Cutaneous and Ocular Toxicology

ISSN: 1556-9527 (Print) 1556-9535 (Online) Journal homepage: http://www.tandfonline.com/loi/icot20

Mercaptobenzothiazole allergenicity—role of the thiol group

Itai Chipinda, Xing-Dong Zhang, Reuben H. Simoyi & Paul D. Siegel

To cite this article: Itai Chipinda, Xing-Dong Zhang, Reuben H. Simoyi & Paul D. Siegel (2008) Mercaptobenzothiazole allergenicity—role of the thiol group, Cutaneous and Ocular Toxicology, 27:2, 103-116, DOI: 10.1080/15569520701713008

To link to this article: http://dx.doi.org/10.1080/15569520701713008

Published online: 10 Oct 2008.

Submit your article to this journal

Article views: 70

View related articles

Citing articles: 10 View citing articles

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=icot20

Download by: [University of Liverpool] **Date:** 17 August 2017, At: 10:27

Cutaneous and Ocular Toxicology, 27: 103–116, 2008
ISSN: 1556-9527 print/1556-9535 online
DOI: 10.1080/15569520701713008

informa
healthcare

Downloaded by [University of Liverpool] at 10:27 17 August 2017

MERCAPTOBENZOTHIAZOLE ALLERGENICITY—ROLE OF THE THIOL GROUP

Itai Chipinda
Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA, and Department of Chemistry, Portland State University, Portland, Oregon, USA

Xing-Dong Zhang
Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

Reuben H. Simoyi
Department of Chemistry, Portland State University, Portland, Oregon, USA

Paul D. Siegel
Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

The rubber accelerator, 2-mercaptobenzothiazole (MBT), is known to cause allergic contact dermatitis (ACD), but the mechanism is unknown. The role of the thiol group in MBT's allergenicity was investigated in the present study. Guinea pigs were sensitized to MBT using a modified guinea pig maximization test (GPMT) and reactivity was assessed toward 2-mercaptobenzothiazole disulfide (MBTS), 2-hydroxybenzothiazole (HBT; thiol-substituted), 2-(methylthio)benzothiazole (MTBT; thiol-blocked), and benzothiazole (BT; thiol-lacking). MBT and MBTS, but not BT, HBT, or MTBT, elicited ACD in MBT-sensitized animals, demonstrating that the thiol group is critical to MBT's allergenicity. In addition, both MBT and MBTS were shown to inhibit both glutathione reductase and thioredoxin reductase, and thus contribute to the stability of MBT-protein mixed disulfides. It is concluded that the probable haptenation mechanism of MBT is through initial oxidation to MBTS with subsequent reduction to form mixed disulfides with proteins.

Keywords: Mercaptobenzothiazole; Thiol; Allergenicity; Contact dermatitis; Vulcanization accelerator

INTRODUCTION

2-Mercaptobenzothiazole (MBT) and its disulfide, 2-mercaptobenzothiazole disulfide (MBTS), are widely used as vulcanization accelerators in the manufacture

This article is not subject to United States copyright law.

Address correspondence to Paul D. Siegel, Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505-2888, USA. Tel.: 304 285 5721; Fax: 304 285 6126; E-mail: pds3@cdc.gov

of natural and synthetic rubber. These mercapto compounds also find wide applications in industry as corrosion inhibitors in cutting oils and greases, as flotation agents in metal ore processing, and as fungicides and bactericides (1). Allergic contact dermatitis (ACD), resulting from dermal contact with rubber products containing MBT and/or MBTS, has been reported in multiple case and clinical studies since the 1950s (2, 3). Even though it is known that MBT produces ACD, the mechanism of MBT's immunogenicity is still a subject for debate. According to Landsteiner and Jacobs' "hapten hypothesis" (4), a low-molecular-weight compound such as MBT can elicit an immune response only after binding to a protein/peptide to form a macromolecular immunogen in the form of hapten-carrier complex. The haptenation of epidermal proteins, via adduct formation between the offending chemical and nucleophilic centers on amino acids, has been demonstrated to be more feasible with electrophilic chemicals (5). Peptide reactivity experiments (6) and data from local lymph node assays (LLNAs) (7) together with the guinea pig maximization tests (GPMTs) (8) have shown electrophilic chemicals to be more potent skin sensitizers, but fail to account for the allergenicity of non-electrophilic compounds such as MBT. Correlation of electrophilicity with occurrence of ACD in humans from 355 randomly chosen chemical allergens demonstrated that only 30%–40% of the contact allergens were electrophilic (9), with the remainder being non-electrophilic compounds like MBT. Cytochrome P450-mediated metabolic activation of MBT to an electrophilic intermediate was not observed in a previous study in our laboratory (10), suggesting that MBT is not a prohapten by its strict definition (i.e., requiring metabolic activation). It was noted that MBT did not react directly with amino acids, but it was easily oxidized to MBTS. Interaction of MBTS with sulfhydryl moieties on proteins produced stable mixed disulfides, supporting the probable haptenation mechanism for MBT through its thiol group. The current biological and biochemical studies were undertaken to further test our hypothesis (10) that the thiol group on MBT is critical for induction of ACD. Guinea pigs were sensitized to MBT and challenged with MBTS as well as thiol-blocked 2-(methylthio)benzothiazole (MTBT), thiol-lacking benzothiazole (BT), and thiol-substituted 2-hydroxybenzothiazole (HBT). The interaction of MBT and these analogues with glutathione and thioredoxin reductases was also investigated.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

MATERIALS AND METHODS

Chemicals

5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB), 2-mercaptobenzothiazole (MBT, 99%), benzothiazole (BT), 2-methylthio(benzothiazole) (MTBT), 2-hydroxybenzothiazole (HBT), 2-mercaptobenzothiazole disulfide (MBTS, 99%), acetone (A), dimethylsulfoxide (DMSO), oxidized glutathione (GSSG), phosphate-buffered saline (PBS), glutathione reductase (GR) from yeast and thioredoxin reductase (TR) from rat liver, thioredoxin reductase assay kit, reduced nicotinamide adenine dinucleotide phosphate (NADPH), ammonium carbonate, and Cremophor were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methohexital and pentobarbital were purchased from Monarch Pharmaceuticals (Bristol, TN, USA). TiterMax adjuvant was from CytRx Corporation (Norcross, GA, USA). Human blood platelets prepared by the method of Worthington

and Rosemeyer (11) with modifications were also used as a source of GR. Dialysis tubing (MWCO 3.5, 12, and 30 kDa) was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). All solutions were prepared using nanopure water from a Millipore system (MilliQ; Bedford, MA, USA).

Animals

Female albino Hartley guinea pigs were purchased from Charles River Laboratories (Wilmington, MA, USA). All animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited animal facility of the National Institute for Occupational Safety and Health (NIOSH), Morgantown, West Virginia, USA. Room temperature was maintained between 18°C and 25°C and the relative humidity between 40% and 60%. A standard 12-hour light/dark cycle was maintained. All protocols were reviewed and approved by the NIOSH Animal Care and Use Committee. Upon arrival, the animals were allowed to acclimate for a minimum of 7 days. They were housed in high-efficiency particulate air (HEPA)-filtered ventilated polycarbonate cages on autoclaved hardwood Beta-chip bedding (Northeastern Products Inc., Warrensburg, NY, USA) and provided with Teklad 7906 guinea pig diet (Harlan, Indianapolis, IN, USA) with autoclaved tap water *ad libitum*. Sentinel animals were free of viral pathogens, parasites, mycoplasmas, and *Encephalitozoon cuniculi*.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

Guinea Pig Maximization Test

The GPMT procedure was adapted from Magnusson and Kligman (8) and the standardized ASTM GPMT assay (12) by substitution of Freund's complete adjuvant (FCA) with TiterMax (TM). Intradermal injection with TiterMax and MBT was conducted on day 0, followed by topical induction on day 7 and subsequent closed-patch challenge tests on day 21 and day 35 on the flanks of the animals.

Induction. ***Day 0:*** Animals (n = 10) were weighed and anesthetized with methohexital (30 mg/kg; i.p.). A 4 × 6 cm area of the shoulder region of each guinea pig was shaved for 3 intradermal injections (1.5 cm apart) consisting of (i) 0.1 ml of TM in water (40% w/v), (ii) 0.1 ml of the MBT in a DMSO/Cremophor (1:9) emulsion (25% w/v), and (iii) 0.1 ml of a mixture of MBT and TM in DMSO/Cremophor, where the concentration for TM was the same as in (i) and the concentration for the test substance was the same as in (ii).

Day 7: Topical booster sensitization was performed by applying MBT in DMSO/Cremophor to a 2.3-cm-diameter patch of Whatman filter paper #3 until it was saturated. The filter paper was then applied to the injection site of guinea pigs and occluded with 3.75-cm occlusive surgical tape. An elastic bandage was wrapped around the torso to secure the tape and left in place for 48 hours.

Challenge procedure. ***Day 21:*** Hair was clipped from the animal's flank and a 25% concentration of MBT in the DMSO/Cremophor was applied to a

2.3-cm-diameter patch of Whatman filter paper and patched onto the animal as described above and left in place for 24 hours.

Evaluation. ***Day 22:*** The minimum criterion for an allergic (positive) reaction was a confluent erythema, and the observations were scored following a previously reported standardized scoring scheme (12). All patched areas were evaluated 1 hour, 24 hours, and 48 hours after removal of the patches.

Cross-reactions. This assay was used to test the reactivity of MBT-sensitized animals to the MBT analogues MBTS, BT, HBT, and MTBT. After the first challenge (with MBT), the animals were allowed to recover for 2 weeks before another challenge was performed (day 35) with MBTS, BT, HBT, and MTBT on the flank area of the animals. The same challenge procedure and vehicle as in the first challenge were used. In each sensitization study, 6 test animals received each test chemical on both the sensitization and challenge sites and 4 non-MBT-sensitized control animals were given exactly the same treatment as described for the test animals. All 10 animals (6 MBT-sensitized and 4 non-sensitized) were challenged with each of the test chemicals.

Enzyme Inhibition: Glutathione Reductase (GR)

Experiments were conducted to test the effect of MBT and its analogues on GR activity. NADPH (8.33 μM) was added to 0.614 nM (76.17 μg/ml) GR at 4°C. The reaction was started by the addition of 10 μM oxidized glutathione (GSSG) with or without 0.568 μM MBT in 1 ml of PBS total volume at 37°C. The same procedure was repeated with the MBT analogues. Michaelis-Menten kinetics were performed using fixed concentrations of MBT, GR, and NADPH with variation of the substrate (GSSG). To generate Lineweaver-Burk plots, 4 variations of GSSG (1.9, 2.5, 5, and 10 μM) were done for each concentration of MBT (5, 7.5, and 15 μM). Loss in NADPH fluorescence (excitation, 340 nm; emission, 450 nm) was measured using a Perkin-Elmer LS 50B Luminescence spectrometer (Beaconsfield, UK) to determine rates of GR-mediated reduction of GSSG. Excitation and emission slit widths were set at 2.5 and 5.5 nm, respectively. The sample holder accommodated 10-mm quartz cuvettes enclosed in a thermostatting circulating water bath (VWR Scientific 1140A, Niles, IL, USA). Data acquisition and processing were performed using the FL Winlab software (Beaconsfield, UK). The control contained GR, NADPH, and GSSG in 0.01 M PBS: 0.5% DMSO (pH 7.4). Other control experiments carried out dismissed the possibility of adventitious reactions occurring between (i) NADPH and MBT/MBTS and (ii) GSSG and MBT/MBTS, and hence the observed NADPH oxidation (leading to loss in fluorescence) can be wholly attributed to the GR-catalyzed reduction of GSSG. To ascertain the nature of the binding (reversible versus irreversible) between MBT and GR, 0.5 U/ml of GR was incubated with 5 μM MBT at 37°C in 0.01 M PBS for 1 hour and dialyzed overnight against 4 L of PBS using a dialysis membrane (molecular weight cut-off [MWCO] = 12 kDa). The activity of the dialyzed GR-MBT mixture on GSSG was then investigated to examine reversibility of the inhibition. A control sample of GR was also dialyzed to adjust for potential dilution during dialysis.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

GR from Human Blood Platelets

Human blood platelets were isolated as a source of GR for inhibition studies. Whole blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes and centrifuged for 10 minutes, at 4°C, at 600 *g*. Plasma was collected and centrifuged for 30 minutes at 2,100 *g*, after which the platelets were washed with PBS and reconstituted to 1 ml of PBS. Freeze/thawed lysed platelets were centrifuged at 2,100 *g* for 30 minutes to remove membranes and supernatant fluid was collected as the source of GR. Protein concentration was determined using the Bradford assay (13). Activity of GR in the supernatant fluid was determined and inhibition studies were carried out as outlined above.

Thioredoxin Reductase (TR) Activity

A previously reported method (14) was used with modifications. Briefly, the TR assay kit from Sigma was used, in which TR was mixed with NADPH in PBS and stored at 4°C. The reaction was started by adding a mixture of DTNB with or without MBT/DMSO. Final concentrations of the reaction mixture contained 3.7 μM (0.2 mg/ml) TR, 0.216 mM NADPH, and 0.3 mM DTNB with or without 0.03 mM MBT. The activity (unit/ml) of TR was derived from the rate of production of 5-thio-2-nitrobenzoic acid (TNB) measured at 412 nm using a Beckman DU800 (Fullerton, CA, USA) UV/Vis spectrophotometer with the DU Series 800 Software being used for data collection. A VWR Scientific 1140A (Niles, IL, USA) circulating water bath coupled to the spectrophotometer was used to control cuvette temperature. Preliminary experiments demonstrated that MBT does not react with DTNB. MBT/DTNB was in excess over the TR to enable enzyme saturation and maximum activity. Ultrapure water was used for all reagent preparations and each assay was run in triplicate. The efficacy of the thiol moiety on MBT was tested by substitution with equivalent molar concentrations of the MBT analogues MBTS, BT, HBT, and MTBT. The concentrations of MBT and MBTS were limited (100 times lower than that of the substrate DTNB) in the assay to maintain the DMSO solvent concentration at $\leq 1\%$ to prevent solvent interference in the assay.

Statistics

Experiments for the GR and TR inhibitions were repeated 5 times with measurement of initial rates. Data were analyzed using SigmaStat 3.1. The p-values were determined using the single-factor analysis of variance (ANOVA), with p-values less than .05 considered significant. Comparison of GPMT results was made using McNemar's test.

RESULTS

MBT Allergenicity and Cross-reactivity of MBT Analogues

The GPMT was used to assess the contribution of the thiol (–SH) functional group in MBT's ability to elicit ACD. The mean scores for erythema for MBT,

Downloaded by [University of Liverpool] at 10:27 17 August 2017

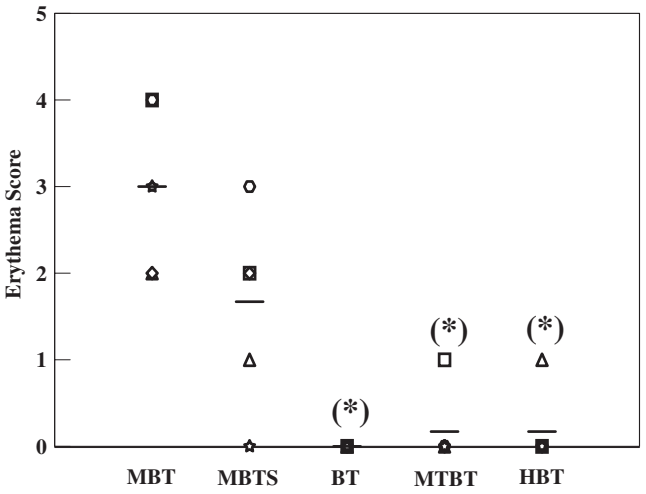

Figure 1 Erythema scores from 2-mercaptobenzothiazole (MBT)-sensitized animals after challenges with MBT, 2-mercaptobenzothiazole disulfide (MBTS), benzothiazole (BT), 2-(methylthio)benzothiazole (MTBT), and 2-hydroxybenzothiazole (HBT). Positive reactions are erythema scores greater than 1. Horizontal bars indicate mean scores. *Significantly different from MBT challenge ($p = .0156$).

Downloaded by [University of Liverpool] at 10:27 17 August 2017

MBTS, and the MBT analogues are shown in Figure 1. The scoring was based on the previously reported criteria (12). Examples of the cutaneous reactions observed are shown in Figure 2. All 6 animals that had been sensitized to MBT reacted to dermal challenge with MBT and 5 of the 6 MBT-sensitized guinea pigs cross-reacted to MBTS. An immediate "wheal and flare" reaction, diagnostic of a type I (immunoglobulin E [IgE]-mediated) hypersensitivity, was observed on the dermis of 1 MBT-challenged animal, in addition to an ACD reaction. Type I allergy to contact sensitizers is not uncommon in guinea pigs and has been reported before (15–17).

The necessity of the –SH functional group on MBT for allergenicity was strongly suggested by the lack of an ACD reaction in MBT-sensitized guinea pigs challenged with BT, HBT, and MTBT (Fig. 1). BT and HBT do not possess the thiol group, whereas the thiol is protected by a methyl group on MTBT. A few animals (both sensitized and non-sensitized) displayed slight irritation reactions from the chemical or DMSO in the vehicle (scored as + 1 reaction). Figure 2 clearly highlights the differences between positive skin allergic reactions due to MBT and MBTS and the lack of cross-reactivity with BT, HBT, and MTBT.

Enzyme Inhibition: Inhibition of Glutathione Reductase

The potential of MBT, MBTS, BT, HBT, and MTBT to inhibit the reduction of GSSG by the NADPH-dependent GR was studied. The initial kinetic GSSG reduction rates are shown in Table 1. The rate of reduction of GSSG was significantly reduced in the presence of 0.568 μM MBT or 0.568 μM MBTS, but not when equivalent amounts of BT, HBT, and MTBT were tested. MBT ($3.3\,s^{-1}$) and MBTS ($3.2\,s^{-1}$) lowered the initial rate of GSSG reduction ($7.7\,s^{-1}$) by more than 50%.

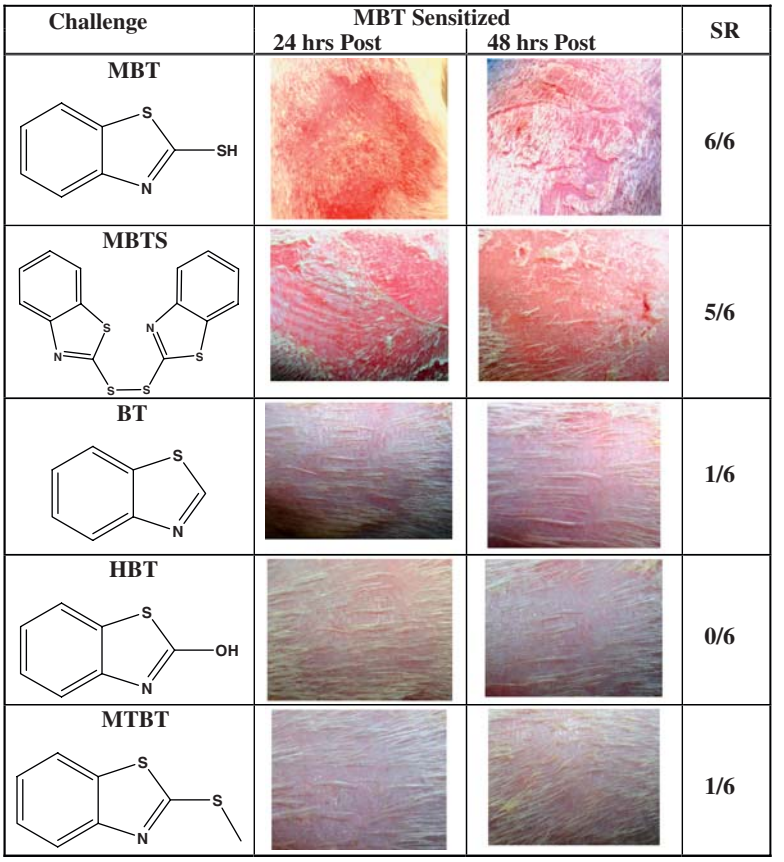

Figure 2 Representative cutaneous reactions of the 2-mercaptobenzothiazole (MBT)-sensitized animals after challenges with MBT, 2-mercaptobenzothiazole disulfide (MBTS), benzothiazole (BT), 2-hydroxybenzothiazole (HBT), and 2-(methylthio)benzothiazole (MTBT). SR indicates the ratio of sensitized guinea pigs displaying allergic contact dermatitis (ACD) to the total number of animals tested. MBT and MBTS elicited ACD in the animals.

Table 1 Initial rates of the GR-catalyzed reduction of GSSG

Test compound (0.568 μM)	Initial rate ± SD (s^{-1})
Control	7.68 ± 0.05
MBT	3.32 ± 0.03[a]
MBTS	3.24 ± 0.04[a]
HBT	7.56 ± 0.05
MTBT	7.10 ± 0.04
BT	7.56 ± 0.04

[a]Significantly different from control ($p < .5$).

GR = glutathione reductase; GSSG = oxidized glutathione; SD = standard deviation; MBT = 2-mercaptobenzothiazole; MBTS = 2-mercaptobenzothiazole disulfide; HBT = 2-hydroxybenzothiazole; MTBT = 2-(methylthio)benzothiazole; BT = benzothiazole.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

Table 2 MBT dose-dependent inhibition of GR-catalyzed GSSG reduction; Michaelis-Menten kinetics performed using fixed concentrations of MBT, 0.614 nM GR, and 8.33 μM NADPH with variation of GSSG [(a) 1.88 μM, (b) 2.5 μM, (c) 5 μM, and (d) 10 μM]

Experiment set	[MBT] μM	[GSSG][a] μM	Initial rate v/s^{-1}
1	0	(a) 1.88	1.17
		(b) 2.50	1.50
		(c) 5.00	2.59
		(d) 10.00	3.50
2	5.0	(a) 1.88	0.58
		(b) 2.50	0.85
		(c) 5.00	1.39
		(d) 10.00	2.26
3	7.5	(a) 1.88	0.44
		(b) 2.50	0.66
		(c) 5.00	1.08
		(d) 10.00	1.84
4	15.0	(a) 1.88	0.30
		(b) 2.50	0.36
		(c) 5.00	0.75
		(d) 10.00	1.35

[a]MBT inhibition of GR was examined at 4 different substrate (GSSG) concentrations.

MBT = 2-mercaptobenzothiazole; GR = glutathione reductase; GSSG = oxidized glutathione; NADPH = reduced nicotinamide adenine dinucleotide.

Further experiments were carried out to investigate the dose-dependent MBT inhibition of reduction of different amounts of GSSG. Table 2 shows the initial reaction rates that were obtained. When reciprocals of the substrate concentration (1/[GSSG]) were plotted against the reciprocals of the instantaneous rates (1/v) in a Lineweaver-Burk plot for varying GSSG concentrations, all the plots for different

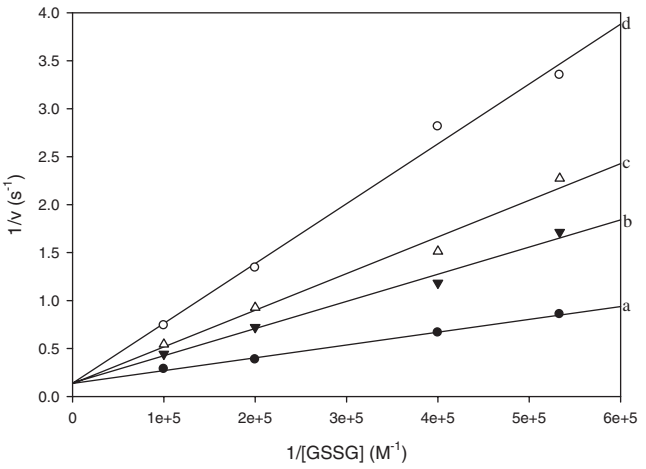

Figure 3 Lineweaver-Burk plots demonstrating competitive inhibition of glutathione reductase (GR) activity by 2-mercaptobenzothiazole (MBT). The concentration of inhibitor was increased for each consecutive set of experiments: (a) 0 μM, (b) 5 μM, (c) 7.5 μM, and (d) 15 μM MBT. GSSG = oxidized glutathione.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

Table 3 Calculated enzyme-inhibitor inhibition constants for MBT; slopes and intercepts taken from the Lineweaver-Burk plots in Figure 3

[MBT] μM	Slope (m_{MBT}) μMs^{-1}	$1/V_{max}$ s^{-1}	K_i μM
0	1.335	0.1368	($K_m = 9.7$)
5	2.832	0.142	4.46
7.5	3.823	0.1349	4.02
15	6.238	0.1389	4.08

MBT = 2-mercaptobenzothiazole; V_{max} = maximum velocity; K_i = inhibition constant; K_m = Michaelis-Menten constant.

MBT concentrations converged into a common intercept on the 1/v axis (Fig. 3). As the MBT concentration changed from 5 μM to 15 μM, the inhibition increased by a factor of 1.7 at a substrate concentration of 10 μM. The dose-related change with a common 1/v axis intercept (Fig. 3) is indicative of a competitive inhibition of the GR activity. Table 3 lists the calculated inhibition constant (K_i) values of the various MBT concentrations. The calculated K_i for MBT was 4.19 ± 0.24 μM, which suggests that MBT is a fairly potent GR inhibitor. To confirm the competitive nature of MBT inhibition, experiments were carried out to determine whether MBT was binding to GR reversibly. The reaction kinetics of the dialyzed reductase-MBT system was not significantly different from that of the dialyzed GR (without MBT) control (data not shown). The reversibility of the GR-MBT binding is consistent with the competitive inhibition kinetics observed. Human platelet GR, similar to the yeast GR, was inhibited dose-dependently by MBT (Fig. 4). The initial reaction rates were reduced to 33% and 27% by 5 μM MBT and 10 μM MBT, respectively.

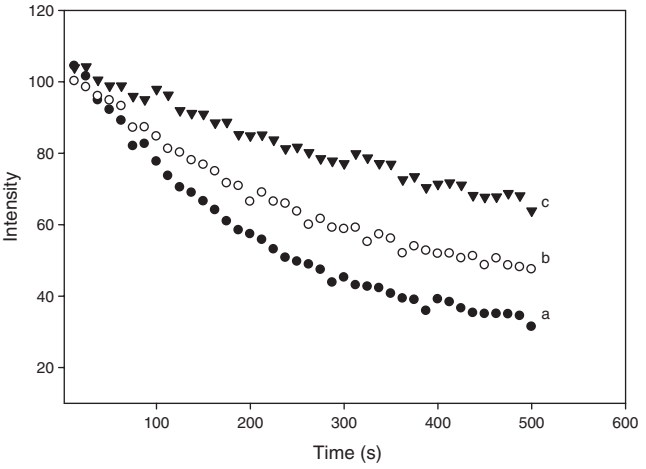

Figure 4 2-Mercaptobenzothiazole (MBT)-mediated inhibition of glutathione reductase (GR) from human blood platelets. The enzyme assays consisted of 0.1 mg/ml of protein + 8.33 μM NADPH + 10 μM GSSG + [(a) 0 μM, (b) 5 μM, or (c) 10 μM MBT]. NADPH = NADPH = reduced nicotinamide adenine dinucleotide; GSSG = oxidized glutathione.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

Table 4 Initial rates of the TR-DTNB assay; initial rate of production of TNB used to calculate initial activity of the enzyme

Test chemical	Initial rate ± SD (ms^{-1})
Control	0.330 ± 0.008
BT	0.32 ± 0.008
MTBT	0.33 ± 0.005
HBT	0.23 ± 0.004
MBTS	0.09 ± 0.003[a]
MBT	0.02 ± 0.002[a]

[a]Significantly different from control ($p < .5$).

TR = thioredoxin reductase; DTNB = 5,5′-dithio-bis(2-nitrobenzoic acid); TNB = 5-thio-2-nitrobenzoic acid; SD = standard deviation; BT = benzothiazole; MTBT = 2-(methylthio)benzothiazole; HBT = 2-hydroxybenzothiazole; MBTS = 2-mercaptobenzothiazole disulfide; MBT = 2-mercaptobenzothiazole.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

Inhibition of Thioredoxin Reductase

Equivalent concentrations of MBT and MBTS inhibited the TR-dependent reduction of DTNB by 90% and 60%, respectively (Table 4). BT and MTBT did not alter TR activity. HBT, however, showed a slight decrease (33%) in TR activity.

DISCUSSION

MBT is commonly used as a vulcanization accelerator in rubber products, and contact allergy has been widely reported from exposure to rubber products. A recent review of ACD to mercapto compounds (2) noted that MBT is a commonly reported cause of contact allergy, but the critical chemical nature conferring allergenicity is still not well understood. The ''electrophilic'' hapten hypothesis developed by Dupuis and colleagues (18) is based on the assumption that the activity of a chemical is directly related to its electrophilic properties. While this electrophilic-based mechanism can be used to explain the activity of about 30%–40% of ACD-inducing skin sensitizers (aldehydes, alkyl halides, sulfones, etc.) (9), and *in silico*-based quantitative structure–activity relationship (QSAR) models have been developed based on this hypothesis, non-electrophilic protein haptenation mechanisms have been invoked to explain ACD associated with exposure to other classes of compounds. MBT and MBTS are typical examples of compounds for which proteins are haptenated by non-electrophilic mechanisms. Although QSAR models such as the Toxicity Prediction by Computer-Assisted Technology (TOPKAT) and Deductive Estimation of Risk from Existing Knowledge (DEREK) identify MBT and MBTS as skin sensitizers based on LLNA/GPMT data (19) and have the ability to utilize the possible structural motifs believed to cause skin sensitization to a certain degree of confidence, they are still unable to quantitatively determine the level of sensitization. QSAR models are also handicapped by the lack of experimental data detailing and confirming the mechanistic pathways of protein haptenation. Identification of haptenation pathways should allow for better predictive *in silico* models, aiding in potential product modification or substitution to decrease the incidence of ACD in the workplace.

In addition to MBT and MBTS, the structurally related compounds morpholinyl mercaptobenzothiazole (MOR) and N-cyclohyxyl-2-benzothiazole (CBS) are also known contact allergens (2). It has been commonly assumed that the "true hapten from all these compounds" was MBT (20). This was based on its likelihood of penetrating the skin, existing in a reduced form due to the presence of excess glutathione (GSH) and the capability of MBT to react with amino acids (20, 21). Wang and Tabor (21) reported that MBT can react with the carboxyl groups of amino acids to form thioester bonds, with amines on lysines and with the thiol group of cysteine (disulfide bonds). In our previous work (10), we were unable to confirm the work of Wang and Tabor (21). Under physiological conditions, MBT did not react directly with amino acids or albumin. It is not known if MBT is oxidized within the skin. Previous studies have shown that MBT is readily oxidized to the dimer by common workplace oxidants like bleach, iodine, and hydrogen peroxide (10). It was found that MBTS was readily reduced by both non-protein (GSH) and protein thiols. The products of the reduction of MBTS by albumin were MBT and the mixed disulfide MBT-(Cys34) albumin. It was concluded that the probable mechanism of protein haptenation by MBT was through oxidation (either prior to or following absorption into the skin), with subsequent protein haptenation through disulfide bond formation.

The present study expands on our previous studies and suggests the critical nature of the thiol moiety and haptenation through disulfide formation for MBT and MBTS. The GPMT, a commonly used, internationally accepted method for identification of chemical allergens, was used in the present study to evaluate the critical nature of the thiol moiety toward MBT and MBTS allergy. Even though the GPMT endpoints are more subjective than the LLNA, the assay has greater sensitivity and allows for chemical allergen cross-reactivity assessment. Thus, the guinea pig model provided a sensitive assay that enabled testing of the thiol-mediated allergenicity. MBTS was implicated in the ACD mechanism by its ability to produce an ACD reaction in MBT-sensitized guinea pigs. The importance of the thiol moiety as the critical chemical functional group was further demonstrated by lack of allergic skin reactions in MBT-sensitized guinea pigs challenged with the thiol-lacking, thiol-substituted, or thiol-blocked MBT analogues BT, HBT and MTBT, respectively. These results are consistent with those reported by Fregert and Skog (22). They found no cross-reactivity, to chemical analogues of MBT that lacked the thiazole, benzene, or thiol group, in MBT-allergic subjects. The semi-quantitative GPMT was thus sufficient to test our hypothesis even though a quantitative GPMT assay would have been able to shed some light on the dose-responsive activity of the animals to MBT/MBTS. A single positive response by one of the thiol-blocked/substituted/lacking analogues would suggest a possible non-thiol-mediated mechanism of allergenicity. The fact that MBT and MBTS have been shown to be moderate sensitizers in the human maximization test (7) together with their non-irritating properties ensured minimal risk of false-positive and false-negative results with the GPMT. The absence of false-positive results in all the animals that had positive reactions at induction was confirmed by the erythema, which persisted for days.

The redox state in the localized area of dermal exposure may be critical in the haptenation potential of MBT. It was found that both MBT and MBTS were potent

Downloaded by [University of Liverpool] at 10:27 17 August 2017

inhibitors of GR and TR. Human TR and GR are 44% homologous with a common conserved redox active catalytic site, –Cys61-Val-Asn-Val-Gly-Cys66-, and the role of both GR and TR as cellular redox regulators derives from this common active site. The fact that sulfhydryl-based inhibition using MBT was observed with both GR and TR might point to the interaction of this redox active site with MBT. Even though the mechanism of MBT inactivation of TR is not known, the observed loss of MBTS might have been due to reduction leading to the more potent MBT. It is, however, not clear whether reduction of MBTS is an enzymatic process or a chemical interaction between MBTS and the accessible Cys 61 and Cys 66 on the redox active peptide. The observed partial TR inhibition with the hydroxyl (–OH)-bearing HBT stands out as the major difference between the GR and TR studies. The availability of a second C-terminal -Cys-SeCys- (where SeCys is selenocysteine) redox active site on TR that is absent in GR might have been responsible for the observed HBT-TR inactivation. The broad substrate specificity of mammalian TR relative to GR has been postulated to be due to this second redox active site (14). Regardless of the mechanism, GR and TR inhibition would be expected to shift the redox status in the area of dermal exposure, producing oxidative stress (potentially enhancing conversion of MBT to MBTS) and enhancing stability of MBT-haptenated proteins. GR inhibition by MBT in a localized area will result in the reduced reduction of MBTS and subsequent mixed disulfide formation with low-molecular-weight thiols like GSH, thereby enabling immunogenic adduct formation between protein thiols and MBTS. Our previous studies have demonstrated the ability of MBTS and not MBT to react with sulfhydryl groups on proteins (10).

Overall, a variety of biomolecular processes result in skin sensitization, thus defining it as a complex immunological phenomenon. A protein haptenated through disulfide bond formation needs to be sufficiently stable to allow for antigen processing and presentation to the T-lymphocytes. Protein haptenation with subsequent proteolytic digestion of the covalently modified biomolecules leading to immunogenic haptenated peptides are prerequisite steps in the cascading reactions that culminate in sensitization. Although *in vitro* microsomal metabolism of MBT was not observed, metabolic activation of an MBT prohapten cannot be completely ruled out. *In vivo* studies to assess the potential for formation of MBT metabolites that are protein reactive would be of value. The results of the present study are consistent with and strongly support our previous MBT studies (10) that suggested that MBT's allergenicity is mediated through initial oxidation to MBTS with subsequent reduction by a free protein thiol, thus forming a haptenated protein via a disulfide. The study confirms the requirement of a thiol moiety for MBT allergenicity and the ability of MBTS to elicit ACD in MBT-sensitized guinea pigs.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

ACKNOWLEDGMENT

This work was supported by an Inter Agency agreement with the National Institute of Environmental Health Sciences (NIEHS) (Y1-ES0001-06). The study was also partially funded from NSF grant number CHE 0614924. The findings and conclusion in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

REFERENCES

1. Reddy CM, Quinn JG. Environmental chemistry of benzothiazoles derived from rubber. Environ Sci Technol 1997; 31:2847–2853.
2. Adams AK, Warshaw EM. Allergic contact dermatitis from mercapto compounds. Dermatitis 2006; 17:56–70.
3. Kaniwa M, Isama K, Nakamura A, Kantoh H, Hosono K, Itoh M, Shibata K, Usuda T, Asahi K, Osada T, Matsunaga K, Ueda H. Identification of causative chemicals of allergic contact dermatitis using a combination of patch testing in patients and chemical analysis. Application to cases from rubber gloves. Contact Dermatitis 1994; 31:65–71.
4. Landsteiner K, Jacobs J. Studies on the sensitization of animals with simple chemical compounds. J Exp Med 1936; 64:625–639.
5. Divkovic M, Pease CK, Gerberick GF, Basketter DA. Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. Contact Dermatitis 2005; 53:189–200.
6. Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP. Development of a peptide reactivity assay for screening contact allergens. Toxicol Sci 2004; 81:332–343.
7. Haneke KE, Tice RR, Carson BL, Margolin BH, Stokes WS. ICCVAM evaluation of the murine local lymph node assay. Data analyses completed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods. Regul Toxicol Pharmacol 2001; 34:274–286.
8. Magnusson B, Kligman AM. The identification of contact allergens by animal assay. The guinea pig maximization test. J Invest Dermatol 1969; 52:268–276.
9. Rosenkranz HS, Klopman G, Zhang YP, Graham C, Karol MH. Relationship between allergic contact dermatitis and electrophilicity. Environ Health Perspect 1999; 107:129–132.
10. Chipinda I, Hettick JM, Simoyi RH, Siegel PD. Oxidation of 2-mercaptobenzothiazole in latex gloves and its possible haptenation pathway. Chem Res Toxicol 2007; 20:1084–1092.
11. Worthington DJ, Rosemeyer MA. Glutathione reductase from human erythrocytes. Catalytic properties and aggregation. Eur J Biochem 1976; 67:231–238.
12. American Society for Testing and Materials. Standard Practice for Testing Guinea Pigs for Contact Allergens: Guinea Pig Maximization Test. F 720-81; West Conshohocken, PA: American Society for Testing and Materials. 2002; Ref Type: Catalog.
13. Sapan CV, Lundblad RL, Price NC. Colorimetric protein assay techniques. Biotechnol Appl Biochem 1999; 29(pt. 2):99–108.
14. Arner ES, Zhong L, Holmgren A. Preparation and assay of mammalian thioredoxin and thioredoxin reductase. Methods Enzymol 1999; 300:226–239.
15. Sjogren F, Anderson C. The spectrum of inflammatory cell response to dimethyl sulfoxide. Contact Dermatitis 2000; 42:216–221.
16. Moore KG, Dannenberg AM, Jr. Immediate and delayed (late-phase) dermal contact sensitivity reactions in guinea pigs. Passive transfer by IgG1 antibodies, initiation by mast cell degranulation, and suppression by soybean proteinase inhibitor. Int Arch Allergy Immunol 1993; 101:72–81.
17. Sjogren F, Anderson C, Groth O. The cellular dermal infiltrate in experimental immediate type cutaneous hypersensitivity. Acta Derm Venereol 1995; 75:417–421.
18. Dupuis G, Benezra C, Schlewer G, Stampf JL. Allergic contact dermatitis to alpha-methylene-gamma-butyrolactones. Preparation of alantolactone-protein conjugates and induction of contact sensitivity in the guinea pig by an alantolactone-skin protein conjugate. Mol Immunol 1980; 17:1045–1051.
19. Fedorowicz A, Singh H, Soderholm S, Demchuk E. Structure-activity models for contact sensitization. Chem Res Toxicol 2005; 18:954–969.

Downloaded by [University of Liverpool] at 10:27 17 August 2017

20. Hansson C, Agrup G. Stability of the mercaptobenzothiazole compounds. Contact Dermatitis 1993; 28:29–34.
21. Wang XS, Tabor MW. Studies of the reactivity of morpholine, 2-mercaptobenzothiazole and 2 of their derivatives with selected amino acids. Contact Dermatitis 1988; 19:16–21.
22. Fregert S, Skog E. Allergic contact dermatitis from mercaptobenzothiazole in cutting oil. Acta Derm Venereol 1962; 42:235–238.

Downloaded by [University of Liverpool] at 10:27 17 August 2017