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LACK OF IN VIVO DNA BINDING OF MERCAPTOBENZOTHIAZOLE TO SELECTED TISSUES OF THE RAT[1,2]

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SUMMARY: In this study, the in vivo binding of 14C-labelled 2-mercaptobenzothiazole (MBT) to DNA was investigated. Male and female Fischer 344 rats were gavaged with 375 mg MBT/kg body weight and killed 8 hours later. DNA was extracted from the liver, adrenal glands, pituitary gland, pancreas, and bone marrow and the amount of radioactivity associated with the DNA was determined. Results from this study indicate that MBT does not significantly bind to DNA from any of the tissues examined. CBI values for liver for the 3 methods of purification were ~1-3 which are on the low end of the covalent binding index. The CBI values for the other tissues were always <1. Other chemicals with similar CBI values include estrone and diethylstilbesterol. Strong hepatocarcinogens such as dimethylnitrosamine and aflatoxin have CBI values ranging from 6000 to greater than 20000.

2-Mercaptobenzothiazole (MBT, Figure 1) is vulcanization accelerator used widely in the rubber industry and is a precursor for other vulcanizers such as sulfenamides. MBT has shown some evidence of carcinogenic activity in a recent rat chronic bioassay completed by the National Toxicology Program (1); however in short term mutagenicity tests, MBT was negative or only weakly positive (2). The covalent binding of some chemicals to DNA is a critical step in the transformation process (3) and it has been proposed that that measurement of DNA binding could provide a short term in vivo test for detecting potential carcinogens (4, 5). DNA binding per unit dose can be described by the covalent binding index (CBI) which is a measure of the amount of chemical bound to the genetic material and is described by:

$$CBI = \frac{\text{micromol chemical bound/mol DNA nucleotide}}{\text{mmol chemical applied/kg body weight.}}$$

More precisely, 1 CBI unit corresponds to 1 DNA adduct per 10^6 nucleotides after a dose of 1 mmol chemical/kg body weight. The CBI value can be easily

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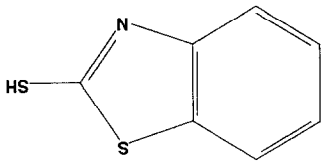

Figure 1. 2-Mercaptobenzothiazole Molecular Weight 162.

calculated using experimental data (6). By expressing the administered dose as dpm of chemical/kg body weight and by expressing DNA binding as dpm of chemical/mg DNA, with 1 mol of DNA weighing 309, the CBI value can be calculated as:

$$CBI = \frac{\text{dpm/mg DNA} \times (3.09 \times 108)}{\text{dpm/kg Body Wt.}}$$

The objective of this study was to determine the extent of covalent binding of MBT to DNA from selected tissues of the rat in order to determine if genotoxicity was involved as a mechanism for the reported carcinogenic activity of MBT in the NTP bioassay (1). DNA was isolated from tissues with exhaustive solvent extraction and the binding of MBT derived radioactivity was determined. Binding was also examined using liver DNA further purified with CsCl ultracentrifugation or hydroxyapatite chromatography. Binding is expressed in terms of pmol MBT bound/mg DNA and a CBI value was generated in order to compare MBT's carcinogenic potential with known carcinogens based on the covalent binding index.

MATERIALS AND METHODS

Radiolabelled 2-mercapto[2-^{14}C]benzothiazole was obtained from Amersham Corporation (Arlington Heights, IL) and had a specific activity of 56 mCi/mmol (331 uCi/mg). The radiochemical purity was 99.7% as determined by HPLC. Unlabelled MBT was purchased from Aldrich Chemical Co. (Milwaukee, WI) and had a stated purity of >98%. All other chemicals and solvents were of the highest purity commercially available.

Male and female Fischer 344 rats obtained from Charles River Breeding Laboratories (Kingston, NY) were quarantined for 10 days prior to initiation of the study. The rats (153-211 g; 9-13 weeks old) were housed in individual suspended stainless steel cages and were maintained at constant temperature (72 + 2 F) and humidity (35-60%) on a 12 hour light/dark cycle. The animals were allowed feed (Purina Certified Rodent Chow 5002, Ralston Purina Co., St. Louis, MO) and tap water ad libitum.

MBT was dissolved in Emulphor EL-719 (GAF Corp., New York, NY) and rats were administered 375 mg (~15 mCi) MBT/kg body weight by gavage in a dosing volume of 5 ml/kg. Eight hours after administration the rats were killed by CO_2 asphyxiation and the liver, adrenal glands, pancreas, pituitary gland, and femurs were removed, washed in cold (4° C) physiological saline (0.9%), weighed, and frozen in liquid nitrogen.

Tissue samples were thawed, minced, and homogenized in 9 volumes of chilled buffer (0.15M NaCl, 0.01M EDTA, 1% SDS) using a glass-glass Dounce Homogenizer (8-10 strokes). DNA was then extracted and isolated from each tissue or tissue pool using chloroform, isoamyl alcohol, and phenol (7). The protein concentration of the supernatant was determined with the dye binding assay (8) and the DNA concentration was determined spectrophotometrically by change in absorption from 260 to 280 nm (Beckman DU-70 Spectrophotometer, Beckman

Instruments, Fullerton, CA). Samples of the DNA solution were mixed with Scintiverse II Scintillation Cocktail (Fisher Scientific, St. Louis, MO) and the radioactive concentration was determined by liquid scintillation spectroscopy (TRI-CARB 460 C Liquid Scintillation Counter, Packard Instrument Co., Inc., Downers Grove, IL). It had been previously determined that the quantitation of DNA with ultraviolet absorbance was in good agreement with the colorimetric assay (9). Tissues from animals administered Emulphor only were extracted in a similar manner and the DNA samples were used to determine levels of background radioactivity. Counting efficiencies were determined by external standardization and cpm were converted to dpm by internal calibration quench curves. Aliquots of each tissue homogenate were mixed with scintillation cocktail and the concentration of radioactivity in each tissue as a percentage of the administered dose was calculated.

Hepatic DNA was further purified by either cesium chloride (Sigma Chemical Co., St. Louis, MO) ultracentrifugation (10, 11) using ethidium bromide (Sigma Chemical Co., St. Louis, MO) as an indicator or by hydroxyapatite chromatography (12). DNA concentrations were measured spectrophotometrically and levels of radioactivity were determined by liquid scintillation counting.

Comparisons of total DNA binding of MBT and the derived CBI values were made between tissues and sexes using one way random factorial ANOVA. Where significant differences occurred ($P<0.05$), a priori multiple comparisons were made with Bonferroni's t test with a fiducial limit of $P<0.05$.

RESULTS AND DISCUSSION

Very little of the administered dose was detected in the 5 tissues of interest 8 hours after single gavage (Table 1). In both male and female

Table 1. Tissue distribution in the male and female rat of 14C MBT derived radioactivity 8 hours after single gavage of 375 mg 14C-MBT/kg (Mean + SEM of (N) animals)

Tissue	Tissue wt. (g)	% Administered Dose	% Administered Dose/g Tissue
Liver (5)			
Male	9.78+0.19	0.51+0.06	0.05+<0.01
Female	6.24+0.28	0.36+0.01	0.06+<0.01
Pancreas (5)			
Male	0.69+0.07	0.03+0.01	0.04+<0.01
Female	0.44+0.07	0.02+<0.01	0.04+<0.01
Adrenal (10)[a]			
Male	0.26+<0.01	<0.01	0.02+<0.01
Female	0.30+0.03	0.01+<0.01	0.05+<0.03
Pituitary (10)[b]			
Male	0.42	<0.01	<0.01
Female	0.40	<0.01	<0.01
Bone Marrow (10)[a]			
Male	0.25+0.12	<0.01	0.01+<0.01
Female	0.17+0.11	<0.01	0.02+<0.01

[a] Two groups were analyzed, each with tissues pooled from 5 animals.
[b] One group was analyzed with tissues pooled from all 10 animals.

Table 2. DNA binding of MBT derived radioactivity to selected tissues of the male and female rat 8 hours after administration of 375 mg 14C-MBT/kg (Mean ± SEM of (N) animals) [a]

Tissue	DNA Extracted (mg)	mg DNA/ gram Tissue	Protein in DNA Extract (mg)	UV Absorbance Ratio[b]	Total DNA Binding (pmol MBT/ mg DNA)	CBI Value
Liver (5)						
Male	14.32±0.06	1.47±0.13	0.07±0.07	1.84±0.003	2.99±0.30*	3.11±0.29*
Female	12.32±0.82	1.98±0.12	ND[c]	2.01±0.01	1.54±0.17	1.65±0.20
Pancreas (5)						
Male	2.38±0.36	3.35±0.26	0.02±0.01	1.79±0.08	ND	ND
Female	1.25±0.36	2.72±0.34	ND	1.75±0.01	ND	ND
Adrenal (10)[d]						
Male	0.23±0.01	0.88±0.01	ND	1.83±0.02	ND	ND
Female	0.30±0.01	1.02±0.15	ND	1.81±0.01	ND	ND
Pituitary (10)[e]						
Male	0.29	0.69	<0.01	1.90	ND	ND
Female	0.19	0.47	<0.01	1.90	ND	ND
Bone Marrow (10)[d]						
Male	0.44±0.20	1.79±0.09	0.02±0.01	1.69±0.12	0.76±0.76	0.77±0.77
Female	0.35±0.19	2.25±0.32	0.03±0.03	1.62±0.14	0.15±0.15	0.16±0.16

[a] DNA was purified by exhaustive solvent extraction, 8 hours after single gavage of 375 mg MBT/kg.
[b] UV absorbance ratio=A 260/A 280.
[c] ND = Below detectable levels.
[d] Two groups were analyzed, each with tissues pooled from 5 animals.
[e] One group was analyzed with tissues pooled from all 10 animals.
* Significantly different from female liver (Table 3).

rats, the liver contained less than 0.6% of the MBT derived radioactivity from the administered dose. The pancreas, adrenal gland, bone marrow and pituitary gland all contained less than 0.03% of the administered dose. The concentration of MBT derived radioactivity (% administered dose/gram tissue) as well as total amount of radioactivity was greatest in the liver followed by the pancreas, adrenal gland, bone marrow, and pituitary gland. Although the amount of total radioactivity in the male livers was 1.4 fold higher than that of the female livers, there was no difference in radioactive concentration on a per gram basis.

The mean amount of DNA extracted from tissues of the male rat ranged from 0.23 mg from the adrenal glands to greater than 14 mg from the liver (Table 2). The amount of DNA extracted from tissues of female rats was similar . Although the greatest amount of DNA was obtained from the liver, the amount of DNA extracted per gram tissue was greatest from the pancreas followed by the bone marrow, liver, adrenal, and pituitary. The average amount of DNA extracted/g tissue for all tissues was approximately 1.6 mg.

The DNA 260/280 UV absorbance ratios ranged from 1.7 to 2.0 for both sexes and less than 70 ug protein was detected in any DNA preparation as contaminant (Table 2). The 260/280 UV absorbance ratio and the yield of DNA are good

Table 3. Comparison of binding of 14-C derived MBT to DNA from livers of male and female rats after differing DNA purification procedures[a] (Mean + SEM of (N) animals)

SEX	PURIFICATION METHOD	PMOL MBT/MG DNA	CBI VALUE
MALE			
	ESE	2.990+0.300 (5)	3.11+0.29 (5)
	ESE + CsCl	2.497+0.672 (3)	2.54+0.71 (3)
	ESE + HA	1.147+0.389 (3)	1.15+0.37 (3)
FEMALE			
	ESE	1.540+0.170 (5)	1.65+0.20 (5)
	ESE + CsCl	1.970+0.374 (3)	2.02+0.31 (3)
	ESE + HA	0.853+0.084 (3)	0.89+0.12 (3)

[a]DNA was purified by exhaustive solvent extraction (ESE) followed by CsCl centrifugation (CsCl) or hydroxyapatite chromatography (HA). Factorial Analysis of Variance indicated significant differences between males and females both in pmol MBT/mg DNA and in CBI values. There was no significant difference in the 3 treatment groups among males or among females regarding either pmol MBT/mg DNA or CBI value.

indicators of the purification process. A 260/280 absorbance ratio of >1.8 is generally regarded as 100% DNA while a ratio of 1.5 is indicative of a 50% protein solution and the extraction of ~2 mg DNA/g tissue is generally regarded to be optimal (13). The purity of these DNA extracts was confirmed by their UV absorbance ratio and the insignificant levels of protein contained in them.

Total DNA binding of MBT derived radioactivity was greatest in the liver of the male rat (Table 2). Less than 1 pmol/mg DNA was detected in the bone marrow and binding was not detectable in the adrenal gland, pancreas, or pituitary gland. As observed in the male rat, DNA binding was greatest in the liver of the female rat. Binding was nondetectable in the pancreas, adrenal, and pituitary glands. The CBI of MBT in the liver of the female rat was significantly less than that of the male.

Calculated CBI values and total binding of MBT derived radioactivity in the liver were not significantly different after further DNA purification with CsCl centrifugation or hydroxyapatite chromatography (Table 3). In all cases, binding was significantly greater in DNA obtained from the livers of male animals compared to female animals.

The objective of this study was to better understand the role of genotoxicity in the induction of tumors observed in the NTP study (1). The levels of genotoxic interactions of 2-mercaptobenzothiazole in selected tissues of the rat after a single gavage of 375 mg/kg were determined and a CBI value was calculated for the observed DNA binding. Results from this study indicate that under these conditions, binding of 14C-MBT derived radioactivity was undetectable in the adrenal glands, pancreas, and pituitary gland of the rat.

Less than 1 pmol MBT/mg DNA was detected in the bone marrow and ~1-3 pmol MBT/mg DNA was detected in the liver. It is not known whether the radioactivity associated with purified DNA is parent MBT or a radiolabelled metabolite. It is unlikely, given the current conditions and the position of the label on the ring, that the low level of binding in the liver is due to incorporation of a radiolabelled fragment of MBT into DNA. There is little evidence in the literature to suggest that ring cleavage of these types of compounds occur. A previous study (14) suggested that MBT is hydroxylated and conjugated prior to elimination.

The carcinogenic potency of more than 80 compounds have CBI values spanning 5 orders of magnitude (4). These chemicals were arbitrarily divided into 3 groups based on their CBI values as follows: Those with CBIs in the range of 1000 to greater than 20000 (aflatoxin B1, dimethylnitrosamine) were shown to be potent carcinogens, those with CBIs on the order of 100 (2-aminofluorene, 2-acetylaminofluorene) were moderate carcinogens, and those with CBIs from 1 to 10 (aniline, estrone, diethylstilbesterol) were weak carcinogens. Therefore based on the results of the present study the carcinogenic potency of MBT is on the very low end of the covalent binding index compared to other well known hepatocarcinogens. This confirms the results from several previous short term bioassays in which MBT was not found to be mutagenic (2). The low binding in the liver is consistent with the observed lack of liver tumors in the NTP chronic rat study (1). It is significant that this low level of DNA binding was only observed in the livers of these animals and no binding could be detected in the other tissues that were examined.

A previous study in which male rats were administered a dose of 0.5 mg unlabelled MBT/kg for 14 days followed by 0.5 mg 14C MBT (total of 7.5 mg/kg) indicated that ~1.5 and ~0.1% of the administered dose had accumulated in the liver and bone marrow respectively, 8 hours after gavage (14). Considerably less than these amounts were obtained in the present study at a dose of 375 mg/kg. Quite possibly the toxicokinetic pathway of MBT is altered at this dose. At doses where nonlinearities occur in absorption, distribution, metabolism, and excretion, DNA binding can be affected. Overloading of DNA repair activities at higher doses will not accurately reflect what occurs at the lower doses pertinent to the human situation (4). To investigate the low level of binding reported in the present study, future investigators should include evaluation of DNA binding at doses where the pharmacokinetic profile is known and with at least 2 timepoints to allow for maximal distribution to occur and to investigate DNA repair mechanisms, if any. Other studies (15) utilize multiple time points in order to reduce the possibility of sampling when the kinetics of covalent interaction with DNA is nonlinear

In conclusion, MBT does not appear to significantly bind to DNA of the liver, pancreas, bone marrow, adrenal, or pituitary glands in the rat. If indeed prolonged, high dose treatment with MBT does promote tumor formation, the results of the present study and previous short term bioassays indicate that MBT acts in a nongenotoxic manner. Future studies should focus on other probable mechanisms such as cell proliferation, pertubations of the endocrine system, or other physiological changes. Clearly no DNA binding was detected in the

endocrine glands investigated in the present study. Therefore, the biochemical and probable nongenotoxic mechanism by which endocrine factors act and their role in tumor promotion should be considered.

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