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Strain differences in the liver microsomal metabolism of the experimental anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid in mice

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Abstract

The experimental anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA) is mainly metabolised by acyl glucuronidation and to a lesser degree by 6-methyl hydroxylation. Strain differences in the maximum tolerated dose (MTD) of DMXAA in mice have been observed. The aim of this study was to compare the kinetics of DMXAA acyl glucuronidation and 6-methylhydroxylation in five various mouse strains, and correlate the in vitro metabolism data with MTD observed. In all mouse strains studied, DMXAA acyl glucuronidation and 6-methylhydroxylation in the liver microsomes followed Michaelis–Menten kinetics. Significant strain variations in the kinetic parameters (K_m, V_{max} and K_m/V_{max}, i.e., CL_{int}) for DMXAA acyl glucuronidation and 6-methylhydroxylation in mouse liver microsomes were observed. A 2–6-fold variation was spanned across strains for K_m, V_{max} and CL_{int}, respectively, for DMXAA glucuronidation and 6-methylhydroxylation. The rank order for total CL_{int} by glucuronidation and 6-methylhydroxylation was BDF1 (1.70 ml/min per g)>wild type of mice lacking IFN-γ receptor (0.80 ml/min per g)>nude mice (0.70 ml/min per g)>Swiss CD mice (0.56 ml/min per g)>C57Bl/6 mice (0.46 ml/min per g), with a 4-fold variation between the mouse strain of the highest and lowest CL_{int}. There was no significant correlation between total CL_{int} and MTD ($r^2=0.88$, $P>0.05$), but the rank order for CL_{int} was consistent with that for MTD. These results suggested that there were significant strain differences in DMXAA metabolism in mouse liver microsomes and the strain-related differences in the metabolism of DMXAA did not provide an explanation for the strain differences in the MTD.

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

1. Introduction

The experimental anti-cancer drug 5,6-dimethylxanthenone-4-acetic acid (DMXAA) (Fig. 1) has recently completed Phase I clinical trials in New Zealand and the UK under the supervision of the Cancer Research Campaign's Phase I/II Clinical Trials Committee [1]. As a biological response modifier, the mechanism of action, toxicological and pharmacological profiles of DMXAA are different

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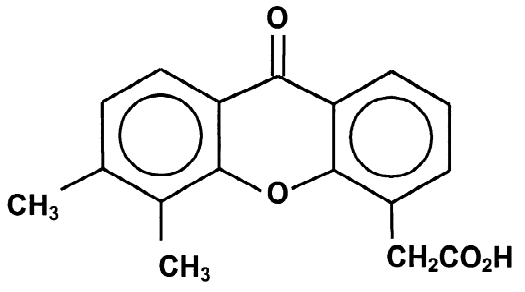

5,6-dimethylxanthenone-4-acetic acid

Fig. 1. Chemical structure of DMXAA.

from conventional chemotherapeutic agents. The induction of cytokines such as tumour necrosis factor-α and interferon (IFN) [2], serotonin [3] and nitric oxide [4,5], anti-vascular [6], anti-angiogenetic [7] and immuno-modulating effects [8,9] are considered to be major mechanisms of action of DMXAA. DMXAA is extensively metabolised by acyl glucuronidation and to a lesser extent by 6-methylhydroxylation in laboratory animals and humans [10–13]. The major metabolites, DMXAA acyl glucuronide (DMXAA-G) and 6-hydroxymethyl-5-methylxanthenone-4-acetic acid (6-OH-MXAA) are excreted into bile and urine. These metabolic pathways have been shown to be catalysed by uridine diphosphate glucuronosyltransferase UGT 1A9 and UGT2B7 [11], and by cytochrome P450 CYP1A2 [13], respectively. DMXAA is extensively bound to plasma proteins [14]. Non-linear pharmacokinetics of DMXAA was observed in animals and patients [1,12], due in part to saturation of elimination process and plasma protein binding.

The mouse is the most commonly used animal model for the preclinical evaluation of DMXAA, and various strains of mice have been used to investigate the toxicity, pharmacological action and disposition of DMXAA [12,15–17]. Remarkable strain differences in the maximal tolerance dose (MTD) for DMXAA in mice have been observed, with 50% higher MTD in C57BL/6J and BDF1 strains than in nude mice (Kestell et al., unpublished data). The aim of this study was to compare the kinetics for DMXAA metabolism in liver microsomes from various strains of mice that have been used for DMXAA studies, in attempt to correlate in vitro metabolism data to MTD and find the appropriate mouse strain for further DMXXA metabolism and toxicity studies.

2. Materials and methods

2.1. Chemicals and reagents

DMXAA and the internal standard, 2,5-dimethylxanthenone-4-acetic acid (SN24350) were synthesised in the Auckland Cancer Society Research Centre [18]. DMXAA was protected from light exposure to avoid degradation [19]. Authentic 6-OH-MXAA was isolated and purified from rat urine by a solid-phase extraction method, and its structure identified by liquid chromatography–mass spectrometry (LC–MS) and ^{1}H nuclear magnetic resonance [12]. Brij 58 and D-saccharic acid 1,4-lactone were purchased from Sigma–Alderich (Auckland, New Zealand). NADPH and UDPGA were purchased from Roche Diagnostics (Auckland, New Zealand). All other reagents were of analytical or HPLC grade as appropriate.

2.2. Preparation of liver microsomes

Healthy male mice (BDF1 strain, C57BL/6J strain, Swiss CD strain, wild-type of IFN-γ receptor knockout mice, and nude mouse strain, $n=15–20$) weighing 24–35 g were housed under constant temperature, lighting and humidity using sterile food and water being provided according to institutional guidelines. Hepatic microsomes were prepared by differential centrifugation, as described [20]. Briefly, Tissue samples were thawed and weighed, and then three volumes of ice-cold homogenisation medium (0.1 *M* sodium phosphate buffer with 0.67 *M* KCl at pH 7.4) were added. The resultant homogenates were transferred to centrifuge tubes, and centrifuged at 9000 *g* for 20 min at 4 °C using a Beckman centrifuge (Beckman Coulter, Fullerton, CA). The supernatant (S9) was collected and centrifuged at 105 000 *g* for 1 h at 4 °C using a L8-70 Beckman ultracentrifuge. The microsome pellet was resus-

pended with homogenisation medium. Hepatic microsomal suspensions were aliquotted (0.5 ml) into a 1.5-ml test tube and stored at −80 °C until used. Microsomal protein concentration was determined by bicinchoninic acid method [21]. The P450 contents were determined as described [22]. All animal procedures were approved by the Animals Ethics Committee of the University of Auckland.

2.3. Microsomal incubations

The kinetics of the in vitro DMXAA glucuronidation and 6-methylhydroxylation with liver microsomes from various strains of mice were investigated using optimised incubation conditions [13]. The typical microsomal incubations for liver microsomal DMXAA glucuronidation contained liver microsomal protein (0.1 mg/ml), 10 m*M* UDPGA, 5 m*M* $MgCl_2$, 0.1 mg/ml D-saccharic acid 1,4-lactone, Brij 58 (0.1–0.4:1, ratio of Brij 58 over microsome, w/w), and DMXAA (5–350 μ*M*) in 0.1 *M* phosphate buffer (pH 6.8), were performed in triplicate. Although UGTs are typically hard to saturate due to its latency issue, the appropriate use of activating detergents will expose enzyme active sites, thus leading to maximal reaction rates. In the present study, Brij 58 as a detergent was used to activate UGTs. D-Saccharic acid 1,4-lactone was used to inhibit the activity of β-glucuronidase in microsomes. The typical incubations for 6-methylhydroxylation contained 1 mg/ml liver microsomes, 5 m*M* $MgCl_2$, 0.5 m*M* NADPH and DMXAA (5–350 μ*M*) in 0.1 *M* phosphate buffer (pH 7.4). The reaction was initiated by the addition of co-factor (UDPGA or NADPH) and conducted at 37 °C for 20 min for glucuronidation and 60 min for 6-methylhydroxylation 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 (SN24350), and vortexing vigorously. Mixtures were centrifuged (3000 *g* for 10 min) to remove the precipitated microsomal protein. The supernatant was removed and evaporated under nitrogen gas using a Speedvac (Solvant Instruments, UK). The residue was reconstituted with 200 μl mobile phase and 50–75 μl injected into the HPLC for measurement of DMXAA-G or 6-OH-MXAA. The formation rate of metabolites, calculated as nmol/min per mg microsomal protein (nmol/min per mg), increased linearly with respect to protein concentration up to 4 mg/ml, and incubation time to 90 min with the given conditions for microsomes from various strains of mice. Within-day assay precision for the formation rate of DMXAA-G or 6-OH-MXAA for five separate incubations of the same batch of microsome from mice was less than 10% at DMXAA concentrations of 30 and 300 μ*M*, respectively.

2.4. HPLC and LC–MS

The methods for the determination of DMXAA-G and 6-OH-MXAA have been described previously [13,23,24]. 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, Torrance, CA) were used. The mobile phase (flow-rate 2.5 ml/min) was acetonitrile: 10 m*M* 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-day coefficient of variations <15%).

The metabolites formed in hepatic microsomes from various species were also identified by linking the HPLC system to the mass spectrometer coupled to an atmospheric pressure chemical ionisation (APCI) or electrospray (ESP) interface (Hewlett-Packard, Avondale, PA, USA). Parameters for APCI interface were as the following: drying gas flow-rate 7 l/min; capillary voltage 4000 V; corona current 5 mA; fragmentor 200 V; nebulising pressure 25 p.s.i.; vaporising temperature 425 °C. The mobile phase flow-rate was 1.0 ml/min. ESP was carried out in the positive ion mode with the needle voltage set at 4000 V, drying gas at 10 l/min, gas temperature at 350 °C, and nebulising pressure at 25 p.s.i. The mobile phase flow-rate was 0.5 ml/min. The mobile phase was the same as used for HPLC. Mass spectra were acquired between m/z 100–750 over a scan duration of 4.91 s.

2.5. Data analysis

Data are presented as mean±SD. Several models to describe the kinetics of DMXAA acyl glucuronidation and 6-methylhydroxylation (single and two binding site, substrate-activator and substrate-inhibitor complex formation, and the sigmoid models) were fitted and compared using the Prism 3.0 program (Graphpad Software, CA, USA). The choice of model was confirmed by comparing and reviewing the relative residuals and the standard error of the parameter estimates from the non-linear regression analysis. The single binding site model $v = V_{max} \times S/(K_m + S)$ gave the best fit, where v is the rate of glucuronidation or hydroxylation; V_{max}, is the maximum velocity; K_m, the Michaelis–Menten constant; S, the substrate concentration. Kinetic constants of DMXAA metabolism in mouse liver microsomes were compared by a two-way analysis of variance (ANOVA) with a post-hoc Newman–Keuls test at a significance level of $P < 0.05$.

3. Results and discussion

Significant strain variations in the kinetic parameters for DMXAA acyl glucuronidation and 6-methylhydroxylation in mouse liver microsomes were observed (Table 1). In all strains studied, DMXAA acyl glucuronidation and 6-methylhydroxylation in the liver microsomes followed Michaelis–Menten kinetics (Fig. 2A,B). For glucuronidation, a 2-, 3- and 6-fold variation was spanned between all mouse strains for K_m, V_{max} and K_m/V_{max} (i.e., CL_{int}), respectively, with a rank order of BDF1 mice > wild type of mice lacking IFN-γ receptor > C57BL/6J mice > Swiss CD mice > nude mice for CL_{int}. For 6-methylhydroxylation, a 2-, 6- and 3-fold variation was spanned between all mouse strains for K_m, V_{max} and CL_{int}, respectively, with a rank order of nude mice > Swiss CD > wild type of mice lacking IFN-γ receptor > C57BL/6J mice > BDF1 mice. Overall, the rank order for total CL_{int} by glucuronidation and 6-methylhydroxylation was BDF1 (1.70 ml/min per g) > wild type of mice lacking IFN-γ receptor (0.80 ml/min per g) > nude mice (0.70 ml/min per g) > Swiss CD mice (0.56 ml/min per g) > C57BL/6J mice (0.46 ml/min per g), with a 4-fold variation between the mouse strain of the highest and lowest CL_{int}. There was no significant correlation between total CL_{int} and MTD ($r^2 = 0.88$, $P > 0.05$), although the rank order for CL_{int} was consistent with that for MTD. The relative resistance of C57BL/6J and BDF1 mice to DMXAA may be due to intrinsic differences in the immune and vascular responses induced by DMXAA, or by differences in tolerance to toxic cytokines that are induced by DMXAA.

It should be noted that the resulting CL_{int} from the present study might not reflect the ''real'' values, as UGTs are usually hard to saturate due to their latency issue. To achieve maximal formation of DMXAA-G, we used Brij 58 to activate UGTs and D-saccharic acid 1,4-lactone to inhibit β-glucuronidase in phosphate buffer at pH 6.8 which will help

Table 1
Comparison of the kinetic parameters for DMXAA metabolism in various mouse strains[a]

Mouse strain	Acyl glucuronidation			6-Methylhydroxylation			
	K_m (μM)	V_{max} (nmol/min per mg)	CL_{int} (ml/min per g)	K_m (μM)	V_{max} (nmol/min per mg)	CL_{int} (ml/min per g)	MTD (mg/kg)
C57BL/6J	144±18	0.05±0.00	0.35±0.04	236±44	0.026±0.002	0.11±0.02	30
BDF1	85±9	0.14±0.01	1.65±0.21	285±56	0.013±0.001	0.05±0.01	30
Swiss CD	144±39	0.04±0.00	0.28±0.08	176.2±43	0.050±0.024	0.28±0.15	N.D.
Wild-type mice lacking IFN-γ receptor	109±18	0.06±0.00	0.55±0.09	329±69	0.083±0.013	0.25±0.09	25
Nude	184±50	0.05±0.01	0.27±0.09	327±86	0.107±0.016	0.33±0.14	20

[a] Kinetics parameters for DMXAA acyl glucuronidation were determined in Brij 58-activated liver microsomes from all strains of mice. Data were obtained from three determinations. $CL_{int} = V_{max}/K_m$. N.D., not determined.

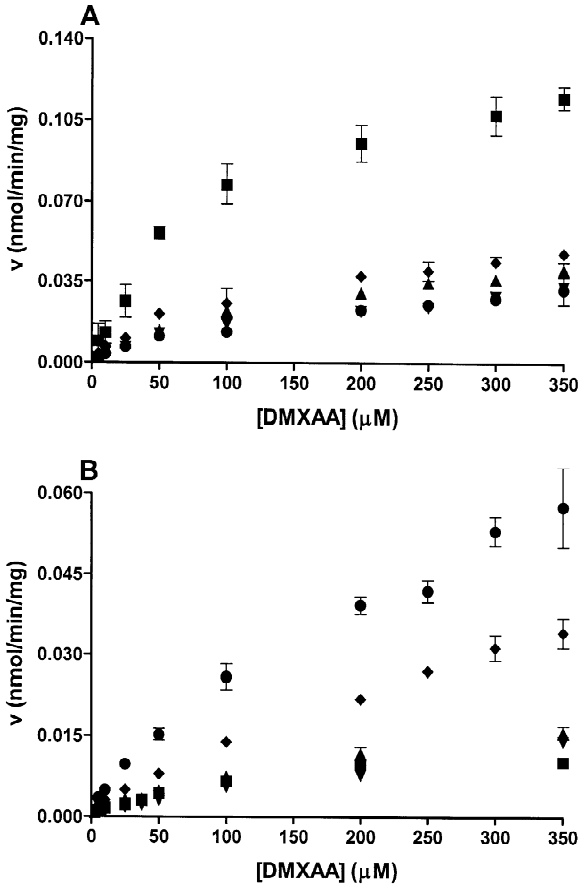

Fig. 2. Typical Michaelis–Menten plots for DMXAA glucuronidation (A) and 6-methylhydroxylation (B) in various strains. Each point represents the mean±SD of three determinations. The curves represent the fit of one-site enzyme model. (■) BDF1, (▲) C57BL/6J, (▼) Swiss CD, (♦) wild type lacking IFN-γ receptor, (●) nude mice.

stabilise DMXAA-G. Hepatocytes are usually considered as a better in vitro model than microsomes for the estimation of CL_{int}, as the latter are subcellular fractions from disrupted cells and thus cofactors (NADPH or UDPGA) are required. Due to the uncertain factors for UGT activities, the in vitro–in vivo extrapolation is difficult. In addition, the lack of correlation between total CL_{int} and MTD may be due partly to that the resulting metabolites (DMXAA-G and 6-OH-MXAA) may also exert some toxicity. It appeared that although DMXAA is highly bound to plasma protein [14], it was bound by microsomal proteins only to a small extent (<15%), resulting in insignificant impact on the utility of in vitro estimation of CL_{int}. However, a high degree of plasma protein binding would affect hepatic extraction and the concentration of free drug in the liver. Therefore, CL_{int} would not be likely to be well modeled in vitro with liver microsomes.

All strains of mice had the ability to metabolise DMXAA to DMXAA-G and 6-OH-MXAA which represented DMXAA metabolic profile in humans. Thus, from a qualitative view, one may conclude that all strains of mice examined are an appropriate model for conducting pre-clinical in vitro metabolism studies. However, significant differences between mice and humans in the kinetic parameters of DMXAA metabolism were observed, as indicated by 10-fold lower CL_{int} values in all strains of mice compared to the human liver microsomal clearance of DMXAA [25].

Similar to humans [24], glucuronidation activity greatly predominated in the BDF1 strain, with minor 6-methylhydroxylation, which is in contrast to all other strains, as approximately equal CL_{int} values were observed for both metabolic pathways. In terms of relative contribution of metabolic pathways, BDF1 appears to the best model representing humans. It would be interesting to compare the metabolic activity of the BDF1 mice to other strains for other compounds such as diclofenac. It would be interesting to see if there were any strain differences in the expression of hepatic UGT1A9/2B7 and CYP1A2 and compared their levels with human livers.

Strain differences in the liver microsomal DMXAA CL_{int} in mice have been demonstrated in this study, which can be due to the differences in both the K_m and its V_{max}. Our previous studies have demonstrated a 19-fold species variation in the total microsomal CL_{int} by glucuronidation and 6-methylhydroxylation between mice, rats, rabbit and humans [25], but a much lower strain variation (6-fold) in total CL_{int} between strains of mice was observed, suggesting the greater differences in the evolution of DMXAA-metabolising enzymes (UGT and CYP) between species than between strain. These in vitro mouse metabolism data for DMXAA may be used to predict the strain differences in the in vivo metabolic clearance. However, extrapolation of drug metabolism from in vitro to in vivo may

encounter difficulties due to the use of liver models based on assumptions.

Significant species differences in the MTD have been observed, and differences in the in vitro metabolism and in vivo pharmacokinetics could not explain these differences [12,25]. Similarly, in the mouse, which is the most sensitive species for DMXAA, the strain-related differences in the metabolism of DMXAA did not provide an explanation for the strain differences in the MTD. Thus, other pharmacokinetic and pharmacodynamic mechanisms involved for the strain differences in MTD should be sought.

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References

[1] M.B. Jameson, P.I. Thomson, B.C. Baguley, B.D. Evans, V.J. Harvey, M.R. McCrystal, P. Kestell, Proc. Annu. Meet. Am. Soc. Clin. Oncol. 19 (2000) 182a.
[2] M. Philpott, B.C. Baguley, L.-M. Ching, Cancer Chemother. Pharmacol. 36 (1995) 143.
[3] B.C. Baguley, G. Cole, L.L. Thomsen, L. Zhuang, Cancer Chemother. Pharmacol. 33 (1993) 77.
[4] L.L. Thomsen, L.-M. Ching, B.C. Baguley, Cancer Res. 50 (1990) 6966.
[5] L.L. Thomsen, L.-M. Ching, L. Zhuang, J.B. Gavin, B.C. Baguley, Cancer Res. 51 (1991) 77.
[6] L.J. Zwi, B.C. Baguley, J.B. Gavin, W.R. Wilson, Oncol. Res. 6 (1994) 79.
[7] Z.H. Cao, B.C. Baguley, L.-M. Ching, Cancer Res. 61 (2001) 1517.
[8] L.-M. Ching, B.C. Baguley, Eu. J. Cancer Clin. Oncol. 25 (1989) 821.
[9] L.-M. Ching, W.R. Joseph, K.E. Crosier, B.C. Baguley, Cancer Res. 54 (1994) 870.
[10] L.K. Webster, A.G. Ellis, P. Kestell, G.W. Rewcastle, Drug Metab. Dispos. 23 (1995) 363.
[11] J.O. Miners, L. Valente, K.J. Lillywhite, P.I. Mackenzie, B. Burchell, B.C. Baguley, P. Kestell, Cancer Res. 57 (1997) 284.
[12] P. Kestell, J.W. Paxton, G.W. Rewcastle, I. Dunlop, B.C. Baguley, Cancer Chemother. Pharmacol. 43 (1999) 323.
[13] S.F. Zhou, J.W. Paxton, M.D. Tingle, P. Kestell, Drug Metab. Dispos. 28 (2000) 1449.
[14] S.F. Zhou, J.W. Paxton, P. Kestell, M.D. Tingle, J. Pharm. Pharmacol. 53 (2001) 463.
[15] J.H. Pang, Z.H. Cao, W.R. Joseph, B.C. Baguley, L.-M. Ching, Eu. J. Cancer 34 (1998) 1282.
[16] P. Kestell, L. Zhao, L.-M. Ching, B.C. Baguley, J.W. Paxton, Cancer Chemother. Pharmacol. 46 (2000) 135.
[17] S.F. Zhou, J.W. Paxton, M.D. Tingle, P. Kestell, L.-M. Ching, Cancer Chemother. Pharmacol. 47 (2001) 319.
[18] G.W. Rewcastle, G.J. Atwell, B.C. Baguley, S.B. Calveley, W. A Denny, J. Med. Chem. 32 (1989) 793.
[19] G.W. Rewcastle, P. Kestell, B.C. Baguley, W.A. Denny, J. Natl. Cancer Inst. 82 (1990) 528.
[20] R.A. Robson, A.P. Matthews, J.O. Miners, M.E. McManus, U.A. Meyer, P.D. Hall, D.J. Birkett, Br. J. Clin. Pharmacol. 24 (1987) 293.
[21] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Garter, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[22] T. Omura, R. Sato, J. Biol. Chem. 23 (1964) 2370.
[23] S.F. Zhou, J.W. Paxton, M.D. Tingle, J. McCall, P. Kestell, J. Chromatogr. (B) 734 (1999) 129.
[24] S.F. Zhou, J.W. Paxton, M.D. Tingle, P. Kestell, M.B. Jameson, P.I. Thomson, B.C. Baguley, Xenobiotica 31 (2001) 277.
[25] S.F. Zhou, J.W. Paxton, M.D. Tingle, P. Kestell, Xenobiotica 32 (2002) 87.