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IN VIVO AND IN VITRO IRREVERSIBLE BINDING OF HEXAMETHYLMELAMINE TO LIVER AND OVARIAN TUMOR MACROMOLECULES OF MICE

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Hexamethylmelamine or 2,4,6-tris-(dimethylamino)-s-triazine (HMM) is known as an antineoplastic substance, especially active in some human malignancies, such as lung and ovarian tumors (1). Its plasma levels and urinary excretion have already been studied in animals and man (2-4) and its metabolism is known to proceed via a series of oxidative N-demethylation steps (5,6).

HMM does not seem to be a direct alkylating agent like its congener triethylene melamine (TEM), but the molecule appears to need metabolic activation to covalently bind to cellular macromolecules. Thus, in view of its metabolism, it is likely that the reactive intermediate(s) responsible for covalent binding is (are) produced by enzymes such as the mixed function oxidases located in the microsomal fraction of the liver and other tissues. From this point of view the liver would be by far the most active organ in operating these metabolic transformations.

However, it is not yet known whether these reactive metabolites are able to leave the activation site (i.e. the liver) or have enough chemical stability to reach the target tissue (i.e. the tumor) important for the therapeutic effect of HMM, or whether they need to be produced in situ by the tumor tissue itself. If the latter were the case, the probability of a reactive metabolite of HMM reaching the biological sites important for its activity would obviously be much greater because of spatial reasons, even if the tumor cells'metabolic capacity were much lower than that of liver cells.

Hence we thought it would be of interest to investigate the following points: 1) The in vivo presence of covalent binding of HMM or its metabolites to macromolecules of liver and tumor cells after injection of labelled HMM to mice bearing an ovarian tumor. 2) The amount of covalently bound radioactivity compared to the total amount of radioactivity present in these tissues. 3) The in vitro capacity of microsomal fractions from liver and tumor to activate HMM to reactive metabolites.

MATERIALS AND METHODS

Chemicals

2,4,6-^{14}C-HMM (specific activity 13.0 mCi/mmol) was obtained from the Division of Cancer Treatment, NCI, NIH, Bethesda, Md., U.S.A. The radiochemical purity of labelled HMM was checked by bidimensional thin layer chromatography on silica gel glass plates which were developed first in a solvent system consisting of chloroform, methanol, acetic acid (88:10:2) and after in ethylacetate, methanol and acetic acid (88:10:2). The compound was found to be more than 99% pure. NADPH was obtained from Boehringer, Mannheim, West Germany; all other chemicals and solvents were of the purest grade commercially available.

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In vitro studies

Preparation of microsomes. (C57Bl/6J) Female mice (27 g, b.w.) from Charles River Italy (Calco, Como, Italy) were used for the preparation of liver and tumor microsomal fractions. Each animal received an intramuscular transplant of 8 x 10^5 cells of M 5076/73 A ovarian cancer. After 20 days, when the tumor weight was about 6g, the animals were killed by decapitation and the livers and tumors were excised, pooled and washed with 0.05 M phosphate buffer pH 7.4. Only the viable part of the tumor was taken. Both liver and tumor tissues were homogenized in 0.05 M phosphate buffer pH 7.4 using a Potter-Helvejehm homogenizer with a Teflon pestle. Microsomes were prepared according to Kato and Takayanaghi (7). The protein concentration was determined by the method of Lowry et al. (8), with albumin fraction V as the standard.

Covalent binding to microsomal proteins. The incubation mixture, in a final volume of 2 ml, contained 2 mg of NADPH, 0.05 M phosphate buffer pH 7.4, magnesium chloride 5 mM, potassium chloride 0.15 M and 10 mg of microsomal liver or tumor protein. Labelled HMM was added in 5 µl of ethanol to obtain a final concentration of 25 µM. Incubation was at 37°C for 2 hours in subdued light. The reaction was stopped by the addition of 3 ml of acetonitrile. The samples were centrifuged, the supernatant was discarded and the precipitate was extracted twice with 5 ml of the following solvent sequence: 5 times with ethanol, once with benzene-ethanol (1:4, v/v), twice with acetone-chloroform (4:1, v/v), once with acetone-ethanol (5:1, v/v), once with diethylether-ethanol (5:1, v/v), twice with ethylacetate-ethanol (5:1, v/v), once with ethanol and ethanol-water (2:3, v/v). All the extraction supernatants were tested for radioactivity. After the last cycle of extraction no further radioactivity could be removed from the proteins. This exhaustive extraction thus removed the original HMM as well as all metabolites reversibly bound to the proteins. Protein loss was very low (≤1%) during the procedure. The precipitate was dissolved in 0.5 ml of Soluene ^{R}-350, Packard, Downers Grove, Illinois, U.S.A., and transferred into a counting vial containing 10 ml of a PPO-POPOP solution in toluene. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter. The values were corrected for quenching by the external standardization method.

In vivo studies

Treatment of animals. Two C57Bl/6J female mice bearing 20-day-old M 5076/73 A ovarian cancer were injected i.p. with radiolabelled HMM (25 mCi/kg corresponding to a dose of 50 mg/kg) dissolved in acetone-corn oil (1:9, v/v). The animals were fasted until killing.

Determination of total radioactivity (HMM + metabolites) present in liver and tumor tissue. For the determination of this parameter, 30 hours after the treatment with radiolabelled HMM, the animals were killed by decapitation, the liver and the viable part of the tumor were excised and homogenized in 4 volumes of cold 0.05 M phosphate buffer pH 7.4 using a Potter-Elvejehm homogenizer with a Teflon pestle. Aliquots (50 µl) of these homogenates were dissolved in 0.5 ml of Soluene ^{R}-350 and transferred into counting vials containing 10 ml of a PPO-POPOP solution in toluene. Determinations were made in triplicate. Radioactivity was measured as described in the previous section.

Covalent binding to tissue macromolecules. From the same homogenates, as in the previous paragraph, 400 µl aliquots were taken and 3 ml of acetonitrile added, the samples were centrifuged, the supernatants discarded and the precipitates were checked for covalently bound radioactivity by the exhaustive extraction procedure described in the in vitro studies. Determinations were made in triplicate.

RESULTS AND DISCUSSION

In this study we used an ovarian tumor (M 5076/73 A intramuscularly transplanted in mice) which is sensitive to HMM (9). Covalent binding experiments were carried out with ring-labelled HMM in order to avoid binding to macromolecules due to the formaldehyde liberated during the process of metabolic N-demethylation.

Data summarized in Table 1 show that ^{14}C-HMM has a very limited capacity to covalently bind to microsomal proteins per se, in both liver and tumor preparations, as can be deduced from the value of covalent binding to denatured microsomes. Binding was stimulated by microsomes in the pre-

sence of NADPH as a cofactor. The absence of the nicotinamide derivative from the microsomal preparations left covalent binding of HMM virtually the same as denatured microsomes.

Table 2 shows that the *in vivo* level of covalent binding of HMM metabolites to tissue macromolecules was about three times higher in liver than in tumor, but the levels of HMM and its metabolites in the two tissues were not different. Therefore the ratio of bound HMM metabolites to total metabolites in the liver 30 hours after HMM treatment reflects this situation.

These preliminary results show that *in vitro* the microsomal fraction from liver and from ovarian tumor both have the capacity to metabolize HMM with the formation of reactive products, capable of covalently binding tissue macromolecules. Furthermore HMM has the *in vivo* capacity to alkylate liver and ovarian tumor macromolecules. This binding is very high as compared to the total radioactivity present in the two tissues at that time, ranging from 9 to 12% in tumor and from 20 to 27% in liver.

Table 1. *In vitro* covalent binding of ^{14}C-HMM and its metabolites catalyzed by mouse liver and ovarian tumor microsomes.

SAMPLE	HMM metabolites irreversibly bound to 1 mg of microsomal protein (pmol ± S.E.)	
	LIVER	TUMOR (M5076/73 A)
Acetonitrile denatured microsomes + HMM + NADPH	1.81 ± 0.12	0.81 ± 0.08
Microsomes + HMM - NADPH	1.89 ± 0.19	0.73 ± 0.06
Microsomes + HMM + NADPH	7.75 ± 0.68°	2.83 ± 0.26°

Each value is the mean ± S.E. of four determinations.

° $p < 0.01$ Compared with denatured microsomes and microsomes - NADPH.

Table 2. Levels of ^{14}C-HMM + metabolites and of ^{14}C-HMM covalently bound to liver and ovarian tumor (M5076/73 A) in mice.

SAMPLE		(A) HMM metabolites irreversibly bound to 1 g of wet tissue macromolecules (nmol)	(B) HMM metabolites present in 1 g of wet tissue (nmol)	%A/B
LIVER	mouse 1	3.51 ± 0.02	12.7 ± 0.1	27
	mouse 2	2.26 ± 0.04	11.0 ± 0.1	20
TUMOR	mouse 1	0.98 ± 0.02	10.6 ± 0.1	9
	mouse 2	0.74 ± 0.01	5.9 ± 0.2	12

Each value is the mean ± S.E. of three determinations.

Previous experiments have shown that tumors do not possess the mixed function oxygenases (10). These experiments were performed by spectrophotometry using classical substrates. In our experiments an homogeneous

substrate was used to check the capacity of tumor to metabolize antitumoral drugs. Metabolic capacity was checked in terms of covalent binding studies which are known to have a higher sensitivity. Further experiments are in progress to structurally characterize the species involved in this binding and to verify if covalent binding to selected cell macromolecules is important for antitumoral activity.

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