# Effects of anticancer drugs on the metabolism of the anticancer drug 5,6-dimethylxanthenone-4-acetic (DMXAA) by human liver microsomes

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*Aims* To investigate the effects of various anticancer drugs on the major metabolic pathways (glucuronidation and 6-methylhydroxylation) of DMXAA in human liver microsomes.

*Methods* The effects of various anticancer drugs at 100 and 500 μM on the formation of DMXAA acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA) in human liver microsomes were determined by high performance liquid chromatography (h.p.l.c.). For those anticancer drugs showing significant inhibition of DMXAA metabolism, the inhibition constants ($K_i$) were determined. The resulting *in vitro* data were extrapolated to predict *in vivo* changes in DMXAA pharmacokinetics.

*Results* Vinblastine, vincristine and amsacrine at 500 μM significantly ($P < 0.05$) inhibited DMXAA glucuronidation ($K_i = 319$, 350 and 230 μM, respectively), but not 6-methylhydroxylation in human liver microsomes. Daunorubicin and *N*-[2-(dimethylamino)-ethyl]acridine-4-carboxamide (DACA) at 100 and 500 μM showed significant ($P < 0.05$) inhibition of DMXAA 6-methylhydroxylation ($K_i = 131$ and 0.59 μM, respectively), but not glucuronidation. Other drugs such as 5-fluoroucacil, paclitaxel, tirapazamine and methotrexate exhibited little or negligible inhibition of the metabolism of DMXAA. Pre-incubation of microsomes with the anticancer drugs (100 and 500 μM) did not enhance their inhibitory effects on DMXAA metabolism. Prediction of DMXAA–drug interactions *in vivo* based on these *in vitro* data indicated that all the anticancer drugs investigated except DACA appear unlikely to alter the pharmacokinetics of DMXAA, whereas DACA may increase the plasma AUC of DMXAA by 6%.

*Conclusions* These results indicate that alteration of the pharmacokinetics of DMXAA appears unlikely when used in combination with other common anticancer drugs. However, this does not rule out the possibility of pharmacokinetic interactions with other drugs used concurrently with this combination of anticancer drugs.

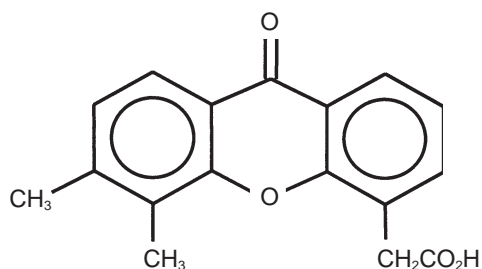*Keywords:* anti-cancer, DMXAA, drug interaction, human liver microsomes

## Introduction

5,6-Dimethylxanthenone-4-acetic acid (DMXAA) (Figure 1) is an anticancer drug with an unusual

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mechanism of action compared with conventional cytotoxic anticancer drugs. DMXAA induces rapid vascular collapse and necrosis in transplantable murine tumours, thought to be due to immune modulation and the induction of cytokines, in particular tumour necrosis factor-α, interferons, serotonin and nitric oxide [1–4]. Co-administration of DMXAA with other drugs has been shown to result in enhanced antitumour activity and alterations in pharmacokinetics, as reported for the combination of DMXAA with melphalan, thalidomide,

**Figure 1** The structure of DMXAA.

and the bioreductive agent tirapazamine, in mouse models [5–8]. These results suggest that coadminstration of DMXAA with other anticancer drugs may be a useful strategy for enhancing their antitumour activity. DMXAA is extensively metabolized, mainly by glucuronidation of its acetic acid side chain and 6-methylhydroxylation [9–11], giving rise to DMXAA acyl glucuronide (DMXAA-G), and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA), which are excreted into bile and urine. Studies have indicated that DMXAA glucuronidation is catalysed by uridine diphosphate glucuronosyltransferases (UGT1A9 and UGT2B7) [10], and 6-methylhydroxylation by cytochrome P450 (CYP1A2) [12].

The aim of this study was to investigate the effects of various anticancer drugs on the metabolism of DMXAA in human liver microsomes, and if significant inhibition is observed, to predict *in vivo* DMXAA–drug pharmacokinetic interactions.

## Methods

### Chemicals and reagents

DMXAA, 2,5-dimethylxanthenone-4-acetic acid (as internal standard), *N*-[2-(dimethylamino)-ethyl]acridine-4-carboxamide (DACA), and amsacrine were synthesized in the Auckland Cancer Society Research Centre (ACSRC) as described [13, 14]. DMXAA was protected from light exposure to avoid degradation [15]. Authentic DMXAA-G and 6-OH-MXAA were isolated and purified by a solid phase extraction method from the bile and urine of rats treated with DMXAA. Both metabolites had a purity of 99% as determined by h.p.l.c, and their structure was confirmed by mass spectrometry and [$^1$H]-nuclear magnetic resonance [11]. Daunorubicin, 5–fluorouracil, paclitaxel, cisplatin, tirapazamine, irinotecan, methotrexate, melphalan, 6-thioguanine, 6-mercaptopurine, cyclophosphamide, folic acid, vinblastine, vincristine and 6-methylguanine were purchased from Sigma-Aldrich Chemical Co. (Auckland, NZ). Uridine diphosphate glucuronic acid (UDPGA) and NADPH were purchased from Roche Diagnostics NZ Ltd. (Auckland, NZ). All

other reagents were of analytical or h.p.l.c grade as appropriate.

### Preparation of human liver microsomes

Human liver samples were obtained under strict ethical conditions from donors, and were stored at −80°C prior to use. Histological examination of the resected livers ensured the use of healthy liver tissue. Relevant details of the donors have been described elsewhere [12]. Ethical approval from the Northern New Zealand Research Ethics Committee and written informed consent for liver tissue to be used for research was obtained. Liver microsomes were prepared by differential centrifugation as described [16] and microsomes were stored at −80°C until used. Microsomal fractions used in this study were from HL6, HL7, HL8, HL12, HL13 and HL14 from our human liver bank. Microsomal protein concentration was determined by the bicinchoninic acid method [17]. The CYP content was determined as described [18].

### In vitro *metabolic inhibition studies*

The effects of a number of anticancer drugs (100 and 500 μM) on DMXAA glucuronidation and 6-methyl-hydroxylation in human liver microsomes were investigated using optimized incubation conditions [12]. Typical incubations (total volume = 200 μl, in triplicate) for DMXAA glucuronidation contained liver microsomal protein (0.1 mg ml$^{-1}$, pooled from HL6, HL7 and HL8), 10 mM UDPGA, 5 mM MgCl$_2$, 0.1 mg ml$^{-1}$ D–saccharic acid 1,4-lactone, Brij 58 (0.1–0.25 : 1, ratio of Brij 58 over microsome, w/w), inhibitor (100 and 500 μM), and DMXAA in 0.1 M phosphate buffer (pH 6.8). D–Saccharic acid 1,4-lactone was used to inhibit the activity of β-glucuronidase in microsomes. Typical incubations (total volume = 200 μl, in triplicate) for 6-methylhydroxy-lation contained 1 mg ml$^{-1}$ liver microsomal protein (from three human livers, HL12, HL13, and HL14), 5 mM MgCl$_2$, 0.5 mM NADPH, inhibitor (100 and 500 μM), and DMXAA in 0.1 M phosphate buffer (pH 7.4). The concentrations of DMXAA were 100 μM for glucuronidation, and 25 μM for 6-methylhydroxylation (the corresponding apparent $K_m$ values for each metabolic pathway). Pooled human liver microsomes were used for DMXAA glucuronidation as this pathway is catalysed by multiple UGT enzymes (UGT1A9/UGT2B7) [10], and the glucuronidation activity for DMXAA was similar between human livers; whereas CYP1A2 was responsible for DMXAA 6-methylhydroxylation, and significant interindividual variation in the activity was observed [12]. The reactions were initiated by the addition of NADPH or UDPGA as appropriate. Pre-incubations were also performed in duplicate in the presence of inhibitor

and cofactor (NADPH or UDPGA) for 0 or 15 min prior to the addition of DMXAA at 37°C in a shaking water-bath. Incubations were stopped by cooling on ice and adding 2 volumes of an ice-cold acetonitrile: methanol mixture (3 : 1, v/v) containing 2 $\mu$M internal standard, and vortexing vigorously. Mixtures were centrifuged (3000 $g$ for 10 min) to remove the precipitated microsomal protein. The supernatant was removed, evaporated under nitrogen, and the residue reconstituted with mobile phase for injection onto the h.p.l.c. All anticancer drugs were dissolved in dimethyl sulphoxide (DMSO), which was used at a final concentration of 1% (v/v) in incubations. DMSO reduced the rate of DMXAA hydroxylation by 22%, but had no significant effect on DMXAA glucuronidation. Each drug was also incubated with microsomes and UDPGA or NADPH in the absence of DMXAA to identify any chromatographic peaks which might interfere with the measurement of DMXAA-G or 6-OH-MXAA.

For those drugs showing significant inhibitory effects, further inhibition kinetic studies were performed to determine the mechanism of inhibition and the apparent $K_i$ values. To construct Dixon plots, DMXAA (25–100 $\mu$M for glucuronidation; 6.25–25 $\mu$M for 6-methylhydroxylation) was incubated at 37°C with human liver microsomes in the presence of inhibitors. The inhibitor concentrations used were 50–400 $\mu$M for amsacrine, for 62.5–500 $\mu$M for vinblastine and vincristine, for 37.5–300 $\mu$M daunorubicin, and 0.625–5 $\mu$M for DACA.

*High performance liquid chromatography (h.p.l.c)*

The determination of DMXAA-G and 6-OH-MXAA has been described previously [19]. Briefly, the h.p.l.c system consisted of a solvent delivery system, a Model SF250 fluorescence detector (excitation and emission wave-length, 345 nm and 409 nm, respectively), a Model 460 autosampler, and a Model D450 data processing system (All from Kontron Instrument Co., Milan, Italy). A Luna C18 guard column and a 5-$\mu$m Spherex C18 analytical column (150 × 4.6 mm; Phenomenex) were used. The mobile phase was acetonitrile: 10 mM ammonium acetate buffer (24 : 76, v/v, pH 5.0) at a flow rate of 2.5 ml min$^{-1}$. The difference between the theoretical and measured concentration, and the coefficient variation, were less than 15% at the low quality control (QC) concentration (0.5 $\mu$M), and less than 10% at the medium (2.5 $\mu$M) and high (10 $\mu$M) QC concentrations. The limit for the determination of DMXAA-G and 6-OH-MXAA was 0.25 $\mu$M for a 75 $\mu$l injection volume. Assay specificity was indicated by the absence of interfering chromatographic peaks in microsomal samples and in incubations with anticancer drugs.

*Prediction of drug interaction based on* in vitro *data*

For the inhibition of a metabolic pathway by a drug, the degree of inhibition (R) can be calculated by the following equation 1 [20]:

$$\% \text{ Inhibition R} = \frac{[I]}{[I]+K_i \times \left(1+\frac{[S]}{K_m}\right)} \times 100 \quad (1)$$

where [I] is the unbound concentration of the inhibitor; $K_i$ is the inhibition constant; and [S] is the unbound therapeutic substrate concentration.

As DMXAA has a low plasma clearance (2–5 ml min$^{-1}$ kg$^{-1}$) in cancer patients [21], the degree of change ($R_c$) in the plasma area under the concentration-time curve (AUC) by the inhibiting drug can be calculated as follows [22]:

$$R_c(\%) = \frac{1}{f_h \times f_m \times \frac{1}{1+[I]/K_i} + 1 - f_h \times f_m} \times 100 \quad (2)$$

where $f_h$ is the fraction of hepatic clearance in total clearance, and $f_m$ the fraction of metabolic pathway in hepatic clearance. Approximated $f_h$ and $f_m$ for DMXAA were estimated from the urine of a patient treated with DMXAA, where 2.4%, 35.9% and 5.5% was excreted as unchanged DMXAA, DMXAA-G and 6-OH-MXAA, respectively [23]. Thus the approximate $f_h$, $f_m$ (glucuronidation) and $f_m$ (6-methylhydroxylation) were 97.6%, 86.7% and 13.3%, respectively, assuming that biliary excretion of DMXAA in humans is similar to that in the urine, as was found in the rat and rabbit [9, 11]. The unbound fraction of DMXAA (2000 $\mu$M) in human plasma was 0.123 as determined by ultrafiltration followed by h.p.l.c [24].

*Data analysis*

Data were expressed as mean ± s.d. The initial estimate of the apparent $K_i$ values and the nature of inhibition were obtained from Dixon plots, where the apparent $K_i$ was given by the intersection point of the linear regression lines for data sets of 1/v against the concentration of inhibitor. Several inhibition models (competitive, uncompetitive, and mixed inhibition), represented by the following equations, were fitted to the data and compared using Prism 3.0 program (Graphpad Software Co., CA).

$$v = \frac{V_{max} \times [S]}{K_m \times \left(1+\frac{[I]}{K_{ic}}\right) + [S]} \quad (3)$$

$$v = \frac{V_{max} \times [S]}{K_m + [S] \times \left(1+\frac{[I]}{K_{iu}}\right)} \quad (4)$$

$$v = \frac{V_{max} \times [S]}{K_m \times \left(1 + \dfrac{[I]}{K_{ic}}\right) + [S] \times \left(1 + \dfrac{[I]}{K_{iu}}\right)} \quad (5)$$

where v is the rate of metabolism, $V_{max}$, is the maximum velocity, $K_m$, the Michaelis-Menten constant, [S], the substrate concentration, [I], the inhibitor concentration, $K_i$; the apparent inhibition constant and subscripts c and u represent competitive and uncompetitive inhibition. The appropriate model was chosen by comparing and reviewing the relative residuals and the standard error of the parameter estimates. The significance of differences in the formation of DMXAA metabolites was assessed by Student's unpaired t-test. Differences were considered statistically significant when P values were < 0.05.

## Results

### Effects on anticancer drugs on DMXAA metabolism in vitro

The effects of various anticancer drugs on DMXAA glucuronidation and 6-methylhydroxylation by human liver microsomes are reported in Table 1. Vinblastine, vincristine and amsacrine at 500 μM significantly ($P < 0.05$) inhibited DMXAA glucuronidation ($K_i = 319$, 350 and 230 μM, respectively), but not 6-methylhydroxylation in human liver microsomes. Daunorubicin and DACA at 100 and 500 μM showed significant ($P < 0.05$) inhibition of DMXAA 6-methylhydroxylation ($K_i = 131$ and 0.59 μM, respectively), but not glucuronidation. Figure 2 illustrates the Dixon plots and the estimated apparent $K_i$ values for inhibition of DMXAA metabolism by various anticancer drugs. The mechanism of inhibition of either DMXAA glucuronidation or 6-methyl-hydroxylation by these anticancer drugs was competitive, as indicated by the smallest mean sum of squares compared with noncompetitive and mixed models. Other drugs such as 5-fluorouracil, paclitaxel, cisplatin, irinotecan, tirapazamine and methotrexate exhibited little or negligible inhibition of the metabolism of DMXAA. Pre-incubation of microsomes with the anticancer drugs (100 and 500 μM) did not enhance their inhibitory effects on DMXAA metabolism.

### Quantitative prediction of DMXAA–drug interaction in vivo

The predicted percentage inhibition (R) and percentage increase in AUC of DMXAA (R$_c$) that might be caused by coadministration of various anticancer drugs based on

**Table 1** Effects of various anticancer drugs on DMXAA glucuronidation and 6-methylhydroxylation by human liver microsomes[1].

| Drugs | Glucuronidation (% enzyme activity remaining) | | 6-Methylhydroxylation (% enzyme activity remaining) | |
| --- | --- | --- | --- | --- |
| | at 100 μM inhibitor | at 500 μM inhibitor | 100 μM inhibitor | 500 μM inhibitor |
| Folic acid | $73 \pm 8$ | $70 \pm 2$ | $102 \pm 5$ | $111 \pm 12$ |
| Vincristine | $74 \pm 14$ | $46 \pm 8$ | $101 \pm 0$ | $67 \pm 14$ |
| Amsacrine | $82 \pm 12$ | $27 \pm 1$ | $102 \pm 19$ | $91 \pm 5$ |
| Daunorubicin | $84 \pm 19$ | $63 \pm 7$ | $59 \pm 2$ | $15 \pm 2$ |
| Paclitaxel | $88 \pm 7$ | $78 \pm 5$ | $98 \pm 8$ | $95 \pm 6$ |
| Cisplatin | $91 \pm 5$ | $67 \pm 2$ | $100 \pm 10$ | $86 \pm 7$ |
| DACA | $96 \pm 1$ | $71 \pm 16$ | $17 \pm 2$ | $6 \pm 1$ |
| Irinotecan | $97 \pm 7$ | $71 \pm 4$ | $95 \pm 9$ | $92 \pm 5$ |
| Tirapazamine | $97 \pm 7$ | $95 \pm 7$ | $93 \pm 3$ | $86 \pm 4$ |
| Vinblastine | $99 \pm 4$ | $40 \pm 0$ | $104 \pm 9$ | $113 \pm 1$ |
| 6-Thioguanine | $100 \pm 2$ | $98 \pm 7$ | $89 \pm 2$ | $64 \pm 1$ |
| Cyclophosphamide | $101 \pm 29$ | $74 \pm 12$ | $95 \pm 2$ | $85 \pm 4$ |
| 6-Mercaptopurine | $102 \pm 5$ | $96 \pm 10$ | $97 \pm 8$ | $94 \pm 6$ |
| 5-Fluorouracil | $104 \pm 11$ | $103 \pm 7$ | $97 \pm 7$ | $96 \pm 10$ |
| Methotrexate | $108 \pm 8$ | $88 \pm 2$ | $109 \pm 8$ | $89 \pm 14$ |
| Melphalan | $113 \pm 15$ | $104 \pm 15$ | $99 \pm 7$ | $101 \pm 5$ |

[1] For glucuronidation, DMXAA (100 μM, $\approx K_m$) was incubated for 20 min at 37°C with 0.1 mg ml$^{-1}$ Brij 58-activated liver microsomes from pooled HL6, HL7 and HL8 in the presence of various anticancer drugs. For 6-methylhydroxylation, DMXAA (25 μM, $\approx K_m$) was incubated for 40 min at 37°C with 1.0 mg ml$^{-1}$ liver microsomes from HL12, HL13 and HL14 in the presence of various anticancer drugs. Results are the mean ± s.d. of at least three determinations from pooled human liver microsomes for glucuronidation and mean ± s.d. of data from three individual livers for 6-methylhydroxylation.
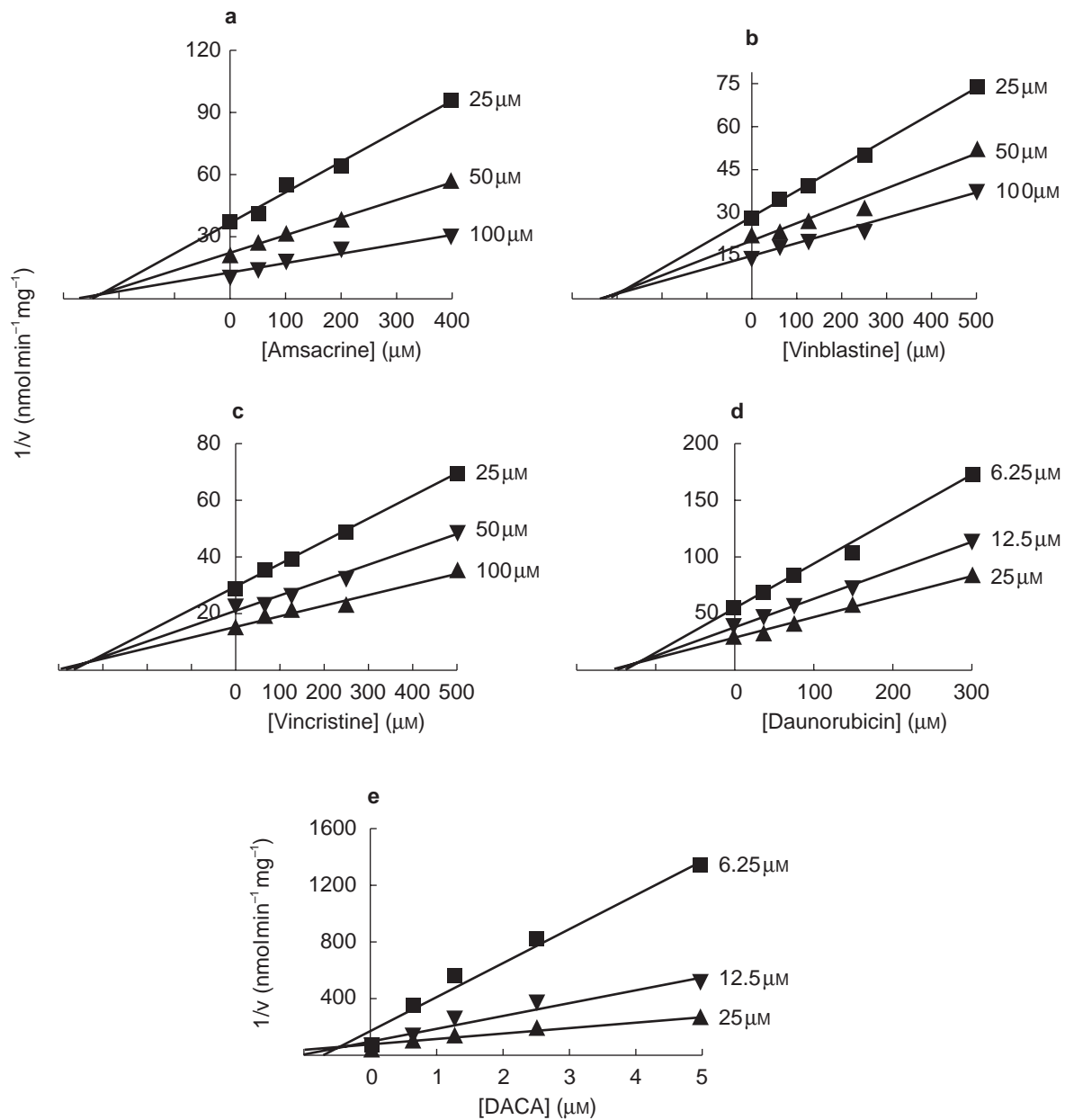
**Figure 2** Dixon plots for the inhibition of DMXAA glucuronidation by amsacrine (a), vinblastine (b) and vincristine (c), and 6–methylhydroxylation by daunorubicin (d) and DACA (e) in human liver microsomes. Concentrations of DMXAA are shown alongside individual plots. Each data point is the mean of at least three determinations.

our *in vitro* studies are shown in Table 2. An increase of 6% in the plasma AUC was predicted with DACA, which is of insignificant magnitude to be of clinical relevance. With amsacrine, daunorubicin, vinblastine and vincristine, there was no predicted *in vivo* inhibition of DMXAA metabolism and change in plasma AUC of DMXAA. Predictions of *in vivo* DMXAA–drug interactions in patients based on these *in vitro* data indicated that all the anticancer drugs tested except DACA appear unlikely to alter the pharmacokinetics of DMXAA.

## Discussion

There is an increasing interest in the drug–drug interaction studies using human liver microsomes or recombinant human drug-metabolizing isozymes (mainly CYPs) in the early stages of drug development, as this may help identify possible drug interactions and avoid drug toxicity *in vivo* [22]. Our results indicate that of the anticancer drugs investigated, only vinblastine, vincristine, amsacrine, DACA and daunorubicin at supratherapeutic concentrations ($\geq 100$ μM) caused a significant inhibition

**Table 2** Predicted DMXAA–anticancer drug interactions *in vivo*.

| Anti-cancer drugs | Apparent $K_i$ value (μM) | Metabolic pathway of inhibition | [I] (μM) | Predicted R (%) | $R_c$ (%) |
|---|---|---|---|---|---|
| Daunorubicin | 131 | Hydroxylation | 0.01–1.00[1] | 0 | 0 |
| DACA | 0.59 | Hydroxylation | 0.21–18.5[2] | 0.1–6.4 | 0.1–6.6 |
| Vinblastine | 319 | Glucuronidation | 0.01–0.17[3] | 0 | 0 |
| Vincristine | 350 | Glucuronidation | 0.01–0.08[3] | 0 | 0 |
| Amsacrine | 230 | Glucuronidation | 0.71–3.70[4] | 0 | 0 |

[1] Paul *et al.* [28]; Galettis *et al.* [29]. Approximately 63% of daunorubicin binds to human plasma [30].

[2] Kestell *et al.* [14]. Approximately 97% of DACA binds to human plasma [14].

[3] Nelson *et al.* [31]. Approximately 75% of vinblastine and vincristine binds to human plasma proteins [32].

[4] Petros *et al.* [33]. Approximately 97% amsacrine binds to human plasma [34].

of either glucuronidation or 6-methylhydroxylation of DMXAA with an apparent $K_i$ of 0.59–350 μM. The inhibition by these anticancer agents appears to be competitive, with no involvement of mechanism-based inhibition. When interpreting the clinical relevance of these inhibition studies, the unbound concentration of both the substrate and inhibitors utilized *in vitro*, and those that exist *in vivo*, must be taken into consideration. The concentrations for DMXAA (25–140 μM) used in this study have been observed as unbound plasma concentrations in patients in a Phase I trial [21].

Numerous models have been used to correlate *in vitro* with *in vivo* drug interactions, with some success [22]. A model for low hepatic clearance drugs administered by intravenous injection was used to extrapolate our *in vitro* drug interaction data to the *in vivo* state. In this model, it is assumed that both inhibitor and DMXAA are metabolized only in the liver, with possible competitive inhibition, and that there are only two metabolic pathways for DMXAA. Our results indicate that none of the anticancer drugs investigated would cause a clinically significant reduction in DMXAA plasma clearance. However, these conclusions must be regarded with caution due to the inherent assumptions within the model. In addition, nonspecific microsomal binding may be another important factor influencing the accuracy of the extrapolation from the *in vitro* to *in vivo* state [25–27]. Non-specific microsomal binding of substrates will result in a higher apparent $K_m$ value determined from the total (added) concentration, rather than the unbound concentration. However, DMXAA as a weak acid is predicted to have insignificant nonspecific microsomal binding, since the microsomal membrane has a net negative charge, and acidic drugs such as caffeine, tolbutamide and naproxen do not bind significantly to it [27]. Furthermore, the model used in this study does not take into account inhibition of metabolism by indirect mechanisms such as alterations in cytokine and/or nitric oxide concentrations, or changes in other drug disposition processes, such as absorption, renal metabolism, or active transport mechanisms.

According to equation 2, 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 [22]. 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 [11, 23]. The intrinsic clearance ratio of glucuronidation: 6-methylhydroxylation was 2.6 from our *in vitro* human studies [Paxton *et al.* personal communication]. Previously Miners *et al.* [10] screened various drugs, mainly substrates of UGTs, for their potential to interact with DMXAA using human liver microsomes and cDNA–expressed UGTs. Significant inhibition of DMXAA glucuronidation was observed with diclofenac, epirubicin, indomethacin, R,S-ketoprofen, lorazepam, S-naproxen, oxazepam, and temazepam with apparent $K_i$ values ranging from 10 to 318 μM. Recently, our mouse studies indicated that diclofenac at 100 mg kg$^{-1}$ administered by intraperitoneal injection increased the plasma AUC of DMXAA by 24% [8]. This was considered to be mainly due to inhibition of DMXAA glucuronidation. DMXAA is 6-methylhydroxylated by CYP1A2 [12]. However, alterations in this relatively minor pathway of DMXAA metabolism would not be expected to have a clinically significant effect on DMXAA disposition. As none of the anticancer drugs investigated are known substrates of the UGTs, it is perhaps of no surprise that there was little inhibition of DMXAA glucuronidation by these drugs, with the exception of amsacrine, vinblastine and vincristine at high concentration.

In conclusion, despite the occurrence *in vitro* of inhibition of DMXAA metabolism by several anti-cancer drugs, such as daunorubicin, DACA and amsacrine, it appears unlikely that combinations of these drugs will result in clinically significant drug interactions. However, this does not rule out the possibility of pharmacokinetic interactions with other drugs used concurrently with this combination of anticancer drugs.

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