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Effects of elevated temperature on misonidazole O-demethylation by mouse liver microsomes: kinetic and stability studies of a model mixed-function oxidase reaction

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Summary. We investigated the effects of a range of temperatures (33° – 44° C) on the stability and kinetics of C3H mouse liver microsomal misonidazole (MISO) O-demethylase in vitro. Microsomal O-demethylase activity was stable for 60 min at 37° C and for 30 min at 41° C but was steadily inactivated with longer incubation times. Inactivation at 44° and 47° C was exponential, with half-lives of 41 and 11 min, respectively. MISO O-demethylation followed Michaelis-Menten kinetics from 33° to 44° C. The apparent V_{max} for desmethylmisonidazole (Ro 05-9963) formation was decreased by 32% (from 2.14 to 1.47 nmol min^{-1} mg^{-1} protein) with a 4° decrease from 37° to 33° C. An increase of 4° from 37° to 41° C enhanced the V_{max} by 47%, but there was only an additional 9% increase for a further 3° rise to 44° C. Apparent K_m values were unaltered at about 1.6 m*M*. These results show that elevated temperatures in the clinically relevant hyperthermia range (41° – 44° C) can enhance a model mixed-function oxidase reaction in vitro. Such effects may be important for the metabolism, activity and toxicity of anticancer drugs combined with hyperthermia in vivo.

Introduction

Many anticancer drugs are oxidatively metabolised to a variety of active and/or inactive species by enzymes in vivo. This process affects the pharmacokinetics and therapeutic efficacy of several clinically used anticancer drugs, including cyclophosphamide [4, 12], N-methyl compounds [24], the chloroethylnitrosourea CCNU [18] and certain 2-nitroimidazole hypoxic cell sensitisers [36]. Despite the increasing interest in drugs combined with hyperthermia for the treatment of neoplastic diseases in man, there have been very few studies of its effects on drug-metabolising enzymes in vitro [3, 5, 8, 28].

In our previous in vivo studies with 2-nitroimidazoles, we have shown that whole-body hyperthermia (WBH; 41° C) enhances pimonidazole (Ro 03-8799) N-oxidation [30] but not misonidazole (MISO) O-demethylation in mice [29]. This difference in metabolic response to hyperthermia may have arisen through the involvement of two or more enzymes with different thermal stabilities and temperature-dependent catalytic activities. However, it is difficult to establish the precise mechanisms involved due to the number of processes likely to be affected by hyperthermia in vivo. Thus, it is important to characterise the effects of hyperthermia on the stability and kinetics of specific enzymes involved in drug activation and metabolism using controlled conditions in vitro.

The hepatic mixed-function oxidase enzyme system (MFO) is involved in the oxidative metabolism of a wide variety of xenobiotic compounds [33], including the O-demethylation of MISO to the metabolite Ro 05-9963 (Fig. 1) [10, 27, 35]. This study examines the effects of temperature (33° – 44° C) on the stability and catalytic activity of mouse-liver microsomal MISO O-demethylases in vitro, as this particular reaction can be regarded as a model MFO reaction [17, 27, 35]. In addition, 2-nitroimidazole radiosensitisers such as MISO might be combined with local hyperthermia and radiotherapy in the clinic. The temperature range used was selected to cover the clinically important hyperthermic temperatures of 41° – 44° C [25], while the lower temperature is similar to those seen systemically in mice treated with high doses of MISO [11, 35].

Materials and methods

Drugs and chemicals. Misonidazole [1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol; Ro 07-0582; MISO; Fig. 1], its O-demethylated metabolite desmethylmisonidazole [1-(2-nitroimidazol-1-yl)-2,3-propandiol; Ro 05-9963; Fig. 1] and the internal standard Ro 07-0741 [1-(2-nitroimidazol-1-yl)-3-fluoro-propan-2-ol] were supplied by Dr. C. E. Smithen (Roche Products Ltd, Welwyn Garden City, UK). Glucose-6-phosphate, β-NADP and yeast glucose-6-phosphate dehydrogenase were supplied by Sigma Chemical Co (Poole, Dorset, UK).

Microsomal preparation. Liver microsomes were prepared by standard techniques [9, 34] from 13-to 15-week-old male C3H/He mice that had been fasted overnight. Microsomes were washed once in 20 m*M* TRIS-HCl buffer containing 1.15% potassium chloride (pH 7.4) and resuspended in 0.1 *M* sodium phosphate buffer (pH 7.4). Microsomal suspensions were stored at −70° C for up to 6 weeks before use with no measurable loss of activity [2]. All liver and enzyme preparations were handled on ice.

Microsomal metabolism. Standard incubation mixtures were based on a modification of those used by May et al. [23] for CCNU monohydroxylation. Each reaction mixture

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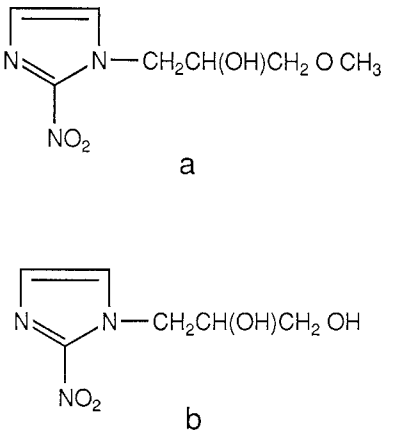

Fig. 1. Structures of *a* misonidazole and *b* desmethylmisonidazole (Ro 05-9963)

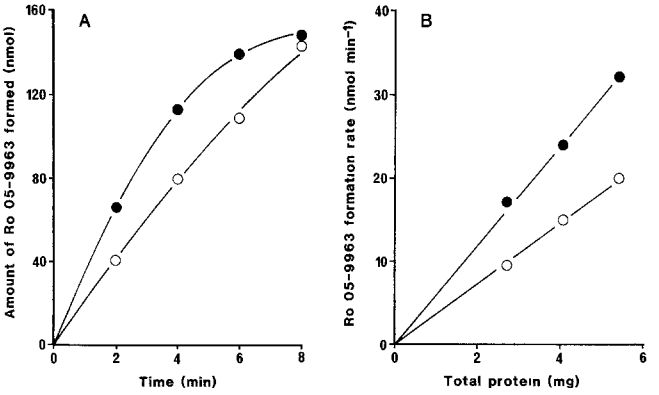

Fig. 2. A Typical progress curves for the formation of Ro 05-9963 from MISO by mouse liver microsomes under aerobic conditions in vitro. *Open symbols*, 37° C; *closed symbols*, 44° C. Each incubation contained an NADPH-regenerating system, 2.72 mg ml^{-1} microsomal protein and 10 m*M* MISO in a final volume of 2 ml 0.1 *M* sodium phosphate buffer (pH 7.4). **B** Effect of microsomal protein concentration and temperature on the rate of Ro 05-9963 formation. *Open symbols*, 37° C; *closed symbols*, 44° C. Results shown were obtained from 2 separate experiments (incubation conditions as in *A*)

contained an NADPH-generating system consisting of 8.3 m*M* glucose-6-phosphate, 360 μ*M* β-NADP, and 0.4 units ml^{-1} yeast glucose-6-phosphate dehydrogenase, as well as 3 mg microsomal protein and 0.5–10 m*M* MISO in a total volume of 2 ml 0.1 *M* sodium phosphate buffer (pH 7.4). Incubations were carried out in 25-ml Erlenmeyer flasks in a shaking water bath (Grant Instruments, Shepreth, Cambridgeshire, UK) set at the desired temperature and shaken vigorously at 150–200 strokes min^{-1} to ensure full oxygenation. Reaction mixtures were preincubated at the appropriate temperature for 3–5 min before the reaction was started by the final addition of MISO in 0.1 *M* sodium phosphate buffer (pH 7.4) (10–200 μl).

Microsomal O-demethylase stability was assayed at 37° C by measuring the activity remaining in an aliquot of preincubation mixture after the appropriate aerobic preincubation in a shaking water bath set at the desired temperature. Preincubation mixtures (20 ml) contained 1.5 mg ml^{-1} microsomal protein in 0.1 *M* sodium phosphate buffer (pH 7.4).

Assays for kinetic and stability studies were carried out in duplicate. Samples (75 μl) were removed consecutively at 2 and 4 min after the start of the reaction. The reactions were stopped by the addition of the samples to methanol (150 μl) containing internal standard (4 mg l^{-1} Ro 07-0741). Samples were centrifuged at 4,000 *g* for 15 min at −15° C and the methanol extracts (15–40 μl) were injected directly onto the column for high-performance liquid chromatographic (HPLC) analysis.

High-performance liquid chromatography. Chromatographic separations were carried out using a modification of our previously published method [37]. All equipment and columns were supplied by Waters Associates (Milford, Mass, USA). The equipment used included 6000 A chromatography pumps, a Model 730 data module, a Model 720 system controller, a Model 710A automated sample processor (WISP) and Model 440 fixed-wavelength UV detectors. Separations were carried out on an octadecylsilane (C18) Resolve or μBondapak Rad-Pak column [8 mm inside diameter (id) × 10 cm; 10 μm bead size] fitted to an RCM-100 or Z-module as appropriate. Columns were eluted isocratically at a flow rate of 2–4 ml min^{-1} with a 25%–35% methanol:water mobile phase. Peaks were detected by UV absorbance at 313 nm. Protein concentration was determined using bovine serum albumin as a standard [20].

Kinetic analysis. Michaelis-Menten kinetics were established using the criteria described by Henderson [13]. Plots of s/v vs s were used to check visually for deviations from Michaelis-Menten kinetics, as this type of analysis has been shown to have minimally distorted experimental errors (see [13]). Apparent K_m and V_{max} values with SE limits were determined by weighted ($W = v^2$; [13]) least-squares linear regression analysis of duplicate values of 1/v vs 1/s [19] using the Generalised Linear Interactive Modelling (GLIM) programme of the Royal Statistical Society of London. Half-lives were calculated from enzyme stability data using a linear-regression analysis programme for a desk-top programmable calculator.

Results

Characteristics of the enzyme reaction

The incubation conditions were demonstrated to be non-rate-limiting at 37° and 44° C with respect to the β-NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase concentrations used. The individual components of the NADPH-regenerating system were required for activity. No O-demethylation was observed in the absence of microsomes or with boiled microsomes.

Progress curves for Ro 05-9963 formation were linear up to 4 min and became non-linear thereafter (Fig. 2, *A*). Initial reaction rates were derived in duplicate by fitting straight lines through the 0, 2 and 4 min time points (Fig. 2, *A*). All reaction rates were obtained before 10% substrate depletion occurred. The Ro 05-9963 formation rate was proportional to microsomal protein concentration in the range 2.7–5.5 mg ml^{-1} at both 37° and 44° C (Fig. 2, *B*), and rates were increased at the elevated temperature (Fig. 2, *A*, *B*).

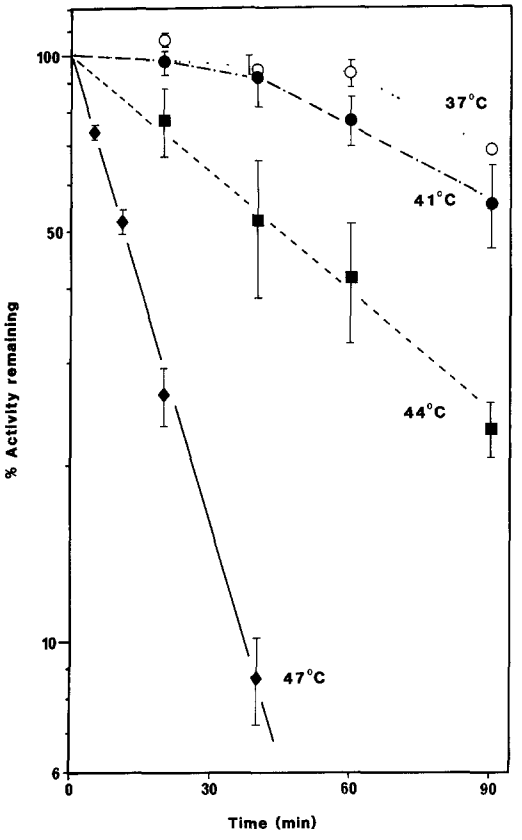

Fig. 3. Effects of temperature and aerobic preincubation time on the residual MISO O-demethylase activity in mouse liver microsomes assayed subsequently at 37° C. Symbols: ○, 37° C; ●, 41° C; ■, 44° C; ◆, 47° C. Incubations contained an NADPH-regenerating system, 1.5 mg ml^{-1} microsomal protein and 10 m*M* MISO in a final volume of 2 ml 0.1 *M* sodium phosphate buffer (pH 7.4). Results represent the mean ±2 SE of 3–4 determinations per point using pooled data from 2 independent experiments. Lines were fitted to the data by least-squares linear regression analysis

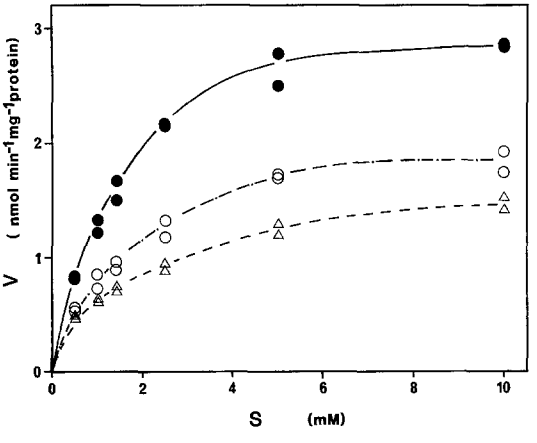

Fig. 4. Representative plots of v vs s for Ro 05-9963 formation by mouse liver microsomes under aerobic conditions in vitro at 33° C (△), 37° C (○) and 44° C (●). Incubation conditions were as described in Fig. 2. Results are duplicate determinations from a single experiment with 6 substrate concentrations per temperature. Units of s and v are expressed as m*M* and nmol min^{-1} mg^{-1} protein, respectively. Lines were fitted to the data by eye. Similar results were obtained in a repeat experiment

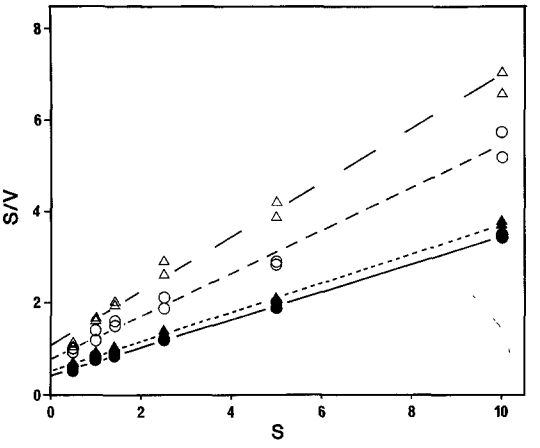

Fig. 5. Representative plots of s/v vs s for Ro 05-9963 formation by mouse liver microsomes under aerobic conditions in vitro. Symbols: △, 33° C; ○, 37° C; ▲, 41° C; ●, 44° C. Incubation conditions were as described in Fig. 2. Results shown are duplicate determinations for 6 different substrate concentrations at each temperature. Units of s and v are expressed as m*M* and nmol min^{-1} mg^{-1} protein, respectively. Lines were fitted to the data by eye. Similar results were obtained in a repeat experiment

Microsomal enzyme stability

Figure 3 shows the microsomal O-demethylase activity remaining after up to 90 min aerobic preincubation at various temperatures from 37° to 47° C. O-Demethylase activity was stable for about 60 min at 37° C, with a 31% loss in activity after 90 min. At 41° C the enzyme activity was stable for about 40 min and thereafter decreased exponentially with a half-life of 69 min, with 55% of the initial activity remaining after 90 min incubation.

O-Demethylases were rapidly inactivated at 44° C and above, with a 48% and 92% loss of activity after 40 min incubation at 44° and 47° C, respectively. The loss of enzyme activity was exponential over the entire time course, with half-lives of 41 and 11 min, respectively.

Microsomal enzyme kinetics

The formation of Ro 05-9963 followed Michaelis-Menten kinetics at all temperatures studied between 33° and 44° C. Plots of v against s were rectangular hyperbolas (Fig. 4), and plots of s/v vs s were linear throughout (Fig. 5). Table 1 shows the apparent K_m and V_{max} values for MISO O-demethylation for two separate microsomal preparations. It can be seen that a decrease in temperature of 4° C from 37° to 33° C resulted in a 32% decrease in V_{max}. Conversely, the 4° rise in temperature from 37° to 41° C resulted in an average 47% increase in V_{max}. At 44° C the V_{max} was enhanced by about 58% compared with 37° C, a rise of only 9% above the value seen at 41° C despite the further 3° rise in temperature. Although the two microsomal preparations exhibited slightly different O-demethylase activities, the apparent K_m values were remarkably similar for each preparation and were clearly unaltered by temperature (Table 1).

Discussion

The MFO enzyme system consists of three major components: the haemoprotein cytochrome P-450, the flavoprotein NADPH:cytochrome P-450 (cytochrome c) reduc-

Table 1. Effects of elevated temperature on the apparent K_m and V_{max} for MISO O-demethylation by C3H/He mouse liver microsomes in vitro

Microsomal preparation	Temperature (°C)	K_m[a] (m*M*)	V_{max}[a] (nmol min^{-1} mg^{-1} protein)
A	44	1.61 (0.119)	4.84 (0.179)
	41	1.58 (0.186)	4.52 (0.299)
	37	1.55 (0.138)	3.18 (0.141)
B	44	1.57 (0.10)	3.39 (0.107)
	41	1.65 (0.162)	3.24 (0.134)
	37	1.63 (0.162)	2.14 (0.106)
	33	1.37, 1.54 (0.169), (0.095)	1.62, 1.31 (0.094), (0.176)

[a] Results were derived from 2 independent experiments. Parameters (±SE) were calculated using pooled data from duplicate determinations, with 6 different substrate concentrations per determination, by weighted least-squares linear regression analysis of 1/v vs 1/s

tase, and phosphatidylcholine [21]. It is involved in the oxidative metabolism of many foreign compounds [6, 17, 33], including the O-demethylation of MISO [27, 35]. Previous work using rat liver microsomes in vitro at 37° C, with both NADH and NADPH cofactors, showed that MISO O-demethylation was reduced markedly under N_2 and in the presence of NADH alone but not with NADPH alone [27]. In the present studies on mouse liver microsomal O-demethylation, all of the individual components of the NADPH-regenerating system were required for enzyme activity. The kinetic parameters K_m and V_{max} reported for uninduced, normal rat liver microsomes were 1.87 ± 0.30 m*M* and 413 ± 14 pmol min^{-1} mg^{-1} protein, respectively (mean ±SE; $n = 4$ or 5) [27]. This is in good agreement with the K_m values described for C3H/He mouse liver microsomes, but the apparent V_{max} was found to be 10-fold higher in mice.

The present results clearly show that increases in temperature over the range 33°–44° C can markedly accelerate the rate of O-demethylation of MISO by mouse liver microsomes under aerobic conditions in vitro. This range of temperature was selected for its biological and therapeutic relevance but was unsuitable for Arrhenius-type analysis. The lower temperature was chosen because mouse core temperatures commonly fall to around 33° C after high doses of 2-nitroimidazoles [35]. The upper temperature is similar to those used in clinical hyperthermia treatments [25].

Microsomal O-demethylase enzymes were relatively stable for up to 40 min at 37° and 41° C, although inactivation rates increased after longer incubation times. At 44° and 47° C O-demethylases were readily inactivated. This may be a result of direct thermal denaturation of the enzyme protein as seen with other enzymes [7], but alternative possibilities exist. For example, it is known that aerobic liver microsomal preparations are readily inactivated by lipid peroxidation in vitro [15–17], and this process may be accelerated at increased temperatures, particularly in the presence of unchelated Fe^{2+} [14].

Ro 05-9963 production from MISO by mouse liver microsomes followed Michaelis-Menten kinetics at 33°, 37°, 41° and 44° C. The apparent V_{max} was accelerated with increasing temperature, by about 32% from 33° to 44° C and by a further 47% from 37° to 41° C. However, at 44° C there was only a further 9% increase in rate relative to 41° C, probably as a result of a substantial increase in enzyme denaturation (Fig. 3). Apparent K_m values were unaltered at these elevated temperatures.

This demonstration that moderate, clinically achievable increases in temperature can substantially enhance MISO O-demethylation in vitro appears to be in contrast with our in vivo results in which whole-body hyperthermia (WBH; 41° C) had no effect on plasma concentrations of Ro 05-9963 compared with unheated controls [29]. This discrepancy may be a result of an equal enhancement of Ro 05-9963 production and metabolic degradation in vivo. Reductive metabolism is a major degradation pathway for nitroimidazoles [26], and we have demonstrated a stimulation of benznidazole nitroreductive metabolism both in vitro under anoxic conditions [31] and in vivo [29, 32]. Alternatively, WBH may inhibit MISO demethylases in vivo through restricting the cofactor and substrate supply and/or denaturing the enzymes involved.

Pimonidazole N-oxidation has been significantly enhanced in vivo by WBH [30], but there is evidence that this is not likely to be a classic MFO-catalysed reaction [1]. Clawson et al. [3] have reported that the production of alkylating species from cyclophosphamide was decreased in aerobic rat liver microsomes and slices at temperatures above 40.5° and 41.8° C, respectively, compared with 37° C. Similarly, Collins and Skibba [5] have shown that the alkylating activity in perfused rat livers was decreased at 42° C compared with 37° C. Both studies concluded that hyperthermia depressed the metabolic activation of cyclophosphamide. It should be noted, however, that the assay used for alkylating activity does not distinguish between the various cyclophosphamide metabolites, and it may not be equally sensitive to the individual alkylating species generated. In addition, cyclophosphamide does not represent an ideal substrate for investigating temperature-MFO enzyme interactions, as one of its metabolites (acrolein) is a potent inhibitor of this enzyme system [22]. Nevertheless, Collins and Skibba [5] also reported that elevated temperatures (37°–43° C) decreased the half-life of antipyrine in perfused rat livers and concluded that hyperthermia markedly depressed hepatic MFO activity. Despite the differences in the systems used and the potential shortcomings of the methods applied, it seems reasonable to conclude that hyperthermia has a complex effect upon MFO drug-metabolising enzymes in vivo and in vitro.

In summary, these studies have clearly shown that moderate hyperthermia enhances the rate of MISO O-demethylation in mouse microsomes under aerobic conditions in vitro. The type of studies described here form an essential part of an attempt to identify the mechanisms involved in heat-induced changes in drug pharmacokinetics and metabolism. Further studies are required to establish the type of enzymes and reactions that might be particularly susceptible to enhancement by hyperthermia in vivo.

This might facilitate the rational development of drugs undergoing preferential bioactivation in locally heated tumours.

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