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Pharmacokinetics and metabolism of hexamethylmelamine in mice bearing renal cell tumors

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Summary. The pharmacokinetics of hexamethylmelamine (HMM) and its main metabolites hydroxymethylpentamethylmelamine (HMPMM), pentamethylmelamine (PMM), and 2,2,4,6, tetramethylmelamine (2,2,4,6 TetrMM) were studied in renal cell (RC) tumor tissues and plasma of CDF_1 mice that had received IP bolus injections of the maximally tolerated dose (200 mg/kg) of HMM. HMM, PMM, and 2,2,4,6 TetrMM concentrations determined in RC tissues were much higher than the plasma values, as indicated by the pharmacokinetic parameters (C_{max} and AUC). On the other hand, very low levels of HMPMM, generally considered to be a potentially active antitumor compound, were detected in the target tissues, whereas this hydroxylated metabolite was stable and easily determined in plasma. High HMM concentrations in RC tissues could correlate with the high sensitivity of the tumor to this drug. However, the behavior of HMPMM remains unclear; related hypotheses are presented in this paper.

Introduction

Hexamethylmelamine (HMM) is an S-triazine derivative that is clinically active [19] against small-cell lung cancer, ovarian cancer, and malignant lymphoma. Its pharmacokinetics and metabolism in animals have been well documented [2, 9, 10, 20, 22, 24, 25], but the mechanism of action of methylmelamines remains unclear [21]. The presence in mouse plasma of a hydroxymethyl metabolite that could account for the antitumor activity of HMM [12] was recently demonstrated in vivo [14, 15]. Studies of HMM and hydroxymethylpentamethylmelamine (HMPMM) pharmacokinetics in mouse plasma [16] have shown that these drugs should be rapidly distributed between the plasma and tissues. However, the failure of the plasma drug concentration to predict the proper drug concentration in the target tumor led us to describe the time course of the concentrations of HMM and its metabolites HMPMM, pentamethylmelamine (PMM), and 2,2,4,6 tetramethylmelamine (2,2,4,6 TetrMM) in the HMM-sensitive murine RC tumor model [23]. The main objective of this pharmacokinetic study is to establish a correlation between drug concentrations in the RC tumor and the pharmacological properties of HMM. The comparison of plasmatic and tumor data provides hypotheses about the mechanism of action of HMM.

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Materials and methods

Chemicals. HMM, PMM, 2,2,4,6 TetrMM, and HMPMM were kindly supplied by Simon Langdon (Aston University, Department of Pharmacy, Birmingham, England). All reagents were of analytic grade (Merck) except acetonitrile (Rathburn Chemicals LTD, Scotland, HPLC grade).

Tumor. The RC renal adenocarcinoma was maintained in CDF_1 mice by serial transfers every 14th day. The ascitic fluid was removed from tumor-bearing animals under aseptic conditions between days 12 and 15 and was diluted in saline. Cell viability, determined by trypan staining, ranged from 90% to 100%. A total dose of 10^6 live tumor cells in 0.1 ml was injected SC in CDF_1 hosts.

Animals studies. On day 8 posttransplantation, the maximally tolerated dose of HMM (200 mg/kg) was injected IP as a suspension in 1 ml Klucel to groups of 6 male CDF_1 (BALB/c × DBA/2) mice weighing 25 ± 2 g. Animals were anesthetized with diethyl ether at various times after drug administration and killed by cervical dislocation. The malignant tissue were quickly excised and stored at $-30°$ C until assayed. At this stage of tumor expansion, the neoplastic tissue was not yet affected by the presence of necrosis. As a result, the concentrations of melamine derivatives could be measured in the whole tumor tissue.

Recovery study. HMM derivatives were extracted from RC tissues by a method similar to that used for plasma extraction, which has recently been reported in great detail [15]. An aqueous solution containing HMM, PMM, HMPMM, and 2,2,4,6 TetrMM was prepared immediately prior to each assay. Of this solution, 0.5 ml was mixed with RC tissues (300–500 mg) that had previously been pulverized with a glass homogenizer at 4° C; 1.5 ml CH_3CN was then added and the mixture was vortex-mixed for 1 min. The tube was left at 4° C for 10 min to allow complete precipitation of the proteins. A 0.1-ml aqueous solution of atrazine was then added as an external standard and mixed with

the tumor suspension for 30 s. The tube was then centrifuged at 4500 rpm for 10 min at 4° C to pelletize the proteins. Aliquots of the supernatant (50–100 µl) were analyzed by chromatography using previously described chromatographic conditions for the separation of *N*-methyl and *N*-methylolmelamines [15].

Data analysis. The data were analyzed by means of an algorithm based on the simplex method, which is used to minimize a nonlinear cost function; its merit is that it guarantees convergence [1]. Depending on the user's wishes, an automatic initialization can be directly obtained by the FADHA program. FADHA provides a solution to the choice of weights by using the concept of a weighing function rather than weights as such. In this study, the analytic standard deviation (SD) served as the weight for the concentration, whereas the times were equiweighed [1]. An empirical pharmacokinetic model was developed to interpret the data. The description of the time courses of drug concentrations in the tissue samples was fit to a sum of exponentials:

$$C_p(t) = \sum_{i=0}^{j} A_i \exp\left[-a_i (t - t_0) \quad ,\right]$$

where $C_p(t)$ represents the tissue concentration at time t after the injection of the tested drug, j is the number of exponential terms required to explain the data, A and a are the coefficients and exponents of the polyexponential equations, and t_0 is the absorption or formation lag time. Pharmacokinetic parameters were estimated when all $C_i j$ were at a same t_i and when the mean arithmetic tissue concentrations at t_i were analyzed. The program uses a statistical criterion based on the cost function to assign the x or $x + 1$ exponential modeling [1]:

$$t = \frac{F_1 - F_2}{F_2} \times \frac{N - m_2}{m_2 - m_1},$$

where m_1 is the number of parameter of an x exponential model; m_2 is the number of parameters of an $x + 1$ exponential model, F_1 is the maximal cost function of the x exponential model, F_2 is the maximal cost function of the $x + 1$ exponential model, and N is the number of experiments, and assuming that F_2 and $(F_1 - F_2)$ are chi-square-distributed, F_2 and $(F_1 - F_2)$ are independent variables, and $N > m_2$.

When the experiments are numerous enough, the latter variable is asymptotically distributed according to Fisher's F-distribution. If the tabulated t^* $(Fm_2 - m_1, N - m_2) > .t$, then the data are fit to an x exponential model. However, when comparing the same data set to assign the x or $x + 1$ exponential modeling, the cost function value is sufficient for the selection of the appropriate model.

Results

Recovery studies

The recovery of HMM and its metabolites was measured by comparing the peak height of standards dissolved in the chromatographic mobile phase kept at 4° C with the peak obtained from standards extracted from RC tissues at 4° C. A volume variation due to protein precipitation could be measured after the addition of atrazine during the extraction procedure, as described in *Materials and methods.* As shown in Table 1, this method led to recoveries of about 100% for HMM, PMM, and 2.2.4.6 TetrMM; the mean values for HMPMM were 91.6% and 83.8% when extraction was carried out at 4° C and 20° C, respectively. The extraction of melamine derivatives from plasma led to recoveries of about 100% for all compounds, with relative SDs generally lower than 5%, as reported by Dubois et al. [15]. The efficiency of the assay was assessed by quadruplicate assay of spiked tissue samples containing about 0.5–2 µg drug/g tumor. The assay was highly reproducible for all compounds, as indicated by the low percentage of relative SDs. However, the values recorded for HMPMM were twice as high as those for the other drugs, and the difference increased when samples were extracted at 20° C. These results illustrated the great instability of the hydroxylated metabolite of HMM [14] and proved it necessary to carry out extraction at low temperatures (4° C). HMPMM recoveries observed after RC tissue extraction were somewhat lower than the values recorded in plasma samples [15], which tends to demonstrate a greater instability of the compound in this tumor tissue. The influence of various parameters (such as solvent composition, pH, *n*-propylamine content, and temperature) on the separation and/or stability of melamine has previously been reported in detail [15].

Table 1. Determination of HMM and its metabolites in RC tumor

Compound	Percentage recovery			
	added (µg/g) tumor	mean	range	RSD[c] (%)
HMM	1.96	100.7	98–102	2.4
PMM	1.28	102.8	100–104	1.7
2,2,4,6 TetrMM	0.88	101.8	99–103	2.4
HMPMM[a]	0.47	83.8	77– 91	6.7
HMPMM[b]	0.47	91.6	87– 97	5.4

[a] Extraction carried out at 20° C
[b] Extraction carried out at 4° C
[c] Relative SD

Typical chromatograms in Fig. 1 show a mixture of standards *[A]* as well as a tissue extract from mice treated with HMM *[B].* The detection limits range from 1 to 17 ng for all melamine derivatives [15]. Although HMM and the metabolites were identified by comparing their retention times with that of the reference solutions, this method has been advantageously coupled with a more specific technique. The same concentration of a detected compound was injected three times at different wavelengths selected from the UV spectra of the reference melamines, in the same solvent systems.

The ratios calculated were similar to those obtained with the reference solutions. The chromatographic absorbance ratio was compared with the ratio measured by spectrophotometry under the same conditions. HMPMM has also been identified by its thermal degradation to PMM and formaldehyde [15].

Pharmacokinetics of HMM and its metabolites

The plasma pharmacokinetic of HMM and HMPMM after the IP bolus injection of 200 mg/kg HMM in mice have previously been presented [16]. Plasma concentrations of

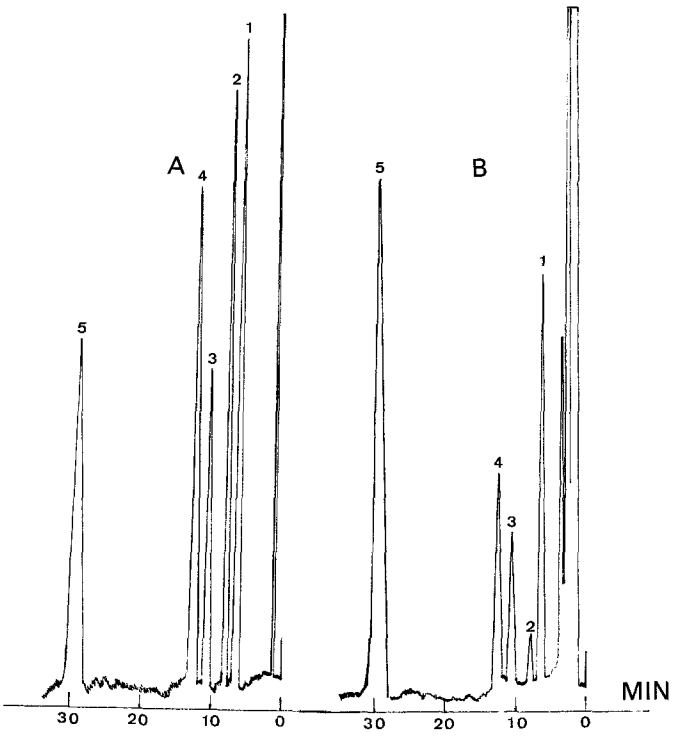

Fig. 1. **A** Elution profile of an aqueous mixture of standards. Peaks: 1 = 2,2,4,6 TetrMM; 2 = HMPMM; 3 = atrazine; 4 = PMM; 5 = HMM. **B** Chromatogram of RC tumor extract 135 min after IP administration of 200 mg/kg HMM. Peaks: 1 = 2,2,4,6 TetrMM; 2 = HMPMM; 3 = atrazine; 4 = PMM; 5 = HMM

HMM and HMPMM vs time data had been fit to one open-compartment model. It was recently possible to complete this work with the pharmacokinetic analysis of the time course of PMM and 2,2,4,6 TetrMM concentrations in plasma. In this case, plasma levels were fit to a sum of two exponentials, the pharmacokinetic parameters being a purely mathematical description of an empirical model. However, to make the reader's comprehension easier, plasmatic parameters of the four melamine derivatives are detailed in Table 2. The tumor pharmacokinetic parameters and their SDs, reported in Table 3, were calculated by considering all c_i and mean c_i data at each t_i. In each case, data were fit to two exponentials ($j = 1$) and to three exponentials ($j = 2$). For all compounds and for all data sets (mean c_i and all c_i at each t_i), the statistical test indicated the two-exponential model. The measures of fit (Σd^2 and cost function value) also gave indications of its quality, as the data best fit to an appropriate model produce the smaller value of Σd^2 for any j and of the cost function for the same j (Σd^2 = cost function value when concentrations and times are equiweighted and the value of the weight = 1) [1].

Moreover, as illustrated in Table 3, it appeared that nonsignificant values were calculated for several parameters (a_1, A_1) when data were fit to three exponentials. The use of the total c_i data set that allowed the assessment of biological intra- and/or interindividual varibility was preferred. The choice of this approach in analyzing pharmacokinetic data should often prevent us from using an inappropriate model that could lead to serious errors in parameter estimates.

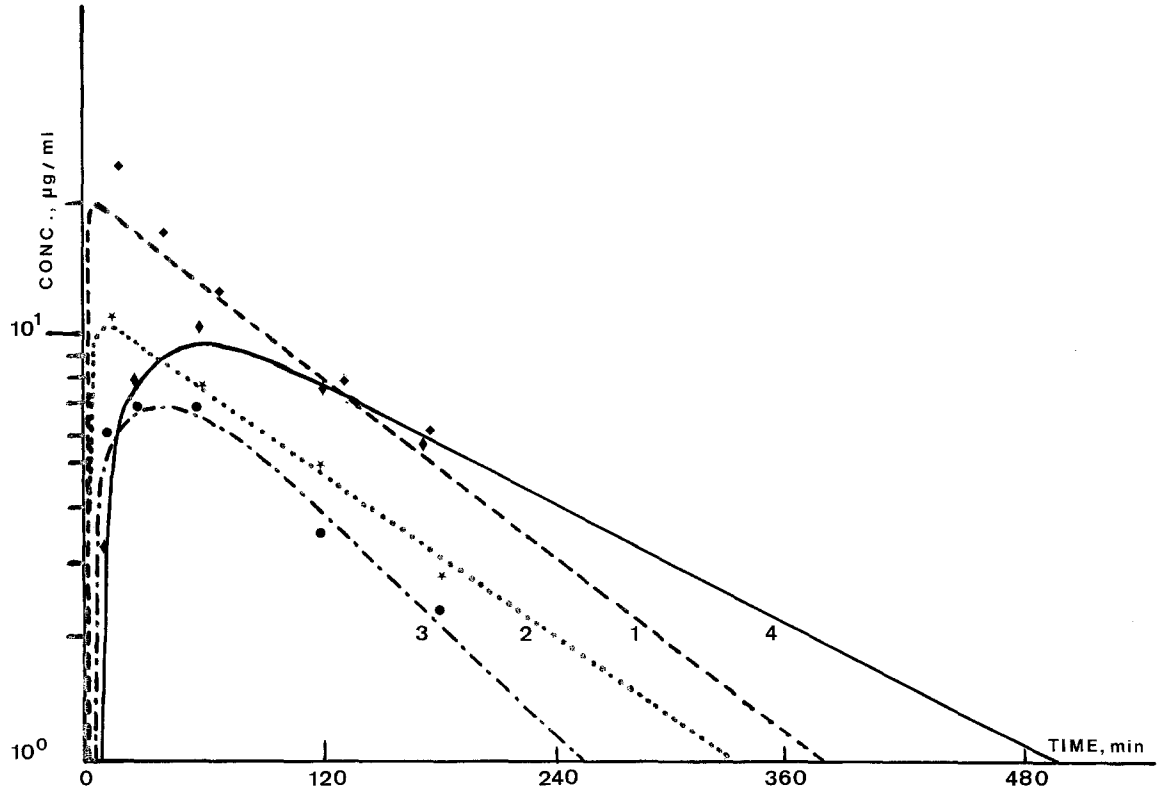

Fig. 2. Plasma levels of HMM (*1*) and its metabolites HMPMM (*2*), PMM (*3*), and 2,2,4,6 TetrMM (*4*) after IP bolus injection of 200 mg/kg HMM. All C_i data have been analyzed to represent these curves, but only mean values are marked to clarify the figure

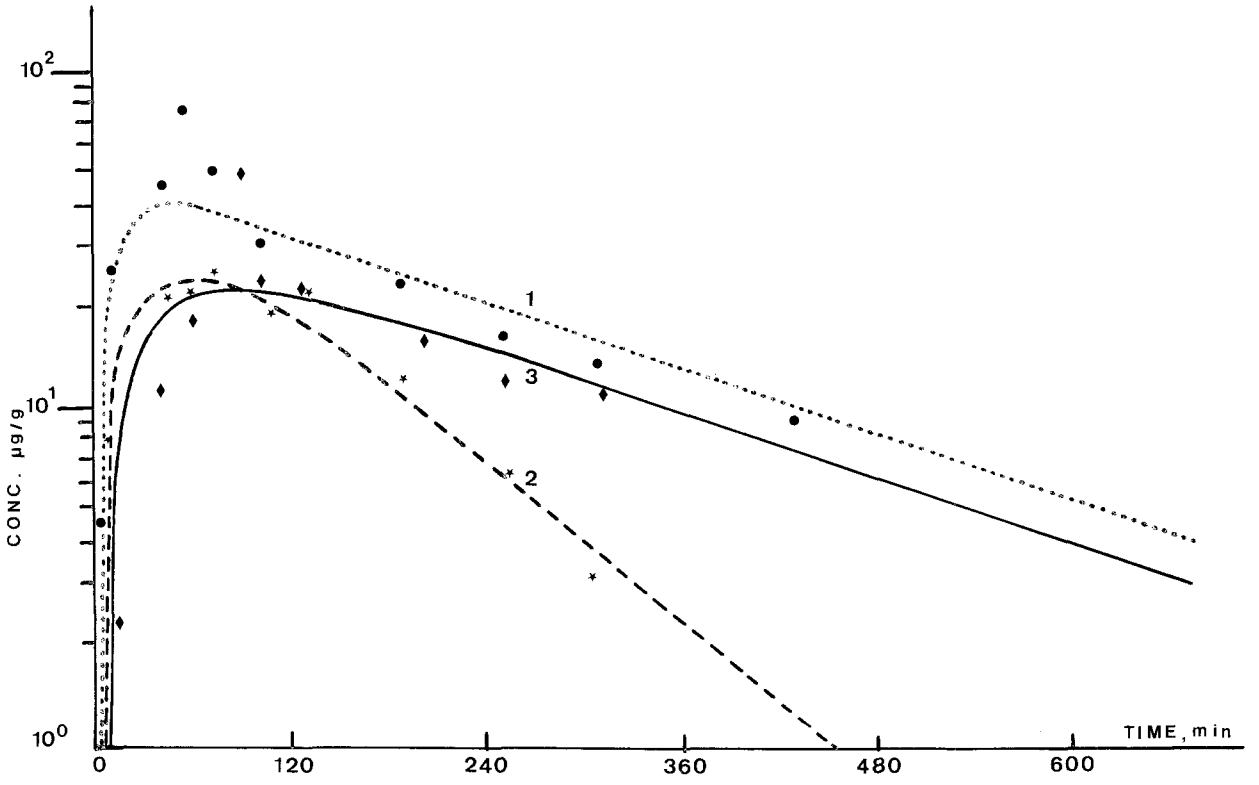

Fig. 3. Tumor levels of HMM (*1*) and its metabolites PMM (*2*) and 2,2,4,6 TetrMM (*3*) after IP bolus injection of 200 mg/kg HMM. All C_i data have been analyzed to represent these curves, but only mean values are marked to clarify the figure

The results reported in Tables 2 and 3 show that HMM is rapidly absorbed from the peritoneal cavity and distributed in the plasma ($t_{1/2}K_A$ = 0.16 min) and tumor tissues ($t_{1/2}$ a_0 = 7 min), where it is detected rather quickly, as indicated by HMM lag times. HMM undergoes rapid and extensive metabolization, as evidenced by the high formation-rate constant of its first metabolite, HMPMM, in the plasma (K_1 = 0.02 min^{-1}).

The three metabolites of HMM were detected in the plasma as well as in the tumor, but the lag times calculated were a few minutes longer for the latter tissue. However, the fitting of HMPMM tumor data to a sum of exponentials was impossible because its concentration levels in RC tissues proved to be rather low and because this metabolite was detected in tumor tissues for too short a period of time. The half-lives and apparent elimination-rate constants obtained in the biexponential fittings show that HMM, PMM, and 2,2,4,6 TetrMM were more slowly eliminated from tumor tissue than from plasma, however this difference was lower for PMM.

The most considerable differences between tumor and plasma pharmacokinetics are illustrated in Table 4, in

Table 2. Plasma pharmacokinetic parameters of HMM and its metabolites

Drug	Pharmacokinetic parameters									
HMM	Lag time (min)	K_A (min^{-1})		K (min^{-1})		$t_{1/2}^{K_A}$ (min)	$t_{1/2}^{K}$ (min)	AUC (min µg/ml)	T_{max} (min)	C_{max} µg/ml
	1.88	4.40		0.02		0.16	43.55	1296	3.16	20.20
HMPMM	Lag time (min)	K_1 (min^{-1})		K_2 (min^{-1})		$t_{1/2}^{K_1}$ (min)	$t_{1/2}^{K_2}$ (min)	AUC (min µg/ml)	T_{max} (min)	C_{max} µg/ml
	1.88	0.02		0.04		43.55	16.06	477	38.73	4.25
PMM	Lag time (min)	a_0 (min^{-1})	A_0 (µg/ml)	a_1 (min^{-1})	A_1 (µg/ml)	$t_{1/2}a_0$ (min)	$t_{1/2}a_1$ (min)	AUC (min µg/ml)	T_{max} (min)	C_{max} µg/ml
	[a]	0.03	−59	0.02	59	27	30	296	41	2.60
2,2,4,6 TetrMM	9	0.04	−10	0.01	10	19	72	789	60	4.67

[a] PMM lag time was not a significant value

K, elimination rate constant; K_a, absorption rate constant; $t_{1/2}^{K_a}$, absorption rate constant half-life; $t_{1/2}^{K}$, elimination half-life; AUC, area under the plasma level curve; C_{max}, maximal plasma drug concentration; T_{max}, time corresponding to the C_{max}

Table 3. Estimated parameters and measures of fit of melamine derivative tumor concentrations

Drug	Estimated parameters							Derived parameters						Measures of fit	
	Lag time (min)	a_0 (min^{-1})	A_0 (μg/g)	a_1 (min^{-1})	A_1 (μg/g)	a_2 (min^{-1})	A_2 (μg/g)	$t^{1/2}$ a_0 (min)	$t^{1/2}$ a_1 (min)	$t^{1/2}$ a_2 (min)	AUC min μ/g	T_{max} min	C_{max} (μg/g)	Cost function	Σd^2
HMM mean C_i data[a]															
j[c] = 1	4±3	0.05 ±0.02	−85±15	0.007 ±0.001	85±15			13	93		9752	48	52	2054	1012
j = 2	4±3	0.05 ±0.02	−98±28	0.02 ±0.02	13±13	0.007 ±0.001	84±14	15	28	92	9740	49	53	2033	987
all C_i data[b]															
j = 1	4±3	0.05 ±0.02	−63±8	0.006 ±0.001	63±8			18	114		9370	47	44	1390	1808
j = 2	4±3	0.0428 ±0.0009	−98±22	0.0420 ±0.0009	14±14	0.008 ±0.001	84±8	16	16	87	8650	52	47	1634	1528
PMM mean C_i data[a]															
j = 1	4.9±0.05	0.01900 ±0.00004	−985±41	0.01800 ±0.00004	985±41			36	38		2880	59	20	295	93
j = 2															
all C_i data[b]															
j = 1	4.9±0.1	0.0019 ±0.0001	−985±91	0.01800 ±0.00009	985±91			36	39		2880	59	20	1203	233
j = 2															
2,2,4,6 TetrMM mean C_i data[a]															
j = 1	11±2	0.021 ±0.002	−42±3	0.0062 ±0.0005	42±3			32	112		4838	93	18	339	60
j = 2	28±21	129 ±129	−90±76	26±26	72±72	0.004 ±0.001	18±3	0.005	0.02	193	5105	29	55	1964	369
all C_i data[b]															
j = 1	11±2	0.021 ±0.002	−42±3	0.0062 ±0.0005	42±3			34	112		4741	94	18	745	101
j = 2	14.9997 ±0.0003	116±96	−77±61	16±16	58±58	0.0033 ±0.0008	19±3	0.006	0.04	213	5749	15	53	2699	628

[a] Estimated parameters and measures of fit of mean C_i data at each t_i
[b] Estimated parameters and measures of fit of all C_i data at each t_i
[c] j, the number of exponential terms required to explain the data

Table 4. Comparison of the bioavailability and maximal concentrations of melamine derivatives in plasma and tumor tissues

Drug	AUC plasma / AUC tumor	C_{max} plasma / C_{max} tumor
HMM	0.13	0.43
PMM	0.10	0.15
2,2,4,6 TetrMM	0.16	0.27

which the bioavailability and maximal concentrations of melamine derivatives in plasma and tumor are compared. It clearly appears that the concentration levels detected were much higher in tumor tissues than in plasma and that the AUC of these drugs was very high in the tumor.

Discussion

HMM is extensively metabolized in mice, rats and humans, as documented by the excretion of demethylated metabolites, the low concentration of the parent drug in urine [11, 24], the high formation-rate constant of its first metabolite, HMPMM. This metabolism, required for antitumor activity [21], has been found to be mediated by cytochrome P-450 in the microsomal and mitochondrial fractions of isolated rat liver and small intestinal cells [3, 4, 6–8, 21]. The oxidative intermediate in HMM demethylation, HMPMM, has been said to play a crucial role in HMM antitumor activity because of its capacity to bind covalently with DNA and with a variety of macromolecules [3, 4, 19, 22]. HMPMM has recently been proven to be a stable carbinolamine in mouse plasma [14, 15].

This finding suggested that this reactive metabolite could leave the activation site (i.e., the liver) and had enough chemical stability to reach the target tissues important for the therapeutic effect. Nevertheless, according to some authors [5, 17, 26], if HMM activation is absolutely required, it may well involve conversion in the target tumor cell. Begleiter et al. [5] have shown that HMM undergoes oxidative metabolism to PMM in L5178Y cells. Furthermore, labelled HMM incubated with microsomes obtained from the M5076 sarcoma was covalently bound with microsomal proteins, a finding which suggests a met-

abolic step transforming the inert HMM into reactive electrophiles [4, 17] by the formation of a reactive iminium species from HMPMM.

Therefore, it was of interest to measure HMM as well as HMPMM, PMM, and 2,2,4,6 TetrMM concentrations in the RC tumor, chosen because of its sensitivity to HMM at the dose used, and to compare the results with those obtained after plasma extraction. The plasma data of HMM and HMPMM, on the one hand, and of PMM and 2,2,4,6 TetrMM, on the other, were fit to a one open-compartment model and to two exponentials, respectively. Consequently, the plasmatic pharmacokinetic behavior of these drugs assumed that any changes occurring in the plasma would reflect changes occurring in tumor drug levels. Our findings support this hypothesis for HMM, PMM, and 2,2,4,6 TetrMM, which were easily detected in tumor tissues. The concentrations measured in RC tissues were much higher than the plasma values. The tumor data were analyzed as described above and the results unequivocally indicated the two-exponential model. However, very low quantities of HMPMM were measured in RC tissues, in spite of the use of a nondestructive one-step extraction method by acetonitrile, illustrated in Table 1. The pharmacokinetic analysis of data concerning HMPMM was thus made impossible. These results suggest, as previously mentioned by Borm et al. [7], that HMPMM could be partially trapped in the tumor cell as a conjugate, or could be bound covalently with protein or DNA after the formation of an iminium derivative, or it could be partially converted into PMM and formaldehyde by an enzymatic factor or hydrolysis, especially if the tumor is slightly acidic. The fact that plasma levels of this metabolite were higher could also reflect an increased stability due to its binding to plasma proteins, which was significant [18]. The study of pharmacokinetic parameters showed that the successive demethylation steps of the HMM metabolism pathway led to a rather constant AUC ratio (0.10–0.16) between plasma and tumor for HMM, PMM, and 2,2,4,6 TetrMM. The maximal concentration ratio (see Table 4) was much lower for PMM and 2,2,4,6 TetrMM than for HMM. These results supported the hypothesis that HMM could be rapidly and partially metabolized in RC cells, producing a high PMM C_{max} value in tumor tissues. The little effect on the PMM tumor AUC of this high C_{max} value could be explained by the short PMM tumor elimination half-life and/or by the possibility that HMPMM could be partially trapped in RC cells.

In conclusion, from the pharmacokinetic results it appears that HMM as well as its metabolites PMM and 2,2,4,6 TetrMM are largely distributed in RC tumor tissues. This could result from their physiochemical properties, due to the nature of the molecular *N*-alkyl substituents [13]. The presence in the target organs of high HMM concentrations could correlate with the great efficiency of this antitumor agent in the treatment of the murine RC tumor [23]. The possible role of HMPMM in HMM activity has been considered, but no definitive conclusion as to the possible metabolism of HMM, HMPMM, PMM, and 2,2,4,6 TetrMM or the binding of HMPMM in RC tissues can be drawn. However, the very low levels of HMPMM determined in the RC tumor is an interesting finding, perhaps representing a first step in the understanding of the mechanism of action of HMM if the hydroxylated metabolite is really the active drug.

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