
V_{max} (nmol/min/mg protein)	0.71 ± 0.52	0.23–1.77	7.7
CL_{int} (mL/min/g)	4.97 ± 6.39	1.83–18.06	9.9
Hydroxylation			
K_m (μM)	21 ± 5	11–31	3.0
V_{max} (nmol/min/mg protein)	0.04 ± 0.02	0.01–0.09	9.0
CL_{int} (mL/min/g protein)	2.05 ± 1.03	0.52–5.50	10
Total CL_{int} (mL/min/g protein)	8.12 ± 4.04	4.33–20.79	4.8

The parameters reported are expressed as the means ± SD from three determinations in microsomal preparations from 14 human livers. Glucuronidation activity was determined in Brij 58-activated microsomes.

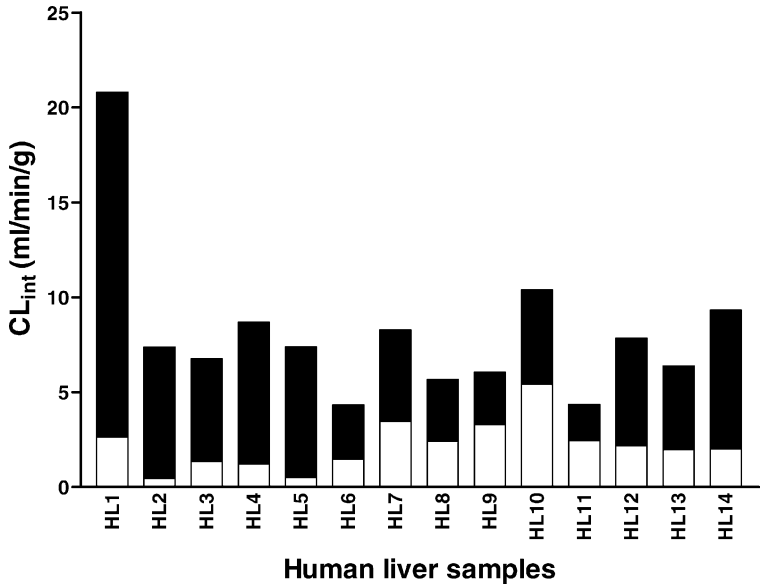


Fig. 3. Interindividual variation in the intrinsic clearance (CL_{int}) values for DMXAA by 6-methylhydroxylation (□), and glucuronidation (■) in 14 human liver samples. Data are the means ± SD of three determinations.

highest level of UGT2B7 was seen in HL1 and HL10, and the lowest in HL2 and HL13.

3.2. Interindividual variation in the metabolism of DMXAA in human liver microsomes

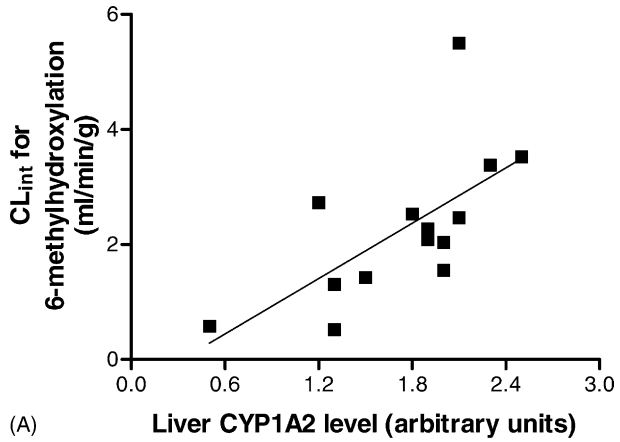
In the 14 human livers analyzed, both glucuronidation and 6-methylhydroxylation of DMXAA followed single-site Michaelis–Menten kinetics, with a wide interindividual variation in kinetic parameters (Table 1). For 6-methylhydroxylation, a 3- and 6-fold interindividual variation in the apparent K_m and V_{max} values, respectively, were observed. In addition, about a 10-fold interindividual variation in the 6-methylhydroxylation CL_{int} was observed. For glucuronidation, 5-, 8-, and 10-fold variations were observed for K_m, V_{max} and CL_{int}, respectively. Fig. 3 shows the interindividual variability in the intrinsic clearance of DMXAA in 14 human livers. When combining the CL_{int} values for both glucuronidation and 6-methylhydroxylation for each liver, a 5-fold variation was observed.

Table 2 summarizes the kinetic parameters and CYP1A2/UGT2B7 protein levels in 14 human liver microsomes. Correlation studies indicated that there was a significant relationship between the CL_{int} of DMXAA by

Table 2
Determined kinetic parameters for DMXAA 6-methylhydroxylation and glucuronidation in 14 human liver microsomes

Human livers	6-Methylhydroxylation				Glucuronidation			
	K_m (μM)	V_{max} (nmol/min/mg)	Cl_{int} (μL/min/g)	CYP1A2 level	K_m (mM)	V_{max} (nmol/min/mg)	CL_{int} (mL/min/g)	UGT2B7
HL1	11 ± 1	0.03 ± 0.00	2.73 ± 0.25	2.9 ± 0.2	62 ± 11	1.12 ± 0.05	18.06 ± 3.10	2.4 ± 0.2
HL2	23 ± 3	0.01 ± 0.00	0.52 ± 0.07	1.3 ± 0.3	258 ± 113	1.77 ± 0.32	6.86 ± 2.71	2.5 ± 0.3
HL3	23 ± 2	0.03 ± 0.00	1.43 ± 0.13	1.5 ± 0.3	109 ± 13	0.58 ± 0.03	5.32 ± 0.56	2.1 ± 0.2
HL4	26 ± 4	0.03 ± 0.00	1.31 ± 0.21	0.7 ± 0.1	234 ± 76	1.73 ± 0.14	7.39 ± 1.36	2.2 ± 0.3
HL5	31 ± 5	0.02 ± 0.00	0.58 ± 0.10	0.5 ± 0.1	166 ± 28	1.13 ± 0.08	6.81 ± 0.93	2.3 ± 0.3
HL6	20 ± 3	0.03 ± 0.00	1.55 ± 0.24	1.3 ± 0.2	108 ± 15	0.30 ± 0.05	2.78 ± 1.38	1.7 ± 0.2
HL7	15 ± 2	0.05 ± 0.00	3.53 ± 0.68	2.5 ± 0.2	80 ± 11	0.38 ± 0.02	4.75 ± 1.74	1.5 ± 0.1
HL8	17 ± 1	0.04 ± 0.00	2.47 ± 0.16	2.1 ± 0.3	72 ± 11	0.23 ± 0.01	3.19 ± 1.50	1.5 ± 0.2
HL9	16 ± 1	0.05 ± 0.00	3.38 ± 0.22	2.3 ± 0.2	86 ± 6	0.23 ± 0.01	2.77 ± 0.39	0.5 ± 0.1
HL10	16 ± 1	0.09 ± 0.00	5.50 ± 0.35	2.1 ± 0.2	86 ± 7	0.42 ± 0.01	4.88 ± 0.59	1.4 ± 0.1
HL11	17 ± 1	0.04 ± 0.00	2.53 ± 0.16	1.2 ± 0.2	317 ± 111	0.58 ± 0.11	1.83 ± 0.99	1.5 ± 0.2
HL12	26 ± 2	0.06 ± 0.00	2.27 ± 0.18	1.9 ± 0.2	133 ± 23	0.74 ± 0.05	5.56 ± 1.34	1.4 ± 0.2
HL13	24 ± 2	0.05 ± 0.00	2.04 ± 0.18	2.0 ± 0.2	131 ± 9	0.57 ± 0.01	4.35 ± 0.38	1.6 ± 0.2
HL14	22 ± 2	0.05 ± 0.00	2.09 ± 0.20	1.9 ± 0.1	83 ± 5	0.60 ± 0.01	7.23 ± 0.56	1.4 ± 0.1
Mean ± SD	21 ± 5	0.04 ± 0.02	2.05 ± 1.03	1.8 ± 0.6	143 ± 79	0.71 ± 0.52	4.97 ± 6.39	1.7 ± 0.5

The parameters reported are expressed as the means ± SD from three determinations in microsomal preparations from 14 human livers. The units for CYP1A2 and UGT2B7 are arbitrary.



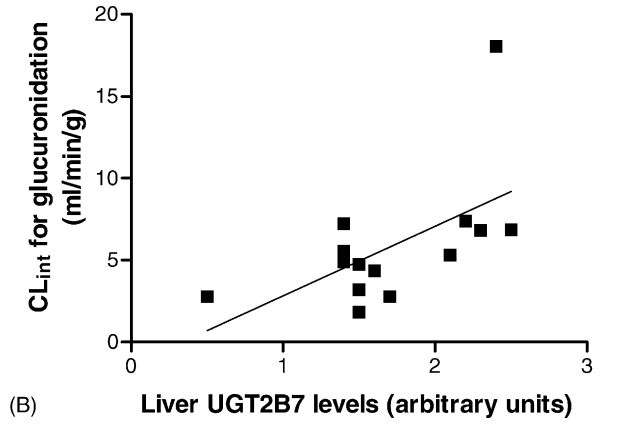


Fig. 4. Relationship between the CL_{int} of DMXAA by 6-methylhydroxylation and CYP1A2 protein levels (A) and between the CL_{int} by glucuronidation and UGT2B7 protein levels (B). Data are the means from three determinations.

6-methylhydroxylation and CYP1A2 levels ($r = 0.6540$, $P = 0.0112$) (Fig. 4), and between the CL_{int} by glucuronidation and UGT2B7 protein levels ($r = 0.5768$, $P = 0.0308$). As shown in Fig. 5, frequency distribution analysis of K_m and V_{max} for DMXAA glucuronidation and 6-methylhydroxylation in 14 human liver microsome samples showed a relatively normal distribution. The median K_m and V_{max} values for DMXAA glucuronidation and 6-methylhydroxylation were 109 μM, 0.58 nmol/min/mg and 21 μM, 0.043 nmol/min/mg, respectively. These values were close to the corresponding mean values.

3.3. Interindividual variation in the inhibition of DMXAA metabolism in human liver microsomes

There were marked interindividual variations in the inhibition of DMXAA glucuronidation by diclofenac and cyproheptadine and in 6-methylhydroxylation by ANF in the 14 human liver samples. A 5-, 6-, and 9-fold variation was observed in the IC_{50} values for diclofenac, cyproheptadine, and ANF, respectively (IC_{50} for diclofenac: 5.5 ± 3.0 μM, range: 2.8–13.5 μM; cyproheptadine: 127 ± 59 μM, range: 35–220 μM; ANF: 0.027 ± 0.017 μM, range: 0.007–0.063 μM). There was a significant correlation between the level of CYP1A2 protein and the ANF IC_{50} for DMXAA 6-methylhydroxylation ($r = 0.5358$, $P < 0.05$). There was also a significant correlation between the level of UGT2B7 protein and the IC_{50} for diclofenac and cyproheptadine for DMXAA glucuronidation (diclofenac: $r = 0.8103$, $P < 0.001$; cyproheptadine: $r = 0.8817$, $P < 0.001$) (Fig. 6).

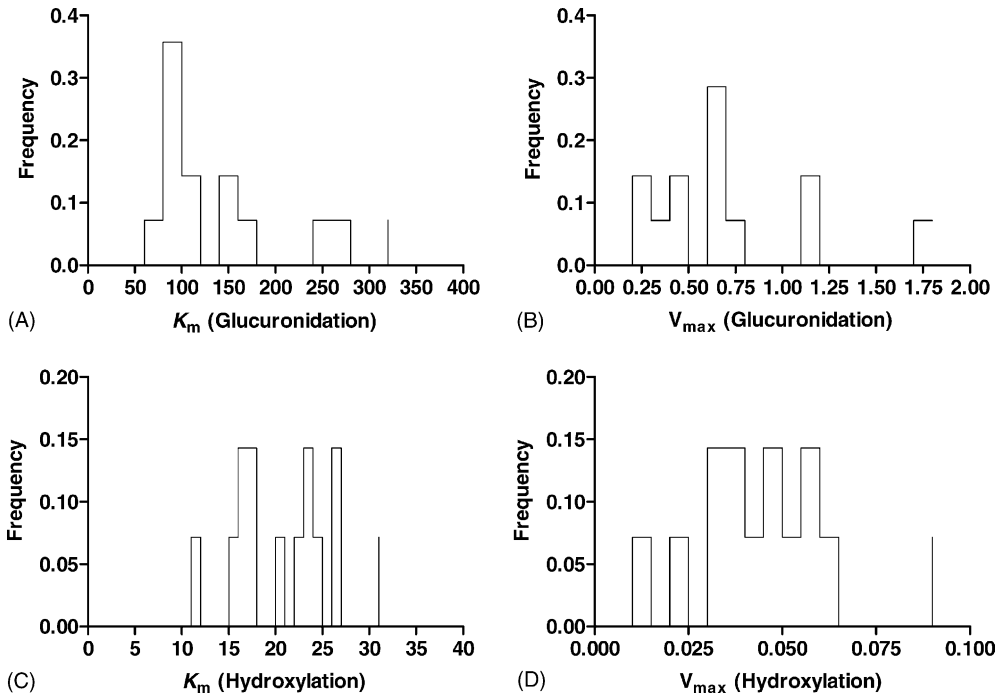


Fig. 5. Frequency distribution of kinetic parameters for DMXAA glucuronidation (A and B) and 6-methylhydroxylation (C and D) in 14 human livers.

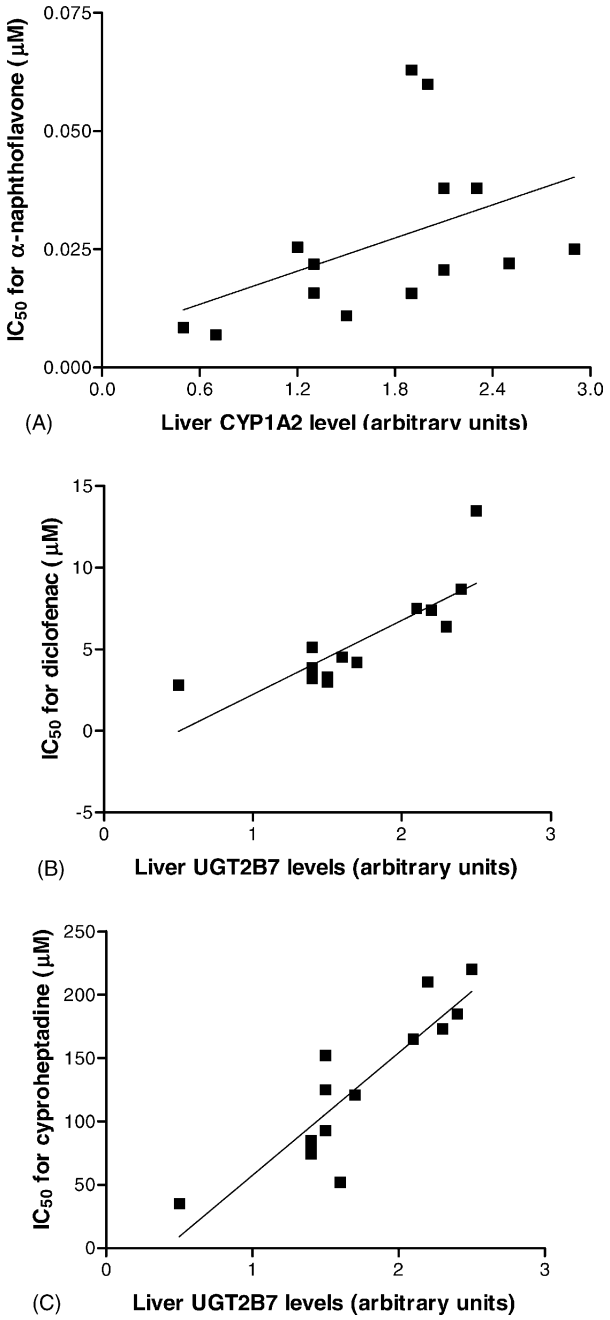


Fig. 6. Relationship between the IC_{50} for α-naphthoflavone and the level of CYP1A2 protein (A) and between the IC_{50} for diclofenac and cyproheptadine and the level of UGT2B7 protein (B and C). Data are means from three determinations.

3.4. Interindividual variation in the hydrolysis of DMXAA acyl glucuronide in human liver microsomes

As shown in Fig. 7A, DMXAA-G was relatively unstable in 0.1 M phosphate buffer at pH 7.4, undergoing fast non-enzymatic hydrolysis and to a lesser extent intramolecular rearrangement, with a degradation half-life of 65.4 min. The addition of 1 mg/mL of human liver microsomes accelerated the degradation of DMXAA-G, with a half-life of 48.1 min, but the accelerated hydrolysis was prevented completely by the addition of the β-glucuronidase inhibitor D-saccharic acid 1,4-lactone (Fig. 7A). Approximately 36% of the DMXAA-G hydrolysis in human liver microsomes was enzymatic. There was a 3-fold variation in the half-life of DMXAA-G among the human liver samples ($t_{1/2} = 47.6 \pm 12.3$ min, $N = 6$; range: 22.6–57.3) (Fig. 7B). In boiled human liver microsomes ($N = 6$), the degradation half-life of DMXAA-G was prolonged to 57.3 ± 5.8 min (range: 45.2–60.1 min), with a smaller variation (1.3-fold) in the half-life (Fig. 7C). In addition, DMXAA-G was readily degraded to form the parent molecule and various isomers in human plasma, with a mean half-life of 36.2 ± 5.1 min and a variation of 1.4-fold (range 31.6–43.4 min) (Fig. 7D).

3.5. Interindividual variation in the binding of DMXAA by plasma proteins

There was a 2- and 3-fold variation in the unbound DMXAA fraction in fresh plasma from healthy humans and cancer patients, respectively (Table 3). The unbound DMXAA fraction in healthy humans was significantly less than that observed in cancer patients. Plasma albumin concentrations varied considerably between healthy humans and cancer patients.

4. Discussion

Our results indicate that there are significant (5- to 6-fold) interindividual variations in the levels of major enzymes involved in the biotransformation of DMXAA (see Fig. 2, CYP1A2/UGT2B7). For a given substrate, the higher the enzyme content, the greater is the catalytic activity. Thus, there is a correlation between DMXAA glucuronidation activity and the UGT2B7 level and between DMXAA 6-methylhydroxylation activity and the CYP1A2 level. Genetic polymorphisms in UGT2B7 [45] and CYP1A2 [8] have been reported, perhaps contributing to the pharmacokinetic variability of drugs that are metabolized by these enzymes. However, UGT2B7 polymorphisms would have a small impact on the variability of DMXAA clearance among patients, as human UGT2B7(H^{268}) and, its allelic variant, UGT2B7(Y^{268}) are distributed equally in the Caucasian population, with a frequency of 26 and 24%, respectively, and at approximately 50% for heterozygotes [45]. However, it remains likely that the marked interindividual variability in the metabolism of DMXAA is due, in part, to the large differences in the glucuronidation activity of the two UGT2B7 variants, to other uncharacterized polymorphisms of the *UGT2B7* gene, or to the UGT1A9 enzyme. UGT1A9, UGT2B7, and CYP1A2 are present also in extrahepatic tissues such as the intestine and kidneys, and interindividual variations in the levels of these extrahepatic enzymes have been reported by others [8,9,46–48]. When these extrahepatic enzymes contribute substantially to the net clearance of

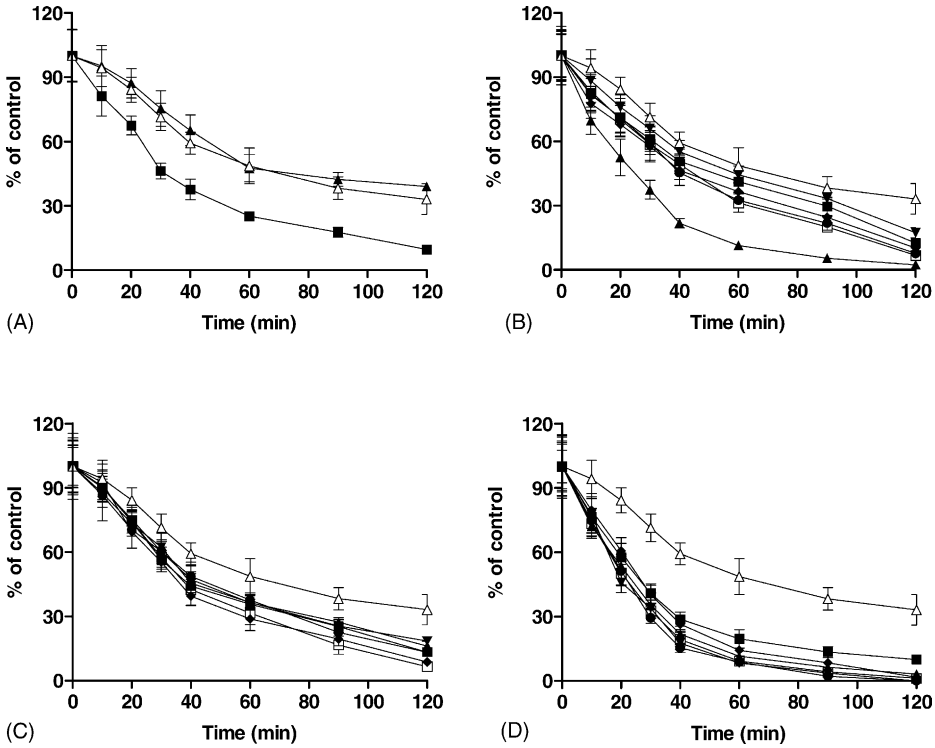


Fig. 7. Degradation of the DMXAA acyl glucuronide (DMXAA-G; 10 μM). (A) DMXAA-G in 0.1 M phosphate buffer, pH 7.4 alone (△) and in the presence of pooled human liver microsomes (1 mg/mL) either with (▲) or without D-saccharic acid 1,4-lactone (■). (B) DMXAA-G in phosphate buffer alone or containing human liver microsomes (1 mg/mL) from HL1–HL6. (C) DMXAA-G in phosphate buffer alone or containing boiled liver microsomes (1 mg/mL) from HL1–HL6. (D) DMXAA-G in phosphate buffer alone and in the presence of human plasma from 6 individuals. DMXAA-G (10 μM) was incubated with human plasma or liver microsomes in 0.1 M phosphate buffer, pH 7.4, at 37° over a 2 hr period. Data are the means ± SD from three determinations. Symbols in (B–D): 0.1 M phosphate buffer, pH 7.4 (△), HL1 (■), HL2 (▲), HL3 (▼), HL4 (◆), HL5 (●), and HL6 (□). The control is defined as the concentration of DMXAA-G at zero time (i.e. 10 μM).

Table 3
Unbound DMXAA fraction (f_u) in healthy human and cancer patient plasma

No.	Sex/age (year)	f_u (%)	Albumin conc. (g/L)	Total protein conc. (g/L)	N
Fresh healthy human plasma					
1	M/38	1.55 ± 0.09	47.87 ± 3.22	75.35 ± 5.89	3
2	M/35	1.65 ± 0.73	47.20 ± 3.51	74.38 ± 2.46	3
3	M/45	2.18 ± 0.18	41.56 ± 0.98	73.21 ± 3.26	3
4	M/25	2.17 ± 0.18	41.73 ± 5.12	72.98 ± 7.75	3
5	F/37	2.97 ± 0.22	37.44 ± 1.80	67.45 ± 3.13	3
6	F/32	1.87 ± 0.38	44.56 ± 7.73	73.21 ± 4.73	3
7	M/47	2.13 ± 0.21	43.29 ± 2.12	72.45 ± 5.12	3
8	F/52	1.86 ± 0.22	46.21 ± 3.12	73.23 ± 3.27	3
9	F/29	2.02 ± 0.17	42.18 ± 1.78	74.83 ± 5.23	3
10	F/32	1.93 ± 0.32	43.28 ± 4.12	71.89 ± 4.27	3
11	F/37	1.75 ± 0.14	47.22 ± 1.85	75.34 ± 3.98	3
Mean ± SD (range)		2.07 ± 0.26 (1.55–2.97)	43.87 ± 6.96 (37.44–47.87)	73.12 ± 4.87 (67.45–75.35)	3
Cancer patient plasma					
1	F/48	5.32 ± 0.72	28.53 ± 3.78	54.35 ± 4.48	3
2	M/57	3.94 ± 0.15	40.01 ± 1.45	67.38 ± 2.21	3
3	F/51	7.29 ± 0.06	30.16 ± 2.23	56.21 ± 3.04	3
4	F/40	2.77 ± 0.58	38.85 ± 6.54	64.98 ± 5.76	3
5	F/37	3.69 ± 0.61	37.58 ± 4.69	62.45 ± 3.88	3
Mean ± SD (range)		4.60 ± 0.42[a] (2.77–7.29)	35.02 ± 9.15[a] (28.53–0.01)	61.07 ± 5.29[a] (54.35–67.38)	3

Data are expressed as the means ± SD from three determination.
[a] $P < 0.05$ compared with fresh healthy human plasma.

DMXAA one would expect to see an increase in the variation of DMXAA clearance among individuals. Pathological conditions such as hepatic and renal diseases may increase the interindividual variability in the disposition of DMXAA, as the expression and activities of various UGT/CYP enzymes may be altered under these conditions [49].

The magnitude (8- to 9-fold) of the interindividual variation in DMXAA glucuronidation or 6-methylhydroxylation activities in 14 human livers was consistent with that for DMXAA-metabolizing enzymes for each reaction. A similar magnitude (0.5 to 1 order) has been observed with other UGT2B7 substrates such as 3′-azido-3′-deoxythymidine [47,50] and (*S*)- and (*R*)-oxazepam [51]. A typical bimodal distribution for the apparent K_m values of (*S*)-oxazepam glucuronidation in 37 human livers has been observed, and consistently 10% of Caucasian individuals with slower glucuronidation of (*S*)-oxazepam has been reported [51]. A normal distribution of DMXAA kinetic parameters may reflect a lack of genetic polymorphisms, but other factors such as the involvement of multiple enzymes and a small number of liver samples also should be considered. The marked (5-fold) variability of K_m values for DMXAA glucuronidation may be due to the fact that DMXAA is glucuronidated by both UGT1A9 and UGT2B7 with K_m values of 33 and 95 μM, respectively [32], but their relative contribution may vary depending on the individual and substrate concentration. The latency issue and potential for a UGT1A9 and 2B7 interaction (e.g. dimerization) also may complicate the interpretation of the glucuronidation kinetic data. The marked interindividual variability in DMXAA 6-methylhydroxylation can be due to the fact that the *CYP1A2* gene is subject to inhibition and/or induction by a number of environmental factors, while genetic polymorphisms may play only a minor role [52–54].

DMXAA, as a biological response modifier, is likely to be combined with other anticancer agents, causing a potential for metabolic drug interactions and the alteration of its clearance. *In vitro* microsomal studies have found that some anticancer drugs such as vinblastine and vincristine inhibited the glucuronidation of DMXAA whereas *N*-[2-(dimethylamino)-ethyl]acridine-4-carboxamide (a mixed topoisomerase inhibitor) inhibited its 6-methylhydroxylation [55]. Thus, it would be expected that the interindividual variability of DMXAA clearance could be enhanced by the co-administration of other anticancer drugs. However, the impact of co-administered drugs on DMXAA clearance will be dependent upon the phamacological properties of the co-administered drugs (e.g. inhibitory potency) and the biochemical profile of the patient (e.g. DMXAA-metabolizing enzyme levels). The present study has indicated that there is approximately one order of magnitude of interindividual variation in the inhibition of DMXAA glucuronidation by diclofenac and cyproheptadine, and 6-methylhydroxylation by ANF, with the inhibition potency (as indicated by the IC_{50}) associated with the levels of DMXAA-metabolizing enzymes (UGT2B7 and CYP1A2). Indeed, a significant correlation between the extent of inhibition by sulfaphenazole or ketoconazole and the protein levels of CYP2C9 and CYP3A4 (catalyze dapsone *N*-hydroxylation) has been reported [56]. However, caution should be taken when suggesting that a greater inhibitory effect of a co-administered drug on DMXAA metabolism is likely to occur in those individuals with low levels of CYP1A2/UGT2B7, since an increase in the IC_{50} value of diclofenac for DMXAA glucuronidation, as a function of UGT2B7 level, might be due to the simultaneous metabolic depletion of diclofenac by this enzyme; although the IC_{50} value for cyproheptadine, which is a UGT1A4 substrate [57], is also correlated with the level of UGT2B7. Genetic polymorphisms can also contribute to the interindividual variability observed in response to enzyme inhibition, e.g. quinidine exhibits a significant inhibitory effect on CYP2D6-mediated encainide metabolism in subjects with the extensive metabolism phenotype for debrisoquine, but it does not inhibit encainide metabolism in subjects with a poor metabolism phenotype, since in poor metabolizers there is no active CYP2D6 enzyme for which quinidine and encainide can compete [58]. In addition to enzyme levels, other factors such as the variability in the concentration of the inhibitor among individuals and the kinetic properties of drugs (high or low clearance) also contribute to the interindividual variations in response to enzyme inhibition [16]. A high-clearance drug is subject to extensive first-pass metabolism and is more sensitive to enzyme inhibition than a low-clearance drug. DMXAA is a low-clearance drug [17], thus, its metabolism might not be sensitive to enzyme inhibition. Therefore, it would be difficult to predict the change in area under the plasma concentration–time curve of DMXAA in patients administered drugs that modulate the metabolism of DMXAA due to marked interindividual variations in enzyme inhibition.

Since the accelerated degradation of DMXAA-G in human liver microsomes was completely inhibited by D-saccharic acid 1,4-lactone, the 3-fold variation in the degradation rate of DMXAA-G in 6 human livers was considered to be due mainly to the variability in the level of microsomal β-glucuronidase. β-Glucuronidase can cleave DMXAA-G, releasing the parent molecule, thereby modifying DMXAA clearance. Indeed, a marked (6-fold) interindividual variability in the activity and expression of human β-glucuronidase in the liver and kidneys has been observed [59]. It is likely that the variations in the activity and expression of β-glucuronidase *in vivo* could increase the interindividual variability in DMXAA disposition. In addition to conjugation–deconjugation recycling, via hydrolysis of the reactive DMXAA-G in the blood (systematic recycling), the variability in DMXAA disposition may be enhanced by entero-hepatic recycling and plasma-mediated hydrolysis [6,60]. However, further studies are required to examine the interindividual variability of DMXAA-G disposition in humans.

Our study has demonstrated that there is a 2- to 3-fold variation in the binding of DMXAA by plasma proteins in healthy humans and cancer patients. This variability in binding among individuals is due mainly to qualitative and quantitative differences in the binding protein (mainly albumin). The magnitude of the interindividual variability in drug binding by plasma proteins is generally lower than that seen in other pharmacokinetic processes such as metabolism [61]. An increase in the plasma f_u of DMXAA in cancer patients will in theory facilitate the distribution of DMXAA out of the plasma compartment and therefore increase the apparent volume of distribution, and also increase the plasma clearance (based on total concentrations). However, the DMXAA free concentration will remain relatively unchanged, in the absence of any alteration in intrinsic clearance. Thus, the pharmacodynamic effect (such as anticancer activity) of DMXAA would not be altered by plasma binding changes.

The results from the present study indicated that variable enzyme activities for DMXAA metabolism were closely correlated to enzyme expression as assessed by immunoblot analysis. The interindividual variability in the DMXAA clearance would be mainly due to the variations from its metabolism and metabolic inhibition by co-administered drugs, and to a lesser extent from the enzyme-catalyzed hydrolysis of its acyl glucuronide, whereas variation in its plasma protein binding may play a minor role. The interindividual variability of DMXAA disposition observed *in vitro* might reflect the greater elimination variability in Phase I cancer patients. Further study is needed to examine the genotype–phenotype relationship, and the results together with therapeutic drug monitoring may provide a useful strategy for optimizing DMXAA treatment. However, the therapeutic outcome of DMXAA not only is governed by its pharmacokinetics, but also by some other factors related to its pharmacodynamics. For example, genetic polymorphisms in TNF-α and its receptor have been reported and have been associated with cancer and autoimmune diseases [62–64]. Given that the induction of cytokines such as TNF-α is pivotal for the anticancer efficacy of DMXAA [17–19], it would be necessary to explore the genetic variations in TNF-α and its receptor in patients if we wish to accurately predict the therapeutic outcome.

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