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High-throughput screening of potential inhibitors for the metabolism of the investigational anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid

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Abstract

By screening potential inhibitors of drug metabolism using the in vitro models, potential drug–drug interactions in vivo may be predicted with the use of appropriate pharmacokinetic principles. This study aimed to develop a rapid screening system using human liver microsomes to efficiently identify the potential inhibitors of DMXAA metabolism. Initial IC_{50} was estimated by using a two-point method, and then K_i values were determined if required and compared with those initial IC_{50} values. More than 100 compounds including known substrates and inhibitors of human uridine diphosphate glucuronosyltransferases (UGTs) and cytochrome P450 (CYP), anti-cancer drugs and xanthenone analogues were screened for their inhibitory effect on DMXAA glucuronidation and 6-methylhydroxylation in human liver microsomes. Both metabolites of DMXAA, DMXAA acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA), formed in human liver microsomes were quantitated by validated HPLC methods. The results indicated that there was a significant relationship ($r^2=0.966$, $P<0.001$) between the two-point IC_{50} values and the apparent K_i values for 20 compounds showing significant inhibitory effects on DMXAA metabolism, suggesting the usefulness of the two-point determination for the initial screening of compounds. This study has been completed using a strategy for rapid HPLC analysis and thus provided early access to detailed information for potential inhibitors of DMXAA metabolism and allows for further DMXAA–drug interaction studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: 5,6-Dimethylxanthenone-4-acetic acid

1. Introduction

It has been found that up to 40% of drug candidates investigated in humans are withdrawn due to serious safety problems [1]. The recent withdrawal of several drugs, including mibefradil (an anti-hypertensive and anti-anginal drug), soruvidine (an anti-viral drug), terfenadine (an anti-histamine) and phenylpropanolamine (a common ingredient in cold drugs), from the market are all due to concerns over or incidences of drug–drug interactions [2]. Unfavorable drug–drug interactions with anti-cancer drugs

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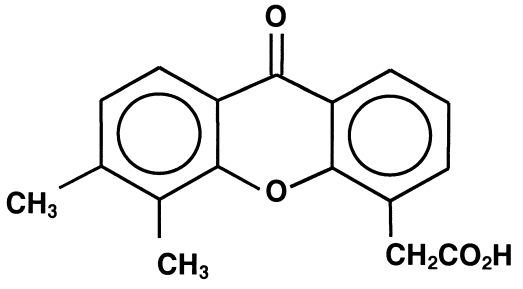
are likely to occur due to frequent poly-pharmacy, but the drug interactions cannot be investigated in healthy volunteers due to ethical reasons [3]. Therefore, appropriate approaches, including animal studies and in vitro models, should be utilized to select against drugs with problematic pharmacokinetic profiles. Increasingly high-throughput screening approaches for this purpose have been used in the pharmaceutical industry [4]. Liver microsomes, precision-cut liver slices, isolated and cultured hepatocytes or liver cell lines, perfused isolated liver, and cDNA-expressed enzymes are all useful in vitro systems [5–7]. By screening potential inhibitors of drug metabolism using these in vitro models, potential drug–drug interactions in vivo may be predicted with the use of appropriate pharmacokinetic principles [8], and thus optimal chemotherapy may be achieved and toxic drug interactions avoided.

The experimental anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA) (Fig. 1) was developed by the Auckland Cancer Society Research Center (ACSRC) and its Phase I trial has recently been completed in New Zealand and the UK [9]. DMXAA mainly has the following pharmacological effects: (a) anti-vascular activity, inducing rapid vascular collapse and necrosis in transplantable murine tumors [10,11]; (b) immuno-modulating activities; (c) cytokine-inducing effects (in particular, tumour necrosis factor-α and interferons); (d) inducing effects on serotonin and nitric oxide [12–14]; and (e) anti-angiogenetic effects [15]. All these effects of DMXAA are considered to contribute to its potent anti-cancer activity. The metabolism of DMXAA has been extensively studied using in vivo and in vitro models including isolated perfused rat liver and hepatic microsomes, and these studies have indicated that uridine diphosphate glucuronosyltransferases (UGT1A2 and UGT2B7)-catalyzed glucuronidation on its acetic acid side chain and to a lesser extent cytochrome P450 (CYP1A2)-catalyzed hydroxylation of the 6-methyl group are its major metabolic pathways [16,17], resulting in DMXAA acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA), respectively. As a biological response modifier, DMXAA has been combined with a variety of drugs with different mechanisms of action in the mouse model, and many of these co-administered drugs, such as thalidomide [18–20], cyclophosphamide [21] and cyproheptadine [22], have been shown to potentiate the anti-cancer activity of DMXAA and also reduced its plasma clearance. However, the mechanisms for the pharmacokinetic DMXAA–drug interactions have not been fully identified.

This study aimed to develop a rapid screening system using human liver microsomes to efficiently identify the potential inhibitors of DMXAA metabolism. Initial IC_{50} was estimated by using a two-point method, and then K_i values were determined if required and compared with those initial IC_{50} values. The two-point IC_{50} estimation was used by Moody et al. [23] for the marker compounds of CYP inhibition, and a significant correlation ($r^2=0.98$, $P<0.001$) between the two-point IC_{50} and the full (seven)-point IC_{50} values was observed.



5,6-dimethylxanthenone-4-acetic acid

Fig. 1. The chemical structure of DMXAA.

2. Materials and methods

2.1. Chemicals and reagents

DMXAA and the internal standard, 2,5-dimethylxanthenone-4-acetic acid (SN24350), *N*-[2-(dimethylamino)-ethyl]acridine-4-carboxamide (DACA), flavone acetic acid (FAA), 5-methyl-xanthenone-4-acetic acid (5-MXAA), 6-methyl-xanthenone-4-acetic acid (6-MXAA), SN23594, SN23882, SN23518, SN23754, SN23598, SN23520, SN23675, SN23599, CB1954, SN21407, SN23933, SN24489, SN24178, SN23862 and amsacrine were synthesised

in the ACSRC [24,25]. DMXAA was protected from light exposure to avoid degradation [26]. 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 HPLC, and their structure was confirmed by mass spectrometry and [^{1}H]-nuclear magnetic resonance [27]. Amitriptyline, bicinchoninic acid (BCA) reagent, Brij 58, chlorzoxazone, cimetidine, cisplatin, cyproheptadine, daunorubicin, diclofenac, diethyldithiocarbamate (DDC), diflunisal, erythromycin, fenclofenac, fenoprofen, 5-fluorouracil, folic acid, indomethacin, irinotecan, medazepam, melphalan, 6-mercaptopurine, methotrexate, 6-methylguanine, α-naphthoflavone (ANF), oxazepam, paclitaxel, paracetamol, phenacetin, quinidine, D-saccharic acid 1,4-lactone, testosterone, 6-thioguanine, tirapazamine, tolbutamide, troleandomycin (TAO), vinblastine, and vincristine were purchased from Sigma–Aldrich (Auckland, New Zealand). Uridine diphosphate glucuronic acid (UDPGA) and NADPH were purchased from Roche Diagnostics (Auckland, NZ). Furafylline was obtained from UFC (Manchester, UK). Other reagents were obtained from the following sources: SKF525A from SmithKline Beecham Pharmaceuticals (Philadelphia, PA, USA); sulphaphenazole from Ciba Geigy (East Hanover, NJ, USA); ketoconazole from ICN Biomedicals (Costa Mesa, CA, USA). 1-Naphthol was from Merck (Darmstadt, Germany). All other reagents were of analytical or HPLC grade as appropriate.

2.2. Preparation of human hepatic microsomes

Human liver samples (HL6, HL7, HL8, HL12, HL13, and HL14) were obtained from our human liver bank [17]. Histological examination of the resected livers ensured the use of healthy liver tissue. Ethical approval was obtained from the Northern New Zealand Research Ethics Committee, and written informed consents from donors or relatives for liver tissues to be used for research. Hepatic microsomes were prepared by differential centrifugation, as described [28]. Livers and microsomes were stored at −80°C until used. Microsomal protein concentration was determined by the bicinchoninic acid binding method using bovine serum albumin as the standard [29]. Total cytochrome P450 contents were measured as described [30].

2.3. In vitro inhibition assay

The effects of more than 100 compounds on DMXAA glucuronidation and 6-methylhydroxylation in human liver microsomes were investigated using optimized incubation conditions [17]. These compounds include UGT substrates such as diclofenac, CYP inhibitors and substrates such as furafylline, anti-cancer agents such as cyclophosphamide, xanthenone analogues such as 6-MXAA, and acridine analogues such as DACA. Xanthenone and acridine analogues were used to identify the structure–activity relationship and specific inhibitors for DMXAA metabolism. Typical incubations (total volume 200 μl, in triplicate) for DMXAA glucuronidation contained liver microsomal protein (0.1 mg/ml, pooled from HL6, HL7 and HL8), 10 m*M* UDPGA, 5 m*M* $MgCl_2$, 0.1 mg/ml 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 μ*M*), 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-methylhydroxylation contained 1 mg/ml liver microsome (from three sources of human livers, HL12, HL13, and HL14), 5 m*M* $MgCl_2$, 0.5 m*M* NADPH, inhibitor (100 and 500 μ*M*), and DMXAA in 0.1 *M* phosphate buffer (pH 7.4). The incubation time was 20 and 40 min for glucuronidation and hydroxylation, respectively. The concentrations of DMXAA were 100 μ*M* for glucuronidation, and 25 μ*M* for 6-methylhydroxylation (the corresponding apparent K_m values for each metabolism pathway). Pooled human liver microsomes were used for DMXAA glucuronidation as this pathway is catalysed by multiple UGT enzymes (UGT1A9/UGT2B7) [16], and the glucuronidation activity for DMXAA is similar in these human livers; whereas for DMXAA 6-methylhydroxylation, CYP1A2 was responsible for this pathway, and significant inter-individual variation in the activity was observed [17], thus three sources of human livers were used. The reactions were initiated by the addition of NADPH or UDPGA as appropriate. Pre-incubations were also performed

in duplicate in the presence of some compounds and co-factor (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 two volumes of ice-cold acetonitrile–methanol mixture (3:1, v/v) containing 2 μ*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 into the HPLC. All compounds were dissolved in dimethyl sulphoxide (DMSO), which was used at a final concentration of 1% (v/v) in incubations. Control incubations with 1% DMSO were undertaken, which showed a 22% reduction in the liver microsomal DMXAA hydroxylation rate, but no significant effect on DMXAA glucuronidation. Each compound was also incubated with microsomes and UDPGA or NADPH in the absence of DMXAA to determine whether they resulted in chromatographic peaks that might interfere with the measurement of DMXAA-G or 6-OH-MXAA.

2.4. HPLC

2.4.1. Chromatographic conditions

The determination of DMXAA metabolites, DMXAA-G and 6-OH-MXAA formed in human liver microsomes by HPLC has been described previously [17,31]. 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, Milan, Italy). A Luna C_{18} guard column and a 5 μm Spherex C_{18} analytical column (150×4.6 mm; Phenomenex) were used. The mobile phase was acetonitrile–10 m*M* ammonium acetate buffer (24:76, v/v, pH 5.0) at a flow-rate of 2.5 ml/min.

2.4.2. Calibration curves

Known amounts of DMXAA-G or 6-OH-MXAA were added to human liver microsomes separately. Calibration curves were constructed with DMXAA-G and 6-OH-MXAA over the concentration range 0.25–20 and 0.5–40 μ*M*, respectively. Sample preparation of standards was the same as for incubated samples. The ratio of the peak area of DMXAA-G or 6-OH-MXAA to that of internal standard was plotted against the known concentration, and linear least-squares regression analysis, weighted according to the reciprocal of peak area ratio squared, was conducted to determine the slope, intercept and coefficient of determination by the program Prizm 3.0 (Graphpad Software, CA, USA).

2.4.3. Sensitivity and selectivity

The limit of quatitation (LOQ) was the minimum concentration which could be determined with acceptable accuracy (i.e. recovery between 80 and 120%) and precision (coefficient of variation (C.V.) $<$20%). The limit of detection was defined as the amount which could be detected with a signal-to-noise ratio of 3. The selectivity of the method was examined by determining if interfering chromatographic peaks were present in human liver microsomes and in incubations with various inhibitors.

2.4.4. Accuracy and precision

Quality control (QC) samples containing DMXAA-G or 6-OH-MXAA were prepared from independent weighings from those used for constructing calibration standards. Final concentrations of low, medium and high QC samples were: 0.5, 2.5, 10 μ*M* for DMXAA-G; and 1, 5, 20 μ*M* for 6-OH-MXAA. These samples were prepared on the day of analysis in the same way as calibration standards. The performance of the HPLC method was assessed by analysis of 12 QC samples (four each of low, medium, and high concentrations) on a single assay day to determine intra-day accuracy and precision, and nine QC samples (three each of low, medium, and high concentrations) on each of four consecutive assay days to determine inter-day accuracy and precision.

2.5. Two-point IC_{50} estimation

The two-point inhibition assay was used initially to estimate IC_{50} [23]. The concentrations of inhibitors were 0.5 and 5 μ*M* for inhibitors with $IC_{50} \leq 0.5$ μ*M*; 10 and 100 μ*M* for inhibitors with IC_{50} of 0.5–250 μ*M*; 100 and 500 μ*M* for inhibitors with $IC_{50} \geq 250$ μ*M* or IC_{50} unknown. The percent

enzyme activity remaining (R) at a certain inhibitor concentration can be expressed as

$$R = \frac{R_0}{1 + ([I]/\mathrm{IC}_{50})^s} - b \quad (1)$$

where R_0 is the enzyme activity without inhibitor, [I] is the concentration of inhibitor, s is the slope factor and b the background (uninhabitable) activity. Assuming $s = 1$ and $b = 0$, then this equation is simplified to give

$$\mathrm{IC}_{50} = \frac{[I] \cdot (100 - I_a)}{I_a} \quad (2)$$

where I_a is the percent inhibition at [I]. Experience shows that this is valid when I_a is between 20 and 80%. The appropriate inhibitor concentrations were used to cover the initially estimated IC_{50} value.

2.6. Determination of apparent K_i values

For those compounds showing significant inhibitory effects, further inhibition kinetic studies were performed to determine the apparent K_i values. To construct Dixon plots, DMXAA (25–100 μ*M* for glucuronidation; 6.25–25 μ*M* for 6-methylhydroxylation) was incubated at 37°C with human liver microsomes in the presence of various inhibitors at a series of concentrations. 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 and compared using the program Prism 3.0 (Graphpad Software):

$$v = \frac{V_{\max} \cdot [S]}{K_\mathrm{m} \cdot [1 + ([I]/K_\mathrm{ic})] + [S]} \quad (3)$$

$$v = \frac{V_{\max} \cdot [S]}{K_\mathrm{m} + [S] \cdot [1 + ([I]/K_\mathrm{iu})]} \quad (4)$$

$$v = \frac{V_{\max} \cdot [S]}{K_\mathrm{m} \cdot [1 + ([I]/K_\mathrm{ic})] + [S] \cdot [1 + ([I]/K_\mathrm{iu})]} \quad (5)$$

where v is the rate of metabolism, $V_{\max}$ 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 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 $P < 0.05$.

For mechanism-based inhibitors, the inhibition kinetic parameters for enzyme inactivation were determined as described [32]. The value of the apparent inactivation rate constant (k_obs) was obtained by the nonlinear least-squares method (Prism 3.0 program) using the following equation:

$$k_\mathrm{obs} = \frac{k_\mathrm{inact} \cdot [I_0]}{K'_\mathrm{app} + [I_0]} \quad (6)$$

where k_obs, k_inact, and K'_app represent the apparent inactivation rate constant of the enzyme at the initial concentration of inhibitor (I_0), the maximum inactivation rate constant, and the apparent dissociation constant between the enzyme and inhibitor, respectively.

3. Results

3.1. Validation of HPLC

Calibration curves were linear over the concentration range used for both analytes with mean r^2 values being greater than 0.999. The mean y-intercepts for DMXAA-G and 6-OH-MXAA were 0.005 and 0.006, respectively. The differences between the calculated and the actual concentration and the relative standard deviation were less than 15% at low QC concentration and less than 10% at medium and high QC concentrations for both metabolites. Under the chromatographic conditions, 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 μ*M*), and less than 10% at the medium (2.5 μ*M*) and high (10 μ*M*) QC concentrations of both DMXAA-G and 6-OH-MXAA. The LOQ of the

assay was 12.5 and 25 pmol for DMXAA-G and 6-OH-MXAA, respectively. In our incubation samples, DMXAA-G and 6-OH-MXAA concentrations were always well above the LOQ. There were no interfering chromatographic peaks in microsomal samples and in incubations with various inhibitors.

3.2. Validation of two-point IC_{50} estimation

The two-point IC_{50} values were estimated for all potential compounds screened. Many compounds, in particular the anti-cancer drugs, had little or negligible inhibitory effects on either DMXAA glucuronidation or 6-methylhydroxylation at an inhibitor concentration of 500 μM, indicating $IC_{50} > 500$ μM. For 20 compounds showing a significant inhibitory effect on either DMXAA glucuronidation or 6-methylhydroxylation, or both pathways, the apparent

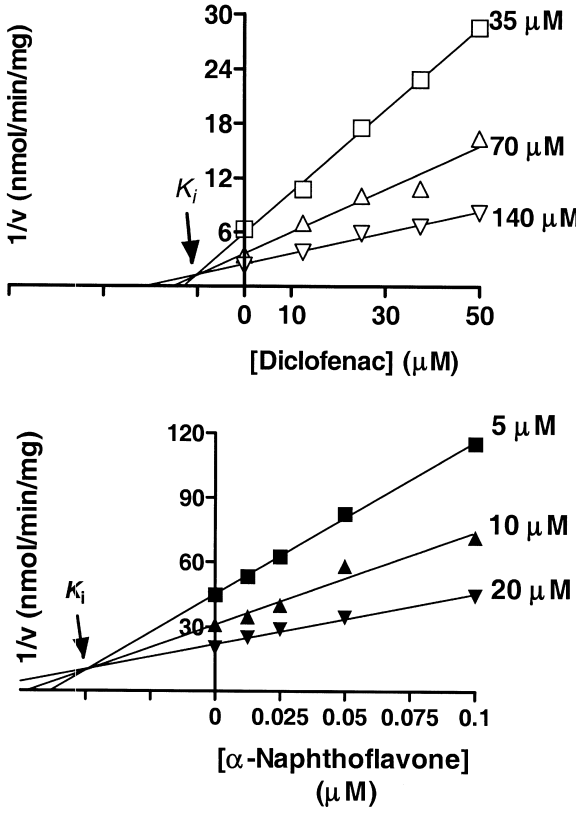


Fig. 2. Dixon plots for the inhibition of in vitro DMXAA glucuronidation by diclofenac and 6-methylhydroxylation by α-naphthoflavone in human liver microsomes.

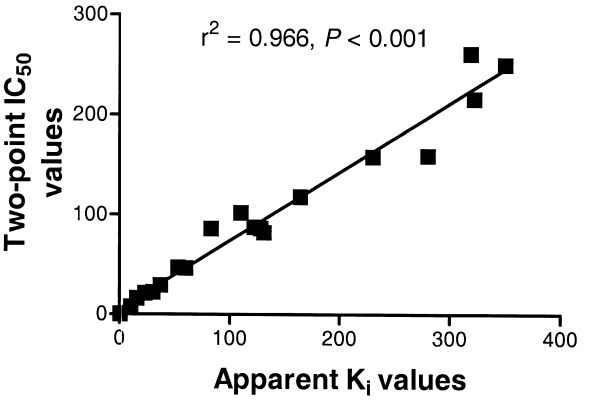


Fig. 3. Relationship between the two-point IC_{50} values and the apparent K_i values of 20 inhibitory compounds for DMXAA metabolism in human liver microsomes.

K_i values were further evaluated. These compounds included diclofenac, ANF, DACA, cyproheptadine, theophylline, phenacetin, etc. Dixon plots for the inhibition of DMXAA glucuronidation by diclofenac and 6-methylhydroxylation by ANF are shown in Fig. 2. There was a significant correlation ($r^2 = 0.966$, $P < 0.001$) between the two-point IC_{50} values and the apparent K_i values for these compounds (Fig. 3).

4. Discussion

Most assays for the routine analysis of in vitro drug–drug interactions involving human drug metabolising enzymes (in particular CYPs) rely on labour- and equipment-intensive sample extraction and HPLC or LC–MS analysis. An ideal screening assay should be chosen based on compatibility for automation, robustness and reproducibility. High-throughput assays are capable of handling the increased number of compounds, and thus offer the opportunity to use the resulting in vitro inhibition data as a criterion for compound progression and monitoring metabolic drug–drug interactions involving human drug metabolizing enzymes before entering clinical trials. This study has been completed using a strategy for rapid HPLC analysis (90 min for sample preparation in batch, automated overnight HPLC analysis, and 15 min per sample for HPLC analysis, thus approximately 90 samples/day), which

is consistent with current pharmaceutical research trends. It should be noted that DMXAA-G is unstable under physiological conditions [37], thus there is a necessity to stabilise it by reducing the pH to 5.0 and adding the β-glucuronidase inhibitor 1,4-saccharilactone. As a result of increased speed and throughput, the strategy we employed here provides early access to detailed information for potential inhibitors of DMXAA metabolism and allows for further DMXAA–drug interaction studies.

Our study indicated that there was a significant relationship between the two-point IC_{50} values and the apparent K_i values for 20 compounds showing significant inhibitory effects on DMXAA metabolism, suggesting the usefulness of the two-point determination for the initial screening of compounds, which may require even higher throughput. Our results are consistent with those reported by Moody et al. [23] for the marker compounds of CYP inhibition, where there was a significant correlation between the two-point IC_{50} and the full (seven)-point IC_{50} values. However, the choice of the initial two concentrations of inhibitory compounds may present a difficulty. For the UGT and CYP substrates and inhibitors, literature data can provide useful clues for the determination of two concentration points. For compounds with unknown information on CYP and UGT inhibition, generally 50–500 μ*M* may be used. For clinical anti-cancer drugs, concentrations relevant to in vivo situations should be used, since inhibitor concentrations are important to interpret the clinical relevance of these inhibition studies [8]. A quantitative prediction for the extent of drug interactions can be achieved if the isozymes related to the metabolism of a drug are identified and the metabolic K_i values of co-administered drugs are determined in liver microsomes and/or cDNA-expressed enzyme systems [33,34]. In fact, the in vitro inhibition data for DMXAA metabolism by anti-cancer drugs have been extrapolated to in vivo and it appears that all the anti-cancer drugs examined had little influence on DMXAA clearance in vivo [35].

When interpreting the clinical relevance of these inhibition studies, the concentration of substrate used in vitro and those that exist in vivo must be taken into consideration [8]. DMXAA is highly bound to plasma proteins [36], but preliminary results from the Phase I clinical trial of DMXAA indicated that free drug concentrations of DMXAA up to 10–200 μ*M* are possible in vivo [9]. Thus it appears that the concentrations of DMXAA used in this in vitro study are relevant to those encountered in vivo.

In summary, this study has been completed using a strategy for rapid HPLC analysis. The results indicate that there is a significant relationship between the two-point IC_{50} values and the apparent K_i values for 20 compounds showing significant inhibitory effects on DMXAA metabolism, suggesting the usefulness of the two-point determination for the initial screening of compounds. The results may also provide early access to detailed information on potential inhibitors of DMXAA metabolism and allow for further DMXAA–drug interaction studies.

5. Nomenclature

DMXAA	5,6-dimethylxanthenone-4-acetic acid
IC_{50}	inhibitor concentration causing 50% enzyme activity inhibition
K_m	the Michaelis–Menten constant
K_i	the apparent inhibition constant
V_{max}	maximal velocity of reaction

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