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The Comparative Pharmacokinetics of Pentamethylmelamine in Man, Rat, and Mouse

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Summary. *The pharmacokinetics of pentamethylmelamine (PMM) have been investigated in mouse (Balb C⁻, CBA/LAC, nude), rat (Wistar), and man. In all three species, PMM was extensively demethylated to N^2,N^2,N^4,N^6-tetramethylmelamine and N^2,N^4,N^6-trimethylmelamine, although marked species differences in the rate of metabolism were observed. PMM metabolism was more rapid in the mouse (plasma $t_{1/2}$ = < 15 min) than in the rat (plasma $t_{1/2}$ = 40 min), and slower in man (plasma $t_{1/2}$ = 102 min) than in either mouse or rat. Furthermore, the peak plasma concentrations of N-methylolmelamines, intermediates generated during oxidative N-demethylation, were correspondingly higher in the mouse (563–773 μM) than in the rat (211 μM), whilst in man they were undetectable (< 50 μM). In view of the highly cytotoxic nature of N-methylolmelamines, we conclude that these pharmacokinetic differences may be related to the antitumour effectiveness of PMM in mouse, rat, and man.*

Introduction

Pentamethylmelamine (PMM) is a water-soluble analogue of hexamethylmelamine (HMM) which has shown activity comparable to HMM against a number of experimental tumours, in particular several human tumour xenografts grown in the mouse [6, 14, 18]. It has been suggested that PMM, like HMM, requires metabolic activation to elicit this antitumour effect [22]. The disappearance of HMM and PMM from the plasma is primarily a result of metabolism [2, 3, 24, 28, 29] involving progressive oxidative *N*-demethylation to *N*-methylmelamine metabolites (Fig. 1).

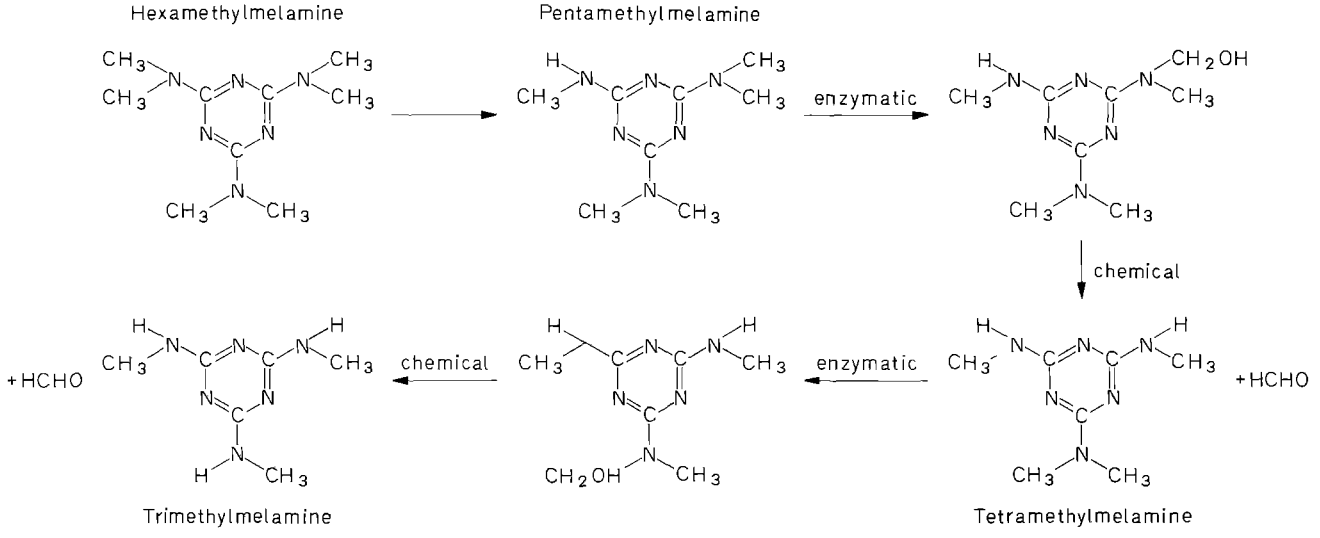

Fig. 1. The metabolism of pentamethylmelamine

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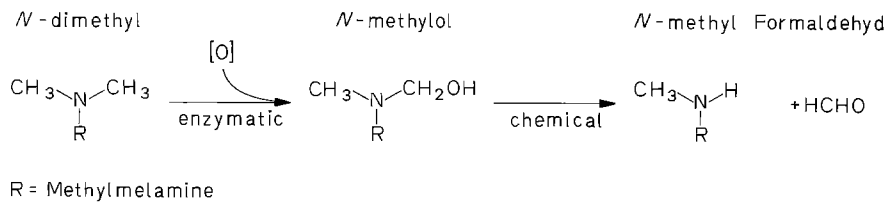

Fig. 2. *N*-Methylolmelamine formation and decomposition

N-Demethylation is thought to proceed via *N*-methylolmelamine intermediates (Fig. 2) [22]. Of interest in this respect is the demonstration of N^2-monomethylol-PMM as an in vitro metabolite of HMM [12]. *N*-Methylolmelamines are chemically unstable and subsequently break down to release formaldehyde and the corresponding *N*-methylmelamine [4, 8]. *N*-Methylolmelamines are considerably more toxic to a number of tumour cell lines in vitro than are PMM and its *N*-methylmelamine metabolites [23]. Furthermore, *N*-methylolmelamines have shown antitumour activity in vivo [6, 14] and therefore are thought to be responsible for the antitumour effect of PMM in vivo.

N-Methylmelamines have previously been estimated in biological fluids primarily by gas-liquid chromatography (GLC) [1, 9, 10, 15]. However, the temperatures utilised in these methods induce breakdown of the *N*-methylolmelamines to *N*-methylmelamines, and can thus give an artificially high estimate of the concentrations of these last compounds. To circumvent this problem, *N*-methylmelamines have been estimated in the present study by high-performance liquid chromatography (HPLC) at room temperature. Although *N*-methylolmelamines can also be estimated by HPLC, the lack of independently synthesised standards precluded the validation of the technique. Thus the total *N*-methylolmelamine content of samples was assayed via the formaldehyde generated in the Nash method [19].

Despite the known activity of PMM in experimental tumour systems, a number of recent phase I clinical studies have failed to demonstrate any complete or partial responses to PMM in man [5, 11, 13, 16, 26]. The present study was therefore designed to determine whether a pharmacological rationale existed for this apparent discrepancy. The pharmacokinetics of PMM were investigated in three strains of mice, i.e., the Balb C^- and the athymic (nu/nu) mouse which carry the PMM-sensitive PC6 tumour and a number of human tumour xenografts, respectively, and the CBA/LAC mouse which carries the PMM-resistant TLX/5 lymphoma. Studies were also performed in the Wistar rat, which is the host for the marginally PMM-sensitive Walker 256 tumour. Finally, the pharmacokinetics of PMM were examined in patients receiving the drug as an IV infusion during a phase I clinical study at the Royal Marsden Hospital [26].

Materials and Methods

Chemicals. HMM, PMM, N^2,N^2,N^4,N^6-tetramethylmelamine, N^2,N^4,N^6-trimethylmelamine, N^2-monomethylol-PMM, N^2,N^4-dimethylol-N^2,N^4,N^6,N^6-tetramethylmelamine, and N^2,N^4,N^6-trimethylol-N^2,N^4,N^6-trimethylmelamine were synthesised by Prof. W. C. J. Ross and Dr D. E. V. Wilman and their colleagues at the Institute of Cancer Research, London, GB, according to published methods [4, 7, 17]. Methanol, analytical grade, was supplied by James Burroughs Ltd (London, GB). All other chemicals, also analytical grade, were supplied by BDH Chemicals Ltd (Poole, Dorset, GB) or by Fisons Scientific Ltd (Loughborough, GB) or Hopkin and Williams Ltd (Romford, Essex, GB).

Patient Treatment. Patients were entered in the phase I trial of PMM under the care of Dr I. E. Smith, Royal Marsden Hospital, Sutton, Surrey, GB, and the study conducted as described previously [26]. PMM was administered as an IV infusion (1–4 h) in 2% ethanolic/saline (250 ml/h) over the dose range 100–1,300 mg/m^2. Blood samples were taken from an indwelling IV cannula in the arm opposite to that of the infusion for up to 4 h after the end of the infusion. Plasma was separated and analysed as described below. *N*-Methylmelamines were analysed (utilising GLC) only in samples from patients receiving 100–500 mg PMM/m^2 where higher sensitivity was required, and where *N*-methylolmelamines were not present and therefore did not interfere with the assay for *N*-methylmelamines. Plasma samples from patients at all dose levels were analysed for *N*-methylolmelamines.

Animal Studies. Groups of three female Balb C^-, female CBA/LAC or male athymic (nu/nu) mice (18–25 g) and male Wister rats (150–200 g) were given a dose of 90 mg PMM/kg IP in 2% ethanolic/saline (0.01 ml/g body weight). A group of three solvent controls was included in each experiment. Animals were anaesthetised with diethyl ether at various times after drug administration and exsanguinated by direct cardiac puncture. Blood was placed in heparinised tubes (10 IU/ml) kept on ice and plasma was prepared by centrifuging at 600 *g* for 10 min at 4° C. One volume of the resulting plasma (routinely 0.25 ml) was precipitated on ice by the addition of 1 vol saturated $Ba(OH)_2$ and 1 vol 0.12 *M* $ZnSO_4$, titrated to give a final pH of 8.5–9.0. Precipitation under alkaline conditions was designed to stabilise possible *N*-methylolmelamine metabolites [21]. The protein precipitate was removed by centrifuging at 1,500 *g* for 20 min at 4° C.

Metabolite Analysis

a) Estimation of N-Methylmelamines in Plasma by HPLC. Aliquots (50 µl) of the supernatant following precipitation with $Ba(OH)_2$ and $ZnSO_4$ were analysed for PMM, N^2,N^2,N^4,N^6-tetramethylmelamine and N^2,N^4,N^6-trimethylmelamine. Analysis was performed on a Waters Associates Model ALC/GPC 204 chromatograph (Waters Associates, Milford, Mass., USA) equipped with a Model 450 variable-wavelength UV detector. Separation was achieved on a 25 cm × 4.6 mm column containing Spherisorb 5 µm hexyl packing (Phase Sep. Ltd, Queensferry, Clwyd, Great Britain) protected with a 6.5 cm × 2.1 mm precolumn containing CO:PELL ODS packing (Whatman, Maidstone, Kent, GB). Compounds were eluted isocratically with 50% methanol/50% 0.05 *M* NH_3HCOOH pH 6.3 (v/v) at a flow rate of 1.5 ml/min and detected in the eluate by absorption at 225 nm and 254 nm. Quantitation was achieved by measurement of peak heights at 225 nm and compounds identified by retention volume and 254/225 nm ratios.

PMM, N^2,N^2,N^4,N^6-tetramethylmelamine and N^2,N^4,N^6-trimethylmelamine estimation was linear over the range 1−200 µ*M* ($r = > 0.99$ in all cases).

b) Estimation of N-Methylmelamines in Human Plasma by GLC. Duplicate 1-ml aliquots of plasma were made alkaline by the addition of 20 µl 1 *M* NaOH. Plasma was then extracted twice with ethyl acetate as previously described [20] and the dried concentrated extract re-dissolved in 100 µl ethyl acetate containing 21 µg/ml HMM as an internal standard. Of the extract, 10 µl was then analysed on a Perkin-Elmer F17 gas chromatograph (Perkin-Elmer Ltd, Beaconsfield, Bucks., GB) fitted with a nitrogen-specific detector supplied with H_2 at 3 ml/min and air at 110 ml/min. Separations were carried out on a 1 m × 4 mm column containing 8% carbowax 20 *M* 2% KOH on Chromosorb W 800-100 mesh (Phase Sep. Ltd, Queensferry, Clwyd, GB) operating at a temperature of 230° C with an injector temperature of 275° C and a carrier gas (N_2) flow rate of 50 ml/min. Compounds were quantitated by measurement of peak height relative to that of the internal standard.

PMM was recovered with an extraction efficiency of 105 ± 4% (SD) over the concentration range 1−100 µ*m*, N^2,N^2,N^4,N^6-tetramethylmelamine with an efficiency of 104 ± 7% (1−100 µ*M*) and N^2,N^4,N^6-trimethylmelamine with an efficiency of 99 ± 4% (5−100 µ*M*). All assays were linear over the concentration range given ($r = > 0.99$ in all cases).

c) Estimation of N-Methylolmelamines in Plasma by the Micro-nash Technique. *N*-Methylomelamines were estimated by measuring the amount of formaldehyde they release on breakdown under the conditions of the Nash assay [19]. $Ba(OH)_2/ZnSO_4$ supernatant (0.3 ml) was mixed with 0.15 ml Nash reagent (0.04 *M* acetyl acetone, 3.9 *M* NH_3CH_3COOH) and incubated at 60° C for 30 min. The resultant colour was measured at 412 nm on a Pye-Unicam SP500 spectrophotometer (Pye-Unicam Ltd, Cambridge, GB) in 1 cm × 0.2 cm microcuvettes.

N^2-Monomethylol-PMM was utilised to calibrate this technique. The assay was linear ($r > 0.99$) over the concentration range 50−1,000 µ*M* formaldehyde equivalents. The assay was equally reproducible when either N^2,N^4-dimethylol-N^2,N^4,N^6,N^6-tetramethylmelamine or N^2,N^4,N^6-trimethylol-N^2,N^4,N^6-trimethylmelamine was used.

d) Estimation of Formaldehyde in Plasma by the MBTH Assay. Of the $Ba(OH)_2/ZnSO_4$ supernatant, 0.5 ml was mixed with 0.5 ml 0.02 *M* 3-methyl-2-benzo thiozolinone hydrazone (MBTH) (Sigma Chemical Co., Poole, Dorset, GB) and left to stand on ice for 30 min, after which 2.5 ml 0.012 *M* $FeCl_3$ was added with thorough mixing and the mixture kept on ice for exactly 5 min. Then 6.5 ml acetone was added and the absorbance read immediately after mixing at 670 nm. Development of the colour on ice substantially reduces the breakdown of *N*-methylolmelamines to formaldehyde during the assay.

The assay for formaldehyde was linear ($r = > 0.99$) over the range 50−100 µ*M*. N^2-Monomethylol-PMM decomposition was marginal (11% at 1.5 m*M*) under the assay conditions used.

Pharmacokinetic Analyses. A mathematical function was fitted to the plasma levels of PMM by means of a non-linear least-squares analysis [25]. Following PMM administration the exponential function

$$C = Ae^{-\beta t}$$

was fitted, where C is the drug plasma concentration, t is the time after dosing (or, in clinical samples, the time after the end of the infusion), A is a concentration constant, and β the first-order disposition rate constant. The plasma terminal phase half-life, $t^{\beta}_{1/2}$, was calculated as

$$t^{\beta}_{1/2} = \frac{0.693}{\beta}.$$

The areas under the *N*-methylolmelamine plasma concentration vs time curves were determined by the trapezoidal rule [27].

Plasma levels of N^2,N^2,N^4,N^6-tetramethylmelamine, N^2,N^4,N^6-trimethylmelamine, and *N*-methylolmelamines were plotted manually, the mean value for each determination being used.

Results

In all the strains and species studied, N^2,N^2,N^4,N^6-tetramethylmelamine and N^2,N^4,N^6-trimethylmelamine were detected as PMM metabolites. However, marked species differences in the rate of metabolism of PMM were observed, as detailed below.

a) Pharmacokinetics of PMM in the Mouse

Figure 3 shows the plasma levels of PMM, N^2,N^2,N^4,N^6-tetramethylmelamine and N^2,N^4,N^6-trimethylmelamine in the Balb C^- mouse following the administration of 90 mg PMM/kg IP. The pharmacokinetics of PMM in CBA/LAC and athymic mice were essentially the same (Table 1). In all three strains PMM metabolism was rapid, as indicated by the plasma $t^{\beta}_{1/2}$ values: Balb C^- = 7.7 ± 0.2 min; CBA/LAC = 14.2 ± 3.8 min; athymic = 12.8 ± 3.2 min. Peak plasma levels of *N*-methylolmelamines were correspondingly high: Balb C^- = 563 ± 24 µ*M*; CBA/LAC = 719 ± 54 µ*M*; athymic = 773 ± 146 µ*M*, occurring soon after administration (< 30 min). The areas under the *N*-methylolmela-

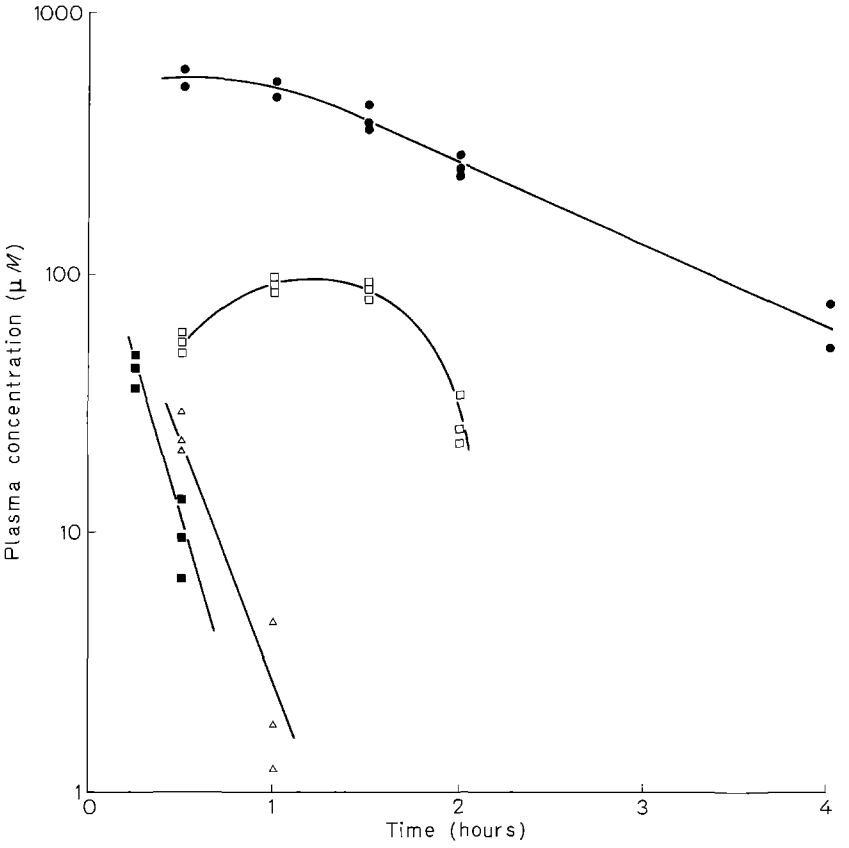

Fig. 3. The pharmacokinetics of PMM in the Balb C^- mouse following IP administration of 90 mg PMM/kg. *N*-Methylmelamines were measured by HPLC. (■———■) PMM; (△———△) N^2, N^2, N^4, N^6-tetramethylmelamine; (□———□) N^2, N^4, N^6-trimethylmelamine; (●———●) total *N*-methylolmelamines. *Lines* were fitted to *points* as described in the *Methods* section under *Pharmacokinetic Analyses*. Each *point* represents a determination made in a single animal

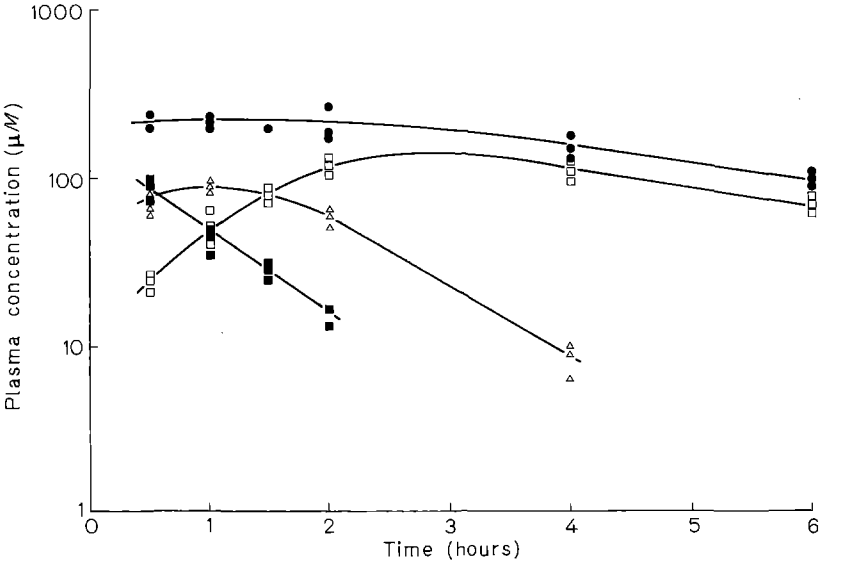

Fig. 4. The pharmacokinetics of PMM in the Wistar rat following IP administration of 90 mg PMM/kg. *N*-Methylmelamines were measured by HPLC. (■———■) PMM; (△———△) N^2, N^2, N^4, N^6-tetramethylmelamine; (□———□) N^2, N^4, N^6-trimethylmelamine; (●———●) total *N*-methylolmelamine. *Lines* were fitted to *points* as described in the *Methods* section under *Pharmacokinetic Analyses*. Each *point* represents a determination made in a single animal

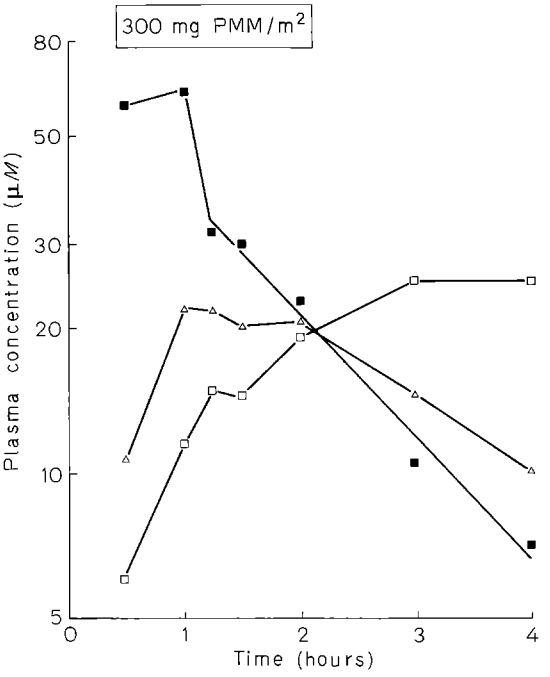

Fig. 5. Pharmacokinetics of PMM in a patient during and after a 1-h IV infusion of 300 mg PMM/m². *N*-Methylmelamines were measured by GC. (■———■) PMM; (△———△) N^2,N^2,N^4,N^6-tetramethylmelamine; (□———□) N^2,N^4,N^6-trimethylmelamine. *Lines* were fitted as described in the *Methods* section under *Pharmacokinetic Analyses*. Each *point* represents the mean of duplicate estimations in a single plasma sample

mine plasma concentration vs time curves were also of a similar order of magnitude: Balb C^- = 1,085 μ*M* h; CBA/LAC = 1,270 μ*M* h; athymic 1,235 μ*M* h. *N*-Methylolmelamines were no longer detectable 6 h after PMM administration. No free formaldehyde was detected in any of the samples as measured by the MBTH method.

b) Pharmacokinetics of PMM in the Rat

Figure 4 shows the plasma levels of PMM, N^2,N^2,N^4,N^6-tetramethylmelamine and N^2,N^4,N^6-trimethylmelamine in the rat following IP administration of PMM at 90 mg/kg. The rate of PMM metabolism is substantially slower in the rat (plasma $t^{\beta}_{1/2}$ = 40.5 ± 4.6 min) than in the mouse, and the peak plasma concentration of *N*-methylolmelamines is correspondingly lower (211 μ*M*). The *N*-methylolmelamine concentration is maintained for a longer period, however, thereby giving a similar area under the plasma concentration vs time curve (955 μ*M* h) to that seen in the mouse. Again, no free formaldehyde was detected in the plasma of rats at any time following PMM administration.

c) Pharmacokinetics of PMM in Man

The detailed analysis of the pharmacokinetics of PMM in man, determined during phase I clinical trial at the Royal Marsden Hospital [26], will be the subject of a separate publication. In brief, following the administration of PMM (100–500 mg/m²) as an IV infusion, plasma levels of the drug decayed with a $t^{\beta}_{1/2}$ of 101.8 ± 16.9 min (six patients). Eight patients were studied in total, but two were excluded because the equation did not fit to the observed data. Of considerable importance is the observation that at no time were *N*-methylolmelamines detected in the plasma of patients receiving PMM (100–1,300 mg/m²). An example of PMM pharmacokinetics is given in Fig. 5.

Discussion

The pharmacokinetics of PMM in man, rat, and mouse, determined in the present study, are summarised in Table 1. The metabolism of PMM was rapid in both mice and rats, notably so in the mouse. The levels of *N*-methylolmelamines thereby generated in the rat and mouse were greatly in excess of those required for cytotoxicity [23]. Therefore, if *N*-methylolmelamines represent the proximally active species, as has previously been suggested [22, 23], sufficiently high levels of these metabolites are formed, in theory, to allow an antitumour response to be obtained. PMM has shown limited but definite activity against some murine tumours [18, 22]. Of more interest is the marked activity of PMM against a number of human tumour xenografts grown in the

Table 1. The pharmacokinetics of PMM in mouse, rat, and man

Species	Strain and sex	PMM dose	PMM plasma $t_{1/2}^{\beta}$ (min ± SE)[a]	*N*-Methylolmelamines		
				Maximum plasma concentration[b]	Time after administration of max. concentration	AUC[c]
Mouse	Balb C$^-$ (♀)	90 mg/kg	7.7 ± 0.2	563 ± 24 μ*M*	30 min	1,085
	CBA/LAC (♀)		14.2 ± 3.8	719 ± 54 μ*M*	30 min	1,270
	nu/nu (♂)		12.8 ± 3.2	773 ± 146 μ*M*	30 min	1,235
Rat	Wistar (♂)	90 mg/kg	40.5 ± 4.6	211 ± 14.7 μ*M*	60–120 min	955
Man	–	100–1300 mg/m^2	101.8 ± 16.9	< 50 μ*M*	–	–

[a] Plasma PMM concentrations were estimated by HPLC except in samples from patients given 100–500 mg PMM/kg
[b] Mean ± SE
[c] AUC = area under the plasma concentration vs time curve (μ*M* × h)

mouse [6, 18]. The sensitivity of such human tumour xenografts to PMM may therefore be a result of the extensive formation of *N*-methylolmelamines in the mouse.

In man PMM metabolism was relatively slow and *N*-methylolmelamines were not detectable. Although further evaluation of the antitumour effectiveness of PMM is required, the initial indications from a number of phase I clinical studies are that PMM has poor activity versus human cancer [5, 11, 13, 16, 26]. Therefore it is possible that the poor clinical response to PMM may be a result of the failure to achieve cytotoxic levels of *N*-methylolmelamines. In those patients where a minor response to PMM has been reported [11] activity is presumably due to the presence of low and possibly sustained levels of *N*-methylolmelamines. In this respect Benvenuto et al. [3] have recently reported that Nash-positive metabolites are in fact observed in patients' plasma following the administration of PMM. Furthermore, they had a prolonged plasma half-life (13.7 h), although these authors do not report either the plasma concentrations of these metabolites or the dose of PMM which gave rise to them. However, since severe gastrointestinal toxicity is dose-limiting for PMM it may not be possible to administer sufficient drug to man to allow the production of cytotoxic levels of *N*-methylolmelamines.

Two factors are probably responsible for the reduced generation of *N*-methylolmelamine metabolites in man reported in the present study. Firstly, the rate of PMM metabolism, as indicated by the plasma $t_{1/2}^{\beta}$, is significantly slower in man than in either the rat or the mouse. Secondly, the highest dose of PMM administered to patients, which in our study was 1.3 g/m^2 (≃ 30 mg/kg), is lower than that used to treat, successfully, tumours in experimental animals (90 mg/kg). However, the possibility of a qualitative metabolism difference has not been excluded.

We are mindful of the fact that the micro-Nash technique employed in the present study does not represent a definitive proof of the presence of *N*-methylolmelamines, and that the sensitivity of the assay is limited. However, we have excluded the possibility that formaldehyde is responsible for the Nash-positivity in rodent plasma. More positive structural identification of these metabolites is currently being sought.

The direct administration of an *N*-methylolmelamine would obviously circumvent the need for metabolic activation. In this respect, N^2,N^4,N^6-trimethylol-N^2,N^4,N^6-trimethylmelamine is a promising candidate. This compound has comparable antitumour activity to HMM and PMM in experimental mouse systems [6, 13]. We are thus investigating the toxicology and antitumour activity of this compound more extensively.

Important parallel studies are those designed to elucidate the mechanisms of *N*-methylmelamine and *N*-methylolmelamine antitumour activity. Although we feel that metabolism is an essential prerequisite of antitumour activity this is obviously not the sole determinant. Thus in the present study the metabolism of PMM in the CBA/LAC mouse, which carries the resistant TLX/5 lymphoma, was essentially the same as in the Balb C$^-$ and athymic (nu/nu) mice, which carry the sensitive PC6 tumour and human tumour xenografts, respectively.

In conclusion, this study reveals some interesting species differences in the metabolism of PMM. The direct relevance of the resultant differences in *N*-methylolmelamine production to antitumour activity requires further examination. However, the results presented in this paper suggest that the rapid

metabolism of PMM in the mouse, leading to high plasma levels of *N*-methylolmelamines, may be responsible for the high degree of sensitivity of certain experimental tumours. Conversely, the slow rate of metabolism of PMM and the low levels of *N*-methylolmelamines in man may be related to the poor clinical activity of this agent. This study further emphasises the importance of pharmacokinetic studies as an adjunct to experimental and clinical cancer chemotherapy, especially when antitumour activity is dependent upon host metabolism.

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