Amelioration of acute mercury toxicity by a novel, non-toxic lipid soluble chelator N,N′bis-(2-mercaptoethyl)isophthalamide: effect on animal survival, health, mercury excretion, and organ accumulation

David Clarke[a], Roger Buchanan[a], Niladri Gupta[bc] and Boyd Haley[bc]*

[a]*Environmental Sciences, Arkansas State University and Graduate Program in Molecular Biosciences, Arkansas Biosciences Institute, Arkansas State University, State University, AK 72467, USA;* [b]*Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA;* [c]*CTI Sciences, Inc., 2430 Palumbo Drive, Lexington, KY 40509, USA*

The toxic effects of mercury are known to be complex with specific enzyme inhibitions and subsequent oxidative stress adding to the damaging effects. There are likely other factors involved, such as the development of impaired metal ion homeostasis and depletion of thiol- and selenium-based metabolites such as cysteine and selenium. Much of the toxicity of mercury occurs at the intracellular level via binding of Hg^{2+} to thiol groups in specific proteins. Therefore, amelioration of mercury toxicity by the use of chelation would likely be enhanced by the use of a chelator that could cross the cell membrane and the blood brain barrier. It would be most favorable if this compound was of low toxicity, had appropriate pharmacokinetics, bound and rendered mercury cation non-toxic and had antioxidant properties. Herein we report on such a chelator, N,N′-bis(2-mercaptoethyl)isophthalamide (NBMI), and, using an animal model, show that it prevented the toxic effects associated with acute exposure induced by injected mercury chloride.

Keywords: chelation; mercury toxicity; NBMI; metal excretion; oxidative stress; antioxidant

Introduction

Heavy metals such as mercury, lead, cadmium, and silver can bind to proteins on the thiol "(–SH)" groups of cysteine. This inhibits or modifies the biological function of these proteins and can lead to several well-defined systemic problems. Further, it is well known that heavy metal toxicity, and specifically mercury toxicity, can induce damage that leads to overproduction of reactive oxygen species (ROSs), including hydroxyl free radicals, which can cause severe damage to cells and tissues (Ercal, Gurer-Orhan, and Aykin-Burns 2001). Concerns about health effects of mercury toxicity-induced oxidative stress has increased with the recent publication based on the 1999–2000 and 2005–2006 NHANES reports that showed that American women with detectable blood Hg levels increased from 2% to 30%, respectively (Laks 2009). A recent review covers the role of mercury toxicity in hypertension, cardiovascular disease, and stroke (Houston 2011). Herein we address the potential of a single lipophilic compound with mercury chelation and hydroxyl free radical

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*Corresponding author. Email: behaley@ctiscience.com

scavenging capability to prevent the toxicity that accompanies subcutaneous injection of mercury chloride at acute levels.

The exact mechanism(s) for mercury's tremendous enhancement of oxidative stress is not known, but the increase in H_2O_2 levels found in mercury toxic fish indicates peroxidase inhibition is involved (Splittgerber and Tappel 1979). Therefore, the conversion of hydrogen peroxide to hydroxyl free radicals (OH•) via a Fe or Cu catalyzed Fenton-type reaction is likely involved. However, it is also likely that a mercury-induced mitochondrial decoupling also contributes to the source of free radicals. Mercury inhibition of glutathione peroxidase, which normally removes H_2O_2, is also likely one cause of this H_2O_2 build up. Increased H_2O_2 leads to increased oxidized glutathione (GSSG) which is one of the first biochemical signals for induction of apoptosis (Franco and Cidlowski 2009). The oxidation of glutathione (GSH) to GSSG by mercury-induced ROSs could lead to health risks associated with an abnormal induction of apoptosis (Franco et al. 2007).

Loss of GSH can also decrease the activity of glutathione-S-transferase (GST) which produces glutathione–organic complexes that are actively transported out of the cell into the blood and then moved into the bile by glutathione-activated receptors in the biliary transport system of the liver (Ballatori and Clarkson 1983). It is well known that exposures to mercury and organic mercury compounds lead to decreased GSH levels by inhibiting the enzymes involved in the synthesis of GSH and the reductive recovery of GSH from GSSG (Chung, Maines, and Renolds 1982)

It has been found to be quite difficult to significantly raise GSH levels by ingesting antioxidants after heavy metal exposure (ESFA 2010). A reasonable approach to reverse oxidative stress and recover GSH levels decreased by mercury toxicity would be to develop a lipophilic chelator with membrane penetrating abilities, hydroxyl free radical (OH•) scavenging ability, and mercury chelating properties that are superior to GSH. With these properties such a compound could dramatically reduce toxic mercury levels, reduce free radical damage, and increase intracellular GSH. This should allow cells affected by mercury to recover to a normal state and would allow GST to remove organic compounds, such as environmental toxins and drugs, built up during the periods of metal toxicity.

Success using currently available putative chelators has been limited because existing agents, e.g. dimercaptopropanesulfonate (DMPS) and dimercaptosuccinic acid (DMSA), are charged compounds that do not effectively enter cells and do not form a stable enough mercury complex to prevent the release of some of the mercury when exposed to kidney proteins containing reactive thiols. This results in relocation of blood mercury into the kidney causing renal toxicity (Brandão et al. 2006). The weaker binding of DMPS and DMSA is due to the fact there is not enough stereo-chemical space between the two thiols located on adjacent carbons to allow for binding a mercury atom. Therefore, DMPS and DMSA are not true chelators as they form "sandwich complexes" with two DMPSs forming single bonds each to a single Hg^{2+} (George et al. 2004). Additionally, DMPS and DMSA chelates of Hg^{2+} are negatively charged and do not effectively enter cells and are rapidly cleared by the kidney with little mercury exiting through the liver into the feces. They are well known to deplete the body of essential metals (Torres-Alanis et al. 2000).

To be effective at treating Hg^{2+} toxicity, a chelator must also efficiently cross the cell membrane and, to prevent mercury toxicity in the central nervous system, cross the blood brain barrier. Inside the cell, the chelator has to be able to penetrate hydrophobic aspects of organelles and interact with proteins that may have Hg^{2+} locked in hydrophobic micro-environments. For example, mercury is known to inhibit porphyrin metabolism at a specific site located on intermembrane space of mitochondria where the terminal porphyrin metabolite is transported from the cytosol into the matrix space for conversion

Figure 1. N,N′-bis-(2-mercaptoethyl)isophthalamide (NBMI).

into heme (Elder and Evans 1978; Woods and Fowler 1987; Woods and Southern 1989). A direct correlation of mercury exposure to abnormal porphyrin metabolism has been reported (Geier et al. 2011). To reverse this aspect of mercury toxicity by chelation, the chelator would have to pass the cell membrane and reach the inter-membrane space of the mitochondria and, possibly need to then enter into a micro-hydrophobic domain.

An effective chelator must be of low toxicity when complexed with Hg^{2+} and not disrupt membranes or biological pathways. Also, the ideal chelator should not be metabolized as that would destroy its structure and decrease its chelation capacity. It should not compete with binding at sites that are meant for natural compounds to prevent any negative biological activity if the chelator is needed at high concentrations. Also, the chelator must be efficiently excreted in a non-toxic form. The optimal chelator should also have an adequate plasma half-life so that regular doses could be administered without the chelator being significantly depleted from the plasma and tissues. A lipid soluble, heavy metal chelator that readily enters cells would be less available for rapid kidney clearance, thereby having a longer half-life while also being able to reach sites where Hg^{2+} binding causes its toxic effects.

N,N′-bis(2-mercaptoethyl)isophthalimide (NBMI, see Figure 1) is a water-insoluble, lipophilic chelator that has exceptionally high affinity for Hg^{2+}, Pb^{2+}, and Cd^{2+} with lower comparative affinity for thiol exchangeable essential metal ions and, due to the lack of charges at physiological pHs, no general ionic attraction to positively charged metals. NBMI was previously synthesized for testing in remediation of mercury contaminated soil and water and found to remove mercury, lead, and cadmium to EPA acceptable levels (Atwood, Howerton, and Matlock 2003).

NBMI readily dissolves in 95% ethanol or dimethylsulfoxide (DMSO), and when added to $HgCl_2$ in water complexes with the Hg^{2+} and precipitates out of water into a white pellet. The ethanol-produced NBMI–Hg complex has been extensively studied *in vitro* and found to be stable to extend exposure to aqueous pHs less than 2 and greater than 12 in leaching experiments (Atwood, Howerton, and Matlock 2003; Zaman et al. 2007). Also, the NBMI–Hg precipitate was found insoluble in 22 organic solvents and solvent–water mixtures including ethanol and DMSO. To date, no standard organic or water/organic mixture has been shown able to dissolve NBMI–Hg precipitate at physiological temperatures (Zaman et al. 2007). Similar data was observed for the Pb^{2+} and Cd^{2+} precipitates with NBMI (Zaman et al. 2007) and indicates exceptionally high affinity for these three metals.

NBMI (Figure 1) has a structure consisting of dicarboxybenzoate (found in apples and cranberries) coupled by an amide linkage to two cysteamines (a metabolite of cysteine and found in flesh and on the terminal end of Coenzyme-A). Both of these natural compounds have hydroxyl free radical scavenging properties and lead to NBMI being

marketed as a dietary antioxidant for 2 years before removal from the market and being submitted for drug approval as recommended by the FDA. NBMI has the following properties: (1) Its structure consists of two non-toxic, natural compounds with apparent antioxidant properties. (2) It has hydrophobic character, indicating it would penetrate lipid bilayers. (3) NBMI binds Hg^{2+} *in vitro* in an exceptionally tight and stable complex. (4) It has no general ionic attraction for metals depending only on thiol-attractive binding properties for chelation which should decrease the loss of essential metals. Therefore, it was decided to test NBMI for its ability to both prevent mercury toxicity and reduce oxidative stress.

Materials and methods

Materials

NBMI Synthesis

NBMI was synthesized using a modification of a previous published method (Atwood, Howerton, and Matlock 2003). An example of a typical synthesis is as follows. 2-Aminoethylthiol hydrochloride (3.0 g) was dissolved in 25 mL of chloroform and 3.7 mL of triethylamine and placed in an ice bath with stirring. Isophthaloyl chloride, 2.68 g, was dissolved in 25 mL of chloroform and slowly added to the solution containing 2-aminoethylthiol and allowed to stir for 2 h on ice. A precipitation of the NBMI was induced by adding about 100 mL of $0.1 \, mol \, L^{-1}$ HCl slowly to the stirring mixture. The resulting precipitate was collected by filtration and washed twice with a water/chloroform (50/50) mixture and then twice with $0.1 \, mol \, L^{-1}$ HCl and thrice with distilled H_2O. The resulting white powder was dried under vacuum and yielded the product NBMI in over 80% yields. Gram amounts of this powder were dissolved in pure ethanol and recrystallized twice, resulting in the final product which contained very low impurities. All chemicals for the synthesis and purity determination were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. NBMI purity was determined (Absorption Systems, LP, Exton, PA) using an LC-MS:MS (Applied Biosystems, Carlsbad, CA) with the following procedure. The column was packed with reversed phase C-18 material (150×3.0 mm, 5 μm particle size, Waters X-bridge). The mobile phase consisted of (A) aqueous with 0.1% formic acid and (B) methanol with 0.1% formic acid. A gradient system was used, and the total run time was 30 min. The elution conditions expressed as % of B is as follows: 0–30 s 10% B, 30 s–10 min 10–90% B, 10–19 min 90% B, 19–20 min 90–10%B, 20–30 min 10%B. Injection volume was 10 μL. Flow rate was $250 \, μL \, min^{-1}$ and the retention time for NBMI was 9.81 min. Analysis was done on a Varian LC 1200 L triple quadruple mass spectrometer (Varian LC 1200 L) using a positive electro-spray ionization source (ESI). Chemicals used in the LC-MS:MS (liquid chromatographic-mass spectrometric) analysis were purchased from Sigma-Aldrich. The resulting product from re-crystallization was greater than 98% NBMI with the major impurity being identified as two NBMIs coupled through a disulfide linkage.

Toxicity studies of NBMI

Injected toxicity. NBMI was first dissolved in DMSO and mixed with a phosphate buffered saline solution (PBS) to obtain a 25% DMSO:75% PBS–NBMI mixture which remained soluble for the time of the experiment. Rats were injected subcutaneously under the loose skin of the abdominal area with the amounts of NBMI indicated in Table 1.

Table 1. Testing of toxicity of subcutaneously injected NBMI.

| Rat # | Micromoles NBMI injected | | | |
	1	2	3	4
Day 1	0	100	200	300
Day 4	0	200	300	400
Day 7	0	300	400	500
Day 10	0	0	1500	1500
NBMI total	0	600	2400	2700

Note: Rats were injected with indicated amounts of NBMI dissolved in DMSO:PBS (25:75) as indicated and given in methods.

Rats were first injected on day one and then again on days 4, 7, and 10 with the indicated increases in NBMI since no toxic effects were observed. Rats were weighed daily and observed for ataxia, food and water intake, and general activity compared to sham-injected controls. Analytical grade sodium phosphate, and DMSO were from Sigma-Aldrich.

Oral toxicity. First, an up/down experiment was done with NBMI suspended in corn oil. Delivery was by a gavage into three female Wistar albino rats of 206–208 g weight. Testing was stopped at $5\,g\,kg^{-1}$ body weight. This was followed by a 28-day study with an oral gavage of NBMI in corn oil with doses of NBMI at 0, 0.1, 0.5, and $1.0\,g\,kg^{-1}$ body weight. The assessment of toxicity was done by weight changes, general appearance, and microscopic and histological evaluation of all organs in a routine approved for drug development (MB Research Laboratories, Spinnerstown, PA).

Endotoxin and mutagenic activity
NBMI was subjected to studies to determine endotoxin activity (Associates of Cape Cod, East Falmouth, MA 02536) and for mutagenic activity (Midwest Bioresearch, LCC Skokie, IL) using the Ames test.

Antioxidant activity
NBMI was tested to determine its oxygen radical absorbance capacity (ORAC) and hydroxyl radical absorbance capacity (HORAC) using the standard Troxlox equivalency method (Brunswick Laboratories, Southborough, MA).

Metabolic profiling of NBMI
To identify metabolites, NBMI was incubated with human and rat liver microsomes and the products were identified by electrospray ionization MS (AB Sciex Instruments, Foster City, CA). Samples of NBMI exposed to microsomal preparations were separated by HPLC and analyzed using MS and MS:MS detection using different scan modes with LightSight[TM] metabolite identification software. The NBMI ion was selected for information dependent acquisition (IDA) and was analyzed using the Enhanced Product

Ions (EPI) scan methodology. The trapped fragment ions were finally scanned using a linear ion trap methodology (Absorption Systems, LP, Exton, PA).

Pharmacokinetic studies

The oral bioavailability of NBMI was evaluated in male Sprague-Dawley rats after an intravenous dose of $1 \, mg \, kg^{-1}$ and oral doses of 7 and $35 \, mg \, kg^{-1}$. NBMI levels were determined at 1, 2, 8, and 24 h after administration in multiple organs and plasma using LC-MS:MS methodology, as described in the bio-distribution studies (Absorption Systems, LP, Exton, PA).

Bio-distribution studies

Four groups of twelve 12 h fasted male Sprague Dawley rats each were given 100 mg NBMI suspended in corn oil kg^{-1} body weight. Blood samples were collected from the rats via a jugular vein cannula at 1, 2, 8, and 24 h and placed into chilled polypropylene tubes containing sodium heparin as an anticoagulant. Samples were centrifuged at a temperature of 4°C and at a speed of 13,000 rpm for 5 min. Samples were maintained chilled throughout processing. Each plasma sample was then transferred into labeled polypropylene tubes, placed on dry ice, and stored in a freezer set to maintain −60°C to −80°C. Liver, kidney, spleen, small intestines, bone marrow, and SC fat were extracted at the indicated time points, weighed, and kept frozen until analysis. Brain tissue samples were collected, rinsed with saline, weighed, patted dry, and kept frozen until analysis.

Levels of NBMI were determined (Absorption Systems, LP, Exton, PA) using a LC-MS:MS system as follows. Prior to analysis, all plasma and homogenized tissue samples were pre-treated with dithiothreitol (DTT) in order to reduce any disulfide bonds. Each plasma and tissue sample was combined with an equal volume of $200 \, mmol \, L^{-1}$ DTT in water prior to extraction. Add $25 \, \mu L$ of samples, standards, or QCs into 2 mL polypropylene centrifuge tubes. Add $25 \, \mu L$ of $200 \, mmol \, L^{-1}$ DTT in water, vortex, and let stand at room temperature for 15 min. Add $150 \, \mu L$ acetonitrile containing $100 \, ng \, mL^{-1}$ tolbutamide as an internal standard. Vortex well and then centrifuge at 13,000 rpm for 10 min. Aliquots of $75 \, \mu L$ of sample were placed into glass HPLC vials for analysis.

A Perkin Elmer series 200 micropumps and LEAP Autosampler equipped with a Phenomenex Synergi Polar RP, $50 \times 2.0 \, mm$ i.d., $4 \, \mu m$ column was used for separation. The column was developed using a mobile phase buffer: $40 \, mmol \, L^{-1}$ ammonium formate, pH 3.5, an aqueous reservoir (A): 10% buffer, 90% water, and an organic reservoir (B): 10% buffer, 90% acetonitrile. The gradient was 2.5 min 100 A to 100% B at a flow rate of $300 \, \mu L \, min^{-1}$. Samples were analyzed using a PE Sciex API4000 QTRAP on Electrospray (turbo ion spray interface in multiple reaction monitoring (MRM) mode). Gases were CUR 20, CAD 7, GS1 20, GS2 30, and the source temperature was 450°C.

NBMI effects on organ-specific accumulation of mercury

Twenty-four mature male Harlan Sprague-Dawley rats (body mass of 389–397 g) were divided into four groups of six and were housed as pairs in a PHS-assured animal facility under 12 h light:dark conditions and given food and water *ad libitum*. All animal use protocols were approved by the Arkansas State University IACUC for the organ-specific

accumulation of mercury study. Adult rats were used as they are less susceptible to the lethal toxicity of mercury chloride.

NBMI effects were determined on mercury excretion and organ-specific accumulation in rats treated with an acute but sub-lethal dose of $HgCl_2$. Groups of animals were injected with Hg and/or NBMI and/or saline as given in methods. After injections, each animal was placed in a metabolic cage and samples of feces and urine were collected for 5 days. Six days after injection, each animal was sacrificed and the brain, kidneys, liver, epididymal (EP), and intraperotineal (IP) fat were collected. Total Hg levels were measured in urine, feces, brain, kidney, liver and EP, and IP fat from all groups using a cold vapor atomic absorption (CVAA) spectrometer.

Animals were weighed before treatment and immediately before sacrifice. The study groups of six rats each were designated as: Group 1 "Unexposed," not injected with Hg and not treated with NBMI, used as the "negative control" group. Group 2 "NBMI Only," not injected with Hg, but treated with NBMI. Group 3 "Hg Only," injected with Hg but not treated with NBMI, used as the "positive control" group. Group 4 "Hg + NBMI," injected with Hg and treated with NBMI.

All exposed animals were subcutaneously (sc) injected with 14.9 µmole (4.06 mg $HgCl_2 \, kg^{-1}$ or 3 mg kg^{-1} Hg) in physiological saline. This dose was equivalent to 29% of the predicted LD_{50} (14 mg $HgCl_2 \, kg^{-1}$ administered by sc injection) for inorganic $HgCl_2$. Total volume of injection was <0.5 mL. During injection animals were briefly anesthetized with isoflurane. After 20 to 30 min of $HgCl_2$ injection, animals that were treated with NBMI received a subcutaneous (sc) injection of NBMI dissolved in DMSO with sufficient PBS to reach a total injection volume of 0.5 mL. The injected NBMI dose was 149 µM (42.3 mg kg^{-1} body weight), a 10-fold excess of molar amount of injected Hg^{2+}. Animals not injected with NBMI or Hg were injected with 0.5 mL physiological saline so that every animal received two 0.5 mL injections. Urine and feces sample collection began 24 h before the Hg injection. Rats were placed in metabolic cages for a total of 4 h per day (2 h at 12 h intervals) while feces and urine samples were collected. If insufficient urine (~0.5 mL) or feces (2 pellets) was not collected after 2 h, the animal remained in the cage until collection was sufficient. Collection continued at 12 h intervals for 5 days after injections. After collection the osmolarity of each urine sample was measured. Urine and feces samples were frozen and stored at −80°C prior to analysis. Feces samples were digested using the protocol used for organs. The osmolarity of each urine sample was measured immediately after collection. To correct for differences in urine concentration, measured mercury levels in all urine samples were normalized to the osmolarity of that sample.

On the 6th day after exposure the animals are sacrificed and liver, kidney, brain, and samples of EP and IP fat were removed. Rats were anesthetized with 0.3 mL of ketamine HCl and 0.4 mL of xylazine before sacrifice. Tissue samples were weighed, immediately frozen in liquid N_2, and stored at −80°C prior to digestion and analysis.

Analysis of mercury in organ tissue, feces, and urine

Organ and feces samples were prepared for analysis by digestion. Samples were thawed, weighed, and dried at 65°C overnight. Each dried sample was weighed and placed in a 50 mL Teflon digestion vessel containing 600 µL of ultrapure hydrofluoric acid and 4.4 mL of ultrapure nitric acid. Each sample was microwave digested at 400 W and 120°C for 110 min. After digestion each sample was transferred to a 15 mL teflon bomb and placed on a ventilated hot plate (~80°C) and the acid was evaporated. Each digest was

dried and re-dissolved in 1 mL of ultrapure nitric acid and 0.833 mL of ultrapure hydrochloric acid, diluted with milli-q water and additional HCl was added to bring the total HCl in each sample to 7% (by vol) before analysis by CVAA spectroscopy. A digestion blank (treated identically, but without a tissue sample) was processed with each batch of tissue samples. A sample of DORM-3 fish protein (National Research Council Canada) used as the matrix matched reference material was also processed with each batch of tissue samples as control. The aqueous samples of digested tissue were CVAA analyzed using a Cetac Quick Trace Mercury Analyzer M-6100 and mercury quantified using external calibration. External precision was assessed by comparing measured DORM-3 Hg with the assayed Hg concentration in this matrix-matched standard.

The measurement of mercury is most often carried out using either cold vapor atomic absorption or cold vapor atomic fluorescence to detect elemental mercury vapor following the reduction of inorganic mercury using tin (II) chloride or other reducing agent (Allibone, Fatemian, and Walker 1999). This technique was originally developed by Thomson and Gooden (1975) and was further refined by others (Godden and Stockwell 1989).

Statistical analysis

Results from CVAA analysis of tissues, feces, and urine were statistically analyzed in several ways. Data used for hypothesis testing was first checked for outliers using the ISO recommended Grubbs test for outliers (Miller and Miller 2005) After elimination of outliers, the data were tested for normality and equal variance using the Kolmogorov–Smirnov test. Data from most experimental groups were not normally distributed. Therefore, the Kruskal–Wallis test (nonparametric *F*-test) were used to determine statistical significance of differences in mean [Hg]. Multiple comparisons were done using pair-wise comparisons using Mann–Whitney rank sums tests and a Bonferroni correction to determine if the multiple comparison tests were significant. Data from Group 4 ("NBMI Only") was normally distributed so a one-way ANOVA was used to analyze those data.

Behavioral observations were recorded for all groups

Acute mercury exposure studies

To determine if NBMI could rescue rats from acute exposures to mercury chloride 36 Sprague-Dawley young male rats (225–245 g weight) were divided into three groups of 12 and fed commercial rat chow and water *ad libitum*. Before all experiments the rats were kept in humane temperature controlled housing, well fed (PHS-assured housing), and at the end of the experiments were euthanized (IACUC of the University of Kentucky). Three groups of 12 were injected subcutaneously under the abdominal skin on the right side with levels of 1.0, 2.0, and 14 mg $HgCl_2$ kg^{-1} body weight dissolved in isotonic 25%DMSO : 75% PBS, respectively. Groups getting 1 and 2 mg $HgCl_2$ were split in half and 6 rats were given a subcutaneous injection of a 528 micromoles of NBMI a 150- and 72-fold excess of NBMI over $HgCl_2$ on a molar basis dissolved in isotonic 25%DMSO : 75% PBS. The rats injected with 14 mg $HgCl_2$ were treated with 880 micromoles of NBMI, a 17.1-fold excess. Injection of NBMI was under the skin of the left

abdominal area 20–25 min after the injection of $HgCl_2$. Rats were weighed daily, observed for ataxia, and any abnormal behavior as well as tremors, convulsions, and death.

Results

Toxicological studies using NBMI

In a preliminary study to determine relative toxicity of NBMI three 2-month-old male Sprague Dawley rats (average 234 g weight) were initially injected subcutaneously in the abdominal area at levels of 0, 100, 200, and 300 μmoles NBMI kg^{-1} body weight, or 0, 3.07, 6.1, and 9.2 mg NBMI kg^{-1} body weight, respectively. Two days later no weight loss, ataxia, apatite suppression, diarrhea, or tremors were noted, so additional injections were done on the same animals as noted in Table 1. None of the test animals showed any effects that would indicate toxicity and the experiment was terminated 3 days after the final dose of 1500 μmoles NBMI kg^{-1} body weight or 426 mg NBMI kg^{-1} body weight in two of the animals. The total exposure to NBMI in the 10-day experiment was 0, 600, 2400, and 2700 μmoles (or 0, 170, 682, and 767 mg) kg^{-1} body weight. The three rats, injected for four times each were kept for an additional 2 weeks for observation and weighing. No signs of weight loss or toxicity appeared and this was considered enough lack of toxicity for NBMI to proceed to the oral dosage testing.

LD-50 determination for oral NBMI

To determine an LD-50 of NBMI, three female Wistar rats of 206–208 g body weight were gavaged with NBMI suspended in corn oil up to 5 g NBMI kg^{-1} body weight. To get 5 g NBMI kg^{-1} body weight, the rats had to be gavaged thrice daily. However, the net effect was no death or other signs of toxicity with the exception of some diarrhea. The LD-50 for NBMI was determined to be in excess of 5 g kg^{-1} body weight. This lack of toxicity was used to determine the maximum level of 1.0 g kg^{-1} body weight in the extended daily dosage toxicity study using 10 male and 10 female rats per each of four dosage groups.

Extended daily dose toxicity study of NBMI

To determine any toxicity with the extended use of NBMI, a 28-day repeated dose of 0, 0.1, 0.5, and 1.0 g NBMI kg^{-1} body weight day^{-1} given by gavage suspended in corn oil was assessed. The study was done on male and female Wistar rats with 10 male and 10 female in each group. No deaths or abnormal weight loss, ataxia, loss of appetite, was caused by NBMI at these levels in the 80 rats used in this study. While some staining of rear extremities occurred in some animals, there was no general diarrhea observed. Therefore, the diarrhea observed in this study at 5 g NBMI kg^{-1} body weight was attributed to the three doses of corn oil needed to deliver such a large amount of NBMI. Organs were evaluated by microscopic and histological analysis (data not shown). None of their organs displayed changes indicating toxic effects. However, some mild to moderate B-cell proliferation was observed in the spleens indicating a low-level immune system stimulation.

Clinical chemistry and hematology of 28-day toxicity study

Since all the chemical and hemotological parameters remained in the normal range, only those of concern due to the chelation nature of NBMI are presented in this research report. Blood was collected and serum was analyzed for Ca^{2+}, Cl^-, Mg^{2+}, K^+, Na^+, and no abnormalities were observed (Table 2). Analysis of red blood cell count, hematocrit, and hemoglobin levels indicated that NBMI did not deplete Fe and there were no indications of low zinc or copper levels. Copper depletion is known to cause anemia, leucopenia, and neutropeania, so these parameters were evaluated and found normal. Zinc depletion is accompanied by diarrhea which did not appear in these animals. However, blood metals were only measured at the end of the 28-day study and a future detailed analysis of thiol binding essential metals will be needed to confirm any direct effect of NBMI on thiol-attracted metals like Zn^{2+} and Cu^{2+}.

Hydroxyl free radical scavenging study and metabolic profiling of NBMI

The antioxidant effects of NBMI was demonstrated by its Trolox equivalency ORAC and HORAC scores of 199,000 and 299,000 per 100 g, respectively. Additionally, metabolic profiling of NBMI incubated with human and rat liver microsomes detected only two

Table 2. Clinical chemistry and hemotology of rats at the end of the 28-day daily dosing toxicity study with three different levels of NBMI.

NBMI Levels (mg kg^{-1})		Na$^+$ (meq L^{-1})	K$^+$ (meq L^{-1})	Cl$^-$ (meq L^{-1})	Mg^{2+} (meq L^{-1})	Ca^{2+} (mg DL^{-1})	Phosphorus (mg DL^{-1})	HGB (gm DL^{-1})	HCT (%)	RBC (mill UL^{-1})
Males										
0	Mean	148	5.28	111	2.51	10.55	8.13	15.7	46.7	8.6
	SD	1.7	0.24	3.7	0.15	0.3	0.89	0.9	2.7	0.5
	n	10	10	10	10	10	10	10	10	10
100	Mean	143	4.83	105	2.58	10.64	8.23	15.6	46.2	8.7
	SD	1.4	0.33	4	0.12	0.3	0.48	0.4	1.2	0.3
	n	10	10	10	10	10	10	10	10	10
500	Mean	144	5.03	104	2.62	10.6	8.42	15.2	45.1	8.3
	SD	1.4	0.31	3.5	0.17	0.4	0.82	0.8	2.2	0.4
	n	9	9	9	9	9	9	9	9	9
1000	Mean	143	5.15	106	2.56	10.53	8.84	15.5	45.5	8.5
	SD	3.9	0.45	4.6	0.12	0.6	0.91	0.6	1.9	0.4
	n	10	10	10	10	10	10	10	10	10
Females										
0	Mean	143	4.94	107	2.68	10.7	7.98	14.6	42.3	7.9
	SD	2.1	0.46	4.1	0.19	0.2	1.1	0.7	2.1	0.3
	n	10	10	10	10	10	10	9	9	9
100	Mean	142	4.81	103	2.64	10.78	7.82	15.2	43.7	8.0
	SD	1.4	0.28	2.3	0.11	0.3	0.59	0.7	2.4	0.4
	n	10	10	10	10	10	10	9	9	9
500	Mean	143	5.08	105	2.72	10.89	8.15	15	42	8.0
	SD	1.4	0.73	3.4	0.2	0.3	0.62	0.5	1.4	0.3
	n	9	9	9	9	9	9	7	7	7
1000	Mean	144	4.89	105	2.82	10.84	8.49	14	40	7.6
	SD	1.5	0.28	4.9	0.25	0.3	1.07	1.6	5.1	0.9
	n	10	10	10	10	10	10	9	9	9

NBMI modified structures. These were further identified by fragmentation mass spectrometry as NBMI with 2 and 3 oxygen atoms attached to a single thiol group.

Plasma clearance of NBMI

After intravenous dosing of $1\,mg\,kg^{-1}$, the average plasma half-life ($t_{1/2}$) for NBMI was determined to be $6.18 \pm 0.98\,h$ with an average plasma clearance rate of $1.01 \pm 0.22\,L\,h^{-1}\,kg^{-1}$. The intravenous dosing showed NBMI had approximately 30% of the liver blood flow of a typical rat ($3.3\,L\,h^{-1}\,kg^{-1}$). The average volume of the distribution of NBMI was determined to be $6.7 \pm 1.0\,L\,kg^{-1}$, which is greater than the total body water of a typical rat ($0.7\,L\,kg^{-1}$), suggesting extensive distribution throughout the body tissues. The data for the plasma clearance is shown in Table 3.

Oral absorption of NBMI

The oral absorption of NBMI was evaluated in 12 male Sprague-Dawley rats per group with three rats sacrificed for each hourly time point NBMI levels were determined. The pharmacokinetic profile of NBMI following oral doses of 7 or $35\,mg\,kg^{-1}$ was similar. Using the same technique used in the plasma clearance study above, the plasma NBMI levels were determined at the same times after oral gavage of the NBMI. Average half-lives ($5.47 \pm 1.75\,h$ and $5.60 \pm 1.02\,h$) and average t_{max} values ($2.3 \pm 1.2\,h$ for both doses) were similar. The amount of NBMI absorbed into the plasma from the oral doses was estimated by the comparative level observed in the above intravenous study. The absorbance of NBMI was also consistent between the two oral doses with average values of $13 \pm 5.9\%$ and $15 \pm 3.6\%$ for the 7 and $35\,mg\,kg^{-1}$ doses, respectively.

Table 3. Individual and average plasma concentrations ($ng\,mL^{-1}$) and pharmacokinetic parameters for NBMI after intravenous administration in male Sprague-Dawley rats at $1\,mg\,kg^{-1}$.

Rat # Time (h)	1	2	3	Mean	SD
0	BLOQ	BLOQ	BLOQ	ND	ND
0.083	522	535	423	493	61.3
0.25	354	320	254	309	50.8
0.5	259	228	203	230	28.1
1	184	155	124	154	30
3	100	84.1	63.1	82.4	18.5
6	39.4	32.7	28.9	33.7	5.32
24	6.6	8.84	3.7	6.38	2.58
C_o ($ng\,mL^{-1}$)	633	691	545	623	73.4
$t_{1/2}$ (h)	5.83	7.28	5.42	6.18	0.98
CL ($L\,h^{-1}\,kg^{-1}$)	0.86	0.91	1.26	1.01	0.22
Vss	5.5	7.2	7.3	6.7	1

Note: BLOQ: Below level of quantification; ND: not determined; C_o: Maximum plasma concentration extrapolated to $t = 0$; $t_{1/2}$: half-life; CL: clearance; Vss: Steady state volume of distribution.

Table 4. Biodistribution of NBMI with time after oral gavage.

| Time (h) | OSR #l plasma (ng mL^{-1}) and tissue (ng g^{-1}) biodistribution vs time, average with standard deviation | | | | | | | |
	Plasma	Brain	Kidney	Liver	Spleen	Bone marrow	Small intestine	Subcutaneous fat
1	588 ± 90	6.1 ± 2.6	61.3 ± 42.7	165.4 ± 143.5	12.1 ± 5.2	12.5 ± 7.4	7432 ± 3201	419.7 ± 418.6
2	883 ± 216	51.8 ± 43.9	144.9 ± 89.9	271.7 ± 274	152.8 ± 128.6	65.7 ± 51.6	8533 ± 5320	683.2 ± 530.6
8	391 ± 14	2.57 ± 0.34	45.1 ± 3.4	23.3 ± 2.2	16.8 ± 1.5	6.88 ± 0.52	1557 ± 82S	17.3 ± 7.4
24	118 ± 29	2.09 ± 0.41	36.9 ± 3.7	14.2 ± 2.3	12.7 ± 2.7	3.76 ± 0.77	1045 ± 396	7.0 ± 0.7

Notes: NBMI suspended in corn oil was given by gavage at zero time. Three animals were used per time point. Conditions were as given in methods.

Pharmacokinetics of NBMI

The pharmacokinetic properties of NBMI were evaluated after oral gavage and showed the highest concentration of NBMI to occur at the 2 h post ingestion time point in plasma and all tissues. The study concluded that the bioavailability of NBMI, evaluated in male Sprague-Dawley rats after an intravenous dose of 1 mg kg^{-1} and oral doses of 7 and 35 mg kg^{-1} was significant. The levels of NBMI in organs decreased from highest in the small intestines > subcutaneous fat > liver > kidney > spleen = bone marrow > brain 2 h after administration. Twenty four hours after administration, the order was: small intestines > kidney > liver > spleen > subcutaneous fat > bone marrow > brain. The data for this evaluation is shown in Table 4. The amount of NBMI remaining after 24 h was about 13.4% of that retained after 2 h. All organs showed this significant drop in NBMI levels between 2 and 24 h post-ingestion.

NBMI reduces clinical observed toxic effects on rats used in organ-specific accumulation of mercury determination

The most significant observation of the acute mercury exposure study was the comparison between Group 3 ("Hg only") and the Group 4 (Hg + NBMI) treatment groups. All animals in Group 3 showed symptoms of significant discomfort by the third day after Hg injection. Animals were lethargic, did not exhibit normal grooming behaviors, and showed reduced food intake. Observers were not blinded to the animals. However, the only two animals that died early in the entire study were in Group 3. Within 3 days of injection blood was visible in the urine of the Group 3 animals which were also showing discomfort. The amount of blood appeared to increase during the collection period. Blood in the urine is symptomatic of acute kidney failure. These animals also exhibited significant amounts of blood in their feces. These symptoms were expected, kidney failure is the most severe symptom of acute mercury chloride poisoning.

In contrast to the toxic effects seen in Group 3, the animals in Group 4 ("Hg + NBMI") that received the same HgCl$_2$ dose as those in the Hg Only group exhibited no signs of discomfort. They remained active and alert, continued normal grooming behaviors and showed no decrease in food consumption. On a few occasions some of these animals did have traces of blood in their urine, but this was inconsistent and, unlike animals in the "Hg Only" group, the amount of blood in their urine did not increase during the collection period.

Table 5. Body and organ weights of test animals after subacute mercury exposure $\pm$ NBMI.

	Body (*g*, mean $\pm$ SD)		Organ mass (post treatment, *g*, mean $\pm$ SD)		
	Pretreatment	Post treatment	Liver	Kidney	Brain
Group 1	397.3 ± 30.9	398.0 ± 26.7	15.4 ± 5.4	3.10 ± 0.28	1.96 ± 0.24
Group 2	389.3 ± 18.9	374.4 ± 27.7	11.7 ± 1.8	2.80 ± 0.56	1.85 ± 0.04
Group 3	389.5 ± 20.5	378.0 ± 33.8	11.3 ± 1.3	3.56 ± 0.66	1.84 ± 0.14
Group 4	396.0 ± 18.9	356.9 ± 46.8	11.0 ± 2.1	3.88 ± 0.69	1.88 ± 0.10

Notes: Average mass of animals and harvested organs in each treatment group. Animals were weighed before injection and again before sacrifice. Organs were removed, blotted dried and weighed before freezing in liquid nitrogen. Groups 1, 2, 3 and 4 represent "unexposed," "NBMI only," "$HgCl_2$ only" and "$HgCl_2$ + NBMI" as described in the Section "Methods."

Weight gain was determined for all animals. Three of the groups lost body mass during the 5-day course of the study. Changes in the weights of the liver, kidney, and brain occurred similar to body mass loss but none of the changes were statistically significant (Table 5).

Mercury accumulation in organs

Mercury accumulation was determined in the organs of all animals. Tissue samples were analyzed using CVAA, after appropriate digestion and dilution, were analyzed to determine if there were tissue-specific differences in mercury levels within each treatment group. Tissues tested were the brain, liver, kidney, EP and IP fat tissues. The masses of IP and EP fat are not shown because these tissues were sampled, the entire amount of fat was not removed.

Within group comparisons

Mean Hg levels for all groups and organs are shown in Figure 2. The most obvious result is that NBMI did not change the organ mercury distribution ratios in any group with the possible exception of the mercury levels in EP and IP fat tissues of the "Unexposed" versus "NBMI only" animals.

Group 1 "Unexposed" animals: Mean mercury levels within this treatment group were the highest in the kidney and then the liver. As expected, mercury levels in this group were very low (less than 200 ppb) in all organs. This shows that unexposed animals had a detectable, but low and uniformly distributed Hg burden.

Group 2 "NBMI Only" animals: Data from this group was similar in Hg levels to the "Unexposed" animals, so one way ANOVA was used to compare means. Mean mercury levels in tissue from the "NBMI Only" treatment group were also highest in liver and kidney tissue (Figure 2). NBMI exposure seemed to increase brain, liver, and EP fat Hg levels and decrease IP fat Hg levels. Due to the low number of rats used in this experiment, additional studies need to be done to further establish significance.

Group 3 "Hg Only" animals: Mean Hg levels in animals of this treatment group were the highest in the kidney and liver and very similar to the "Hg + NBMI" group. Hg levels were higher than in the "Unexposed" and "NBMI Only" groups (Figure 2). A Mann–Whitney

Figure 2. Organ mercury levels after subacute mercury exposure ±NBMI.
Notes: Hg levels in each organ was measured 5 days after $HgCl_2$ injection and/or treatment with NBMI. Animals in the "Unexposed" group were treated with saline only. After elimination of outliers in-group comparisons were made using the Kruskal–Wallis test (nonparametric F-test) or one-way ANOVA (group 2 only) to determine statistical significance of differences in mean Hg levels. Between-group comparisons were made using pairwise comparisons using the Mann–Whitney rank sums tests and a Bonferroni correction. # Mean Hg levels in these organs were significantly higher in "Hg Only" or "Hg and NBMI" groups than in that organ other groups ($p < 0.0001$). *Mean Hg level in the "Hg only" group was not significantly different from the Hg levels in the "Hg + NBMI" group. It was significantly higher than mean Hg levels in other groups ($p < 0.001$). Mean Hg level in the "Hg + NBMI" group was greater than in the "Unexposed" and "NBMI Only" groups.

pair-wise comparison with a Bonferroni correction of Hg levels within each organ showed that the liver and kidney contained higher Hg than other organs in this group ($p < 0.001$). *Group 4 "Hg + NBMI" animals*: Mean Hg levels in kidney and liver were similar to those of the "Hg Only" group and higher than in other tissues from animals in this group. These results suggest that the single dose of NBMI did not affect distribution, accumulation, or the excretion of Hg within 5 days.

Between group comparisons

The most significant finding was that NBMI treatment did not reduce the mercury levels in the organs of rats treated with NBMI. As expected, the rats receiving mercury injections had higher levels of mercury than those not receiving mercury injections. Exposure to NBMI in both the unexposed rats and mercury exposed rats seemed to have no major effect on organ Hg accumulation. Minor changes were noted, such as the mercury levels in the EP and IP fat tissues of the unexposed and NBMI only treated rats (Figure 2), but further study would be needed to validate these observed changes.

Brain: Mean Hg concentration in brain tissue was highest in the Group 3 ("Hg Only") animals (Figure 2). A Mann–Whitney pair-wise comparison with a Bonferroni correction used to compare brain Hg levels from each treatment group showed brain Hg levels from animals in the "Hg Only" group were not statistically different from animals in the "Hg + NBMI" group. Brain Hg levels from Group 3 animals were different from those in the "Unexposed" and "NBMI Only" groups ($p < 0.0001$). Brain Hg levels in animals of the "NBMI Only" groups appeared higher than in the "Unexposed" group. However, these were small differences and it would take additional studies to confirm if they were significant.

Liver: Mean liver Hg levels were highest in rats of the "Hg Only" group. Comparing treatment groups mean liver Hg levels in animals of the "Hg Only" and "Hg + NBMI" groups were not significantly different from each other but were significantly different from those in the other 2 groups ($p < 0.0001$). Liver Hg levels in animals of the "Control" and "NBMI Only" groups appeared slightly higher in the NBMI group.

Kidney: Mean kidney Hg levels were highest in animals of the "Hg Only" group. Applying the Mann–Whitney pair-wise comparison with a Bonferroni correction showed that kidney Hg levels in animals of the "Hg Only" group were not statistically different from that measured in animals from the "Hg + NBMI" group. However, kidney Hg levels in animals from these groups were significantly different from those measured in animals of the "Unexposed" and "NBMI Only" groups ($p < 0.0001$). Kidney Hg levels in animals of the "Unexposed" and "NBMI Only" groups were not significantly different.

Intraperotineal fat: Normality testing of this dataset showed that it was very close to being normally distributed (p-value $= 0.049$), therefore, normality was assumed for hypothesis testing and a one-way ANOVA followed by pair wise comparisons was used. This revealed that Hg levels were higher in animals of the "Hg only" and "Hg + NBMI" groups than in the other two groups ($p = 0.001$) but were not significantly different from each other. IP fat Hg levels in animals of the "Control" and "NBMI Only" groups appeared significantly lower in the NBMI treated. It was observed that the ratios of IP/EP fat Hg levels were quite different in the "Unexposed" versus the "NBMI only" groups with an increase in the EP and a decrease in the IP Hg levels with NBMI treatment, respectively. The similar ratio change occurred in the "Hg only" versus "Hg + NBMI" but the differences were not significant.

Epididymal fat: Mean Hg concentration in EP fat tissue was the slightly higher in animals of the "Hg + NBMI" group. Comparing EP fat Hg levels in animals of the four treatment groups with a one way ANOVA followed by pair wise comparisons showed that Hg levels were higher in animals of the "Hg Only" and Hg + NBMI" groups than in animals of the other groups ($p = 0.031$). Hg levels in animals of the "NMBI" only group was slightly higher than in the "Unexposed" group. Comparing Group 1 to Group 2 and in comparing Group 3 to Group 4, it was consistent that NBMI seemed to slightly elevate Hg levels in the EP tissue while slightly decreasing it in the IP fat. Statistical confirmation of this observation would require further study.

Mercury excretion patterns

Hg level data from all feces and urine samples were hypothesis tested using the nonparametric Kruskal–Wallis test and are shown in Table 6. Mercury excretion by animals of the "Hg Only" and "Hg + NBMI" groups were significantly greater than by the animals of the other two groups ($p = 0.0001$) as expected. But the difference between

Table 6. Urine and feces excretion of mercury from rats after subacute $HgCl_2$ exposure $\pm$ NBMI.

	Unexposed Ppb	Hg only Ppb	Hg and NBMI Ppb	NBMI only Ppb
Urine	0.72 ± 0.2	392 ± 70.6*	242 ± 52.7*	0.30 ± 0.1
Feces	132 ± 66.8	$31,418 \pm 5906$*	$23,447 \pm 4877$*	110 ± 30

Notes: Mean mercury concentration measured in urine and feces collected during the 5-day exposure period. Values are expressed as mean (in parts per billion) $\pm$ SEM.
*Indicates [Hg] is statistically different from that measured in other groups, but are indistinguishable from each other.

excretion by animals of the "Hg Only" and the "Hg + NBMI" groups were not significant. The feces excretion in all comparisons was much higher than the urinary excretion.
Urine: As expected, excretion rates by animals in the "Unexposed" and "NBMI only" groups were very low. Although excretion by animals in the "NBMI Only" group was lower than from those in the "Unexposed" group, the difference was not significant. Mean urine Hg levels were highest in animals from the "Hg Only" treatment group (Table 6). Urine Hg levels from animals in the "Hg only" and Hg + NBMI groups were not statistically different. Excretion from animals in the "Unexposed" and "NBMI only" groups were not statistically different.
Feces: Results from the analysis of feces were similar to those from urine analysis. As expected, feces contained much higher Hg levels than urine, but the same trends were observed (Table 6). NBMI seemed to decrease the total Hg being excreted in acutely Hg exposed rats, but the rate was not statistically different from the "Hg Only" rats.

Acute mercury survival with NBMI treatment

The daily percent survival of young male rats treated with lethal doses of $HgCl_2$, plus and minus NBMI treatment, are shown in Table 7. Each mercury dose level group contained 12 rats, with six serving as controls and six receiving a single dose of NBMI. Young rats were selected as they are more susceptible to inorganic mercury toxicity and indeed did display a higher rate of death than the older rats used in the organ mercury retention experiments. The rats given 1 and $2\,mg\,kg^{-1}$ body weight and NBMI treatment all survived, whereas those without NBMI did not survive.

At the highest mercury exposure level ($14\,mg\,kg^{-1}$ body weight), the single-dose treatment of NBMI was only able to save four of six of the rats. One-third of the rats given the highest lethal dose of mercury chloride plus NBMI died. However, they survived significantly longer than those without NBMI treatment. The surviving 16 rats from all groups were kept under humane conditions and observed for 3 months, then euthanized. No adverse effects were noted in these rats and they remained healthy and active with no apparent signs of illness.

Discussion

The preliminary evaluation of NBMI's possible toxicity was done in a 10 day plus testing period where from 100 up to 2700 micromoles or $767\,mg\,kg^{-1}$ body weight of NBMI were subcutaneously injected (Table 1) and had no discernable toxic effects on rats weighing

Table 7. Survival of young rats with and without NBMI treatment after subcutaneous injection of lethal doses of mercury chloride.

HgCl$_2$	Percent survival						
	0 h	6 h	12 h	24 h	48 h	72 h	168 h
NBMI treatment							
1 mg kg^{-1a}	100	100	100	100	100	100	100
2 mg kg^{-1a}	100	100	100	100	100	100	100
14 mg kg^{-1b}	100	100	100	100	67	67	67
No treatment							
1 mg kg^{-1a}	100	100	100	100	100	50	0
2 mg kg^{-1a}	100	100	67	17	0	0	0
14 mg kg^{-1b}	100	50	0	0	0	0	0

Notes: Each group consisted of 6 rats that were first injected subcutaneously with the indicated amount of mercury chloride dissolve in PBS under the right side of the stomach area. After 20–25 min an injection of NBMI dissolved in isotonic DMSO/PBS (25/75 ratio), was injected subcutaneously under the left side of the stomach area. 1, 2, and 14 mg of HgCl$_2$ are equivalent to 3.5, 7.3, and 51.4 micromoles of Hg^{2+} kg^{-1} body weight, respectively.
[a]NBMI treatment subjects received 528 micromoles or 150 mg NBMI kg^{-1} body weight.
[b]NBMI treatment subjects received 880 micromoles or 250 mg NBMI kg^{-1} body weight. The NBMI/HgCl$_2$ molar ratios were 150, 72, and 17.1 for the 1, 2, and 14 mg HgCl$_2$ groups, respectively.

about 234 g, up to 7 days after the last injection. This implies that NBMI can be injected subcutaneously at high doses for multiple days without significant toxic effects occurring. Additionally, NBMI was found non-mutagenic by an Ames analysis, whose purpose was to evaluate the potential of NBMI to induce the reversion of mutations in the histidine operon of *Salmonella typhimurium* strains TA1537, TA98, TA100, and TA1535 and the tryptophan operon of *Escherichia coli* strain WP2 *uvr*A. However, longer term animal studies would be needed to confirm the total lack of mutagenicity. NBMI was prepared to 1 mg mL^{-1} and tested undiluted to 1 : 512 dilution and found to contain less than one endotoxin unit (EU) mg^{-1}.

An oral toxicity study with NBMI to attempt the determination of an LD-50 could not reach a level of NBMI that would cause death or illness in test animals. Therefore, the LD-50 of NBMI was reported to be greater than 5 g kg^{-1} body weight. This implies a lack of significant toxicity from a single daily oral dose. This observation on oral delivered NBMI is supported by the data in Table 1 where NBMI was injected subcutaneously and did not cause any observable toxic effect. Diarrhea was observed in the oral toxicty study where a maximum of 5 g NBMI kg^{-1} body weight day^{-1} was given. Due to the volume of NBMI suspended in corn oil, it had to be gavaged in a multiple of three doses to deliver 5 g. This resulted in increased corn oil ingestion which likely caused the diarrhea as this factor disappeared in the 28 day daily oral toxicity study.

The safety of extended use of NBMI is supported by the 28-day oral toxicity study using NBMI at up to 1000 mg kg^{-1} body weight daily for 28 days. None of the rats in this 80 rat study died due to NBMI exposures and they maintained their weight throughout the 28 days. This study did not uncover any toxic effect, organ weight loss, or abnormality on microscopic evaluation. The only observed effect was a mild to moderate increase in B-cell

proliferation in the spleen of some of the test animals. This was reported not to be indicative of toxicity but as a result of immune system stimulation by antioxidant activity, which is also caused by cocoa (Ramiro-Puig, Perez-Cano, et al. 2007; Ramiro-Puig, Urpi-Sarda, et al. 2007). The instances of this B-cell proliferation increased with the higher levels of NBMI from 100 to 500 to 1000 mg kg^{-1} body weight, with only minimal effects seen at the lowest dose and, at worst, some moderate effects at the highest dose.

Also, the use of NBMI to study its effects on mercury toxicity on aortic endothelial membranes in culture has shown no toxicity on membrane integrity and to be a very effective prevention of the damage induced by mercury exposure (Secor et al. 2011). However, these studies do not eliminate the possible toxic effects that could occur with much longer exposures to ingested NBMI and this will require further long-term studies.

At the end of 28 days of high-level NBMI (1000 mg kg^{-1} body weight) exposure, there was no depletion of the tested essential metal ions needed for health (Table 2). No significant depletion was seen in Na$^+$, K$^+$, Ca^{2+}, or Mg^{2+}. Phosphate (phosphorous) levels also remained unchanged. Iron binding proteins appeared unchanged as the hematocrit percentage and hemoglobin levels remained unchanged with increasing NBMI. Also, red blood cell levels remained unchanged. While the mechanism(s) underlying the depletion of essential metals by chelation are not well described, they are thought be related to electrostatic factors, rapid kidney clearance into the urine, as well as attraction to thiol groups. It is reasonable to assume that a hydrophobic, uncharged chelator like NBMI would not have high affinity for any metal based on ionic attraction. Thus, it is not surprising that Na$^+$, K$^+$, Ca^{2+}, and Mg^{2+} levels were not negatively affected by NBMI as they might be by high levels of other charged compounds, e.g., DMPS and DMSA used for chelation of lead and mercury. These compounds carry negative charges and have an ionic attraction for all positively charged metal ions in addition to their thiol interacting sites, remain mostly in the blood, and are efficiently cleared by the kidneys. In contrast, interaction between metals and NBMI would be predominantly through binding to the terminal thiol groups.

It is appropriate to be concerned that NBMI could result in Zn^{2+}, Cu^{2+}, and Fe^{2+} depletion as these metals could interact with the thiol groups. Previous research has shown that at pH 4 and 6 both Cu^{2+} and Fe^{2+} are effectively chelated by NBMI (Atwood, Howerton, and Matlock 2003). However, lowering of hemoglobin and hematocrit was not observed in the 28-day study using oral delivery of NBMI at 1.0 g kg^{-1} body weight demonstrating that Fe^{2+} was not depleted (Table 2). Fe^{2+} has higher affinity for thiol containing compounds like NBMI than would Cu^{2+} or Zn^{2+} and the lack of depletion of Fe^{2+} binding proteins supports the observation of no toxic effects associated with depleted Cu^{2+} or Zn^{2+}. Most likely, the preponderance of these metals are tightly bound by specific tight binding carrier and storage proteins that are mostly located in the hydrophilic environment of the serum and cytoplasm and not available to interact with NBMI located in hydrophobic areas. The removal of these metals by hydrophobic NBMI would depend on a complex competition for binding that does not seem to occur. Experimentally, the 28-day study on the effects of large daily NBMI doses suggests that NBMI does not effectively compete with the native binding moieties for these essential metals. However, the only way to verify this would be by detailed experiments to determine the fate of these metals in the presence of NBMI by extended fecal and urine analysis.

The presence of two thiols on NBMI indicates the ability to scavenge hydroxyl free radicals. As observed by the reported Oxygen Radical Absorbance Capacity (ORAC) scores, NBMI may be exceptional in this regard as nothing is currently available in oral form that has ORAC and HORAC scores in the 199,000 to 299,000 Trolox units 100 g^{-1}.

The ORAC and HORAC scores of many food particles can be found in government sponsored data (U.S. Department of Agriculture 2009, www.ars.usda.gov/nutrientdata). For example, an acia fruit, pulp, and skin powder has a HORAC of 99,700 and blackberries, blueberries, and cherries have HORAC scores of 5802, 4633, and 3730, respectively. The HORAC and ORAC scores are not fail proof indicators of the ability of a compound to scavenge hydroxyl free radicals *in situ*. Many compounds with antioxidant properties do not work effectively to treat oxidative stress and this is most likely due to rapid blood clearance and the inability to penetrate cell membranes to get where the oxidative stress is occurring chemically.

The metabolic profiling of NBMI determined that the major metabolites of NBMI after incubation with rat and human liver microsomes was NBMI with two or three oxygen atoms attached to one of the thiol groups. This supports the concept that NBMI can scavenge hydroxyl free radicals $(OH^•)$ on a 3 to 1 molecular basis and this may account for the high ORAC scores. Therefore, NBMI may also serve as a treatment for oxidative stress and this may add to NBMI's ability to ameliorate mercury toxicity as oxidative stress is a symptom known to accompany this toxicity.

The results of the oral bioavailability and pharmacokinetics studies indicate that NBMI penetrates the cells of several critical tissues and is effectively excreted (Table 4). NBMI delivered intravenously was used to determine the plasma half-life to be 6.18 ± 0.98 h. The absorption of NBMI was found to be $13 \pm 5.9\%$ and $15 \pm 3.6\%$ for 7 and 35 mg NBMI kg^{-1} doses, respectively. The plasma and organ NBMI levels peak at roughly 2 h post-ingestion and after 24 h only about $13-15\%$ of the peak levels of NBMI remain. This indicates that NBMI enters the cytoplasm of cells and is effectively cleared from the cells and does not build up in any of the organs tested.

The relatively efficient excretion of NBMI is in contrast to the slow excretion of the NBMI–Hg complex if one assumes that the retention with the lack of toxicity observed in the acute mercury toxic rats treated with NBMI is due to the formation of such a complex, rendering the existing tissue mercury levels non-toxic or of greatly reduced toxicity. The concept of NBMI–Hg greatly reducing the toxicity of the injected Hg^{2+} is supported by the observation in *in vitro* testing of this complex being exceptionally stable to extremes of pH and many organic solvents and high temperatures.

The observation of high Hg levels in tissues treated with Hg and NBMI without toxic effects being observed is supportive of NBMI's ability to decrease the intracellular toxic effects of mercury since the sites of binding of this metal that induce its toxic effects are mostly intracellular, as is the damage done by the increased levels of hydroxyl free radicals.

The data from the acute but sub-lethal dose mercury exposure study showed that subcutaneously injected NBMI was effective at reducing or eliminating the behavioral effects associated with acute Hg poisoning, including death as two animals in the "Hg only" group died. Also, comparison of excretion rates in feces Hg of animals from the "Hg Only" and "Hg + NBMI" groups showed that NBMI treatment did not significantly affect Hg retention time during this 5-day study (Table 6). However, NBMI induced little to no significant changes in organ-specific Hg distribution, accumulation (Figure 2), or excretion (Table 6) in rats exposed to acute $HgCl_2$ levels. Therefore, the protective effects of NBMI appear to not be directly related to increased excretion or any change in the organ distribution of mercury.

Comparing the organ Hg levels and the amount of Hg excreted in urine and feces of "Unexposed" animals and those of the "NBMI only" group suggests that NBMI alone slightly decreased Hg excretion in both the urine and feces and slightly raised the Hg levels in the brain, liver, kidney, and EP fat tissues, while decreasing the Hg levels in the IP fat.

Although this data is indicative of NBMI causing redistribution, it is not significant enough to draw a conclusion and will require further study. Especially, since it was not observed in the acutely toxic rats. In this study animals were not fed mercury-free rat chow, so NBMI may be effective at chelating mercury ingested in the diet or from previously existing internal stores that were not identified. To ascertain if these observations are significant will require further study. The body mass changes observed (Table 5) were found not significant in this study and body weight changes were not observed in the 28-day toxicity trials even at $1000\,\mathrm{mg\,kg^{-1}}$ body weight.

Because NBMI treatment administered to rats acutely exposed to toxic levels of Hg did not significantly alter Hg excretion in urine or feces, its protective mechanism seems to be based on the formation of a non-toxic complex within the animal and not on rapid excretion of the metal from the body. These data also suggest that NBMI chelates the available Hg^{2+} even when present at small amounts and that the formation of NBMI–Hg complex may even slow the excretion. However, NBMI did not significantly decrease fecal excretion within the 5-day test. Testing is underway to determine the rate of Hg excretion with NBMI treatments in exposed rats over more extended periods of time and to determine the ability of NBMI to mitigate the effects of chronic mercury exposure and its effects on exposure to more environmentally relevant amounts of Hg^{2+}. Also, the bioavailability of NBMI and chelation capacity could be improved with better formulations and delivery regimens, but identifying these conditions will require additional testing.

The formation of an NBMI–Hg complex that is non-toxic and slow to be excreted would be expected since the formation of this complex and solubility studies *in vitro* show it to be insoluble in aqueous solutions at pHs between 2 and 12 and in over 20 organic and aqueous solutions commonly used to dissolve similar precipitates. To date, we know of nothing that will dissolve NBMI–Hg precipitate *in vitro*. However, we do know that the body has the ability to remove Hg postulated to be in the NBMI–Hg complex through a process that involves excretion into the feces. Further research on gavaged NBMI–Hg complex will be required to support this hypothesis.

Mercury toxicity is very difficult to treat due to the variations in forms of the toxic mercury species from metallic (Hg^0), mercurous (Hg^{+1}), mercuric (Hg^{2+}), and the various forms of organic mercury such as methyl-mercury. Exposures to these different forms of mercury are responsible for the observed variances of organ distribution, severity, and biological effects of the toxic exposure. The most neurotoxic and difficult forms of mercury to treat are those that are most hydrophobic (e.g., methylmercury, ethylmercury, dimethylmercury, and Hg vapor) with increased ability to cross plasma membranes and the blood brain barrier. It seems plausible that to access and render mercury delivered by these hydrophobic sources less or non-toxic would require a chelator that itself was hydrophobic. The pharmacokinetics (Table 4) show that NBMI has promise to be effective in this regard.

In most cases the final major toxic form of mercury found in the affected tissues is Hg^{2+}. Metallic mercury (Hg^0) is oxidized to Hg^{2+} by catalyase in the blood (Magos, Halbach, and Clarkson 1978) and other cells and organic mercury in the brain is partially converted to mercuric mercury by deacylation (Burbacher et al. 2005). Today, there are no FDA or EMA approved drugs for treating mercury toxicity. Acute mercury toxicity requires extended medical intervention for effective treatment, e.g., chelation with hemodialysis. Drugs such as DMPS and DMSA are used but they are negatively charged and do neither effectively cross the cell membrane nor the blood brain barrier (Aposhian 1983). Use of these compounds can reduce blood mercury levels by about 20%

temporarily (Risher and Amier 2005). This indicates a much larger body storage of mercury that DMPS and DMSA cannot access. Also, these mercury binding compounds have been reported to redistribute blood mercury into the kidney and increase renal toxicity (Risher and Amier 2005).

DMPS and DMSA are not true chelators as there is not enough space between the thiols on adjacent carbons to allow one molecule of this type of compound to form two bonds to the Hg^{2+} (George et al. 2004). This causes DMPS and DMSA to bind Hg^{2+} in weaker binding ''sandwich'' complexes with two molecules of binding agent bound per mercury atom (George et al. 2004). This makes the use of these compounds susceptible to translocation of mercury from other tissues to reactive protein-thiols in the kidneys which preferentially bind the Hg^{2+} (Brandão et al. 2006; Flora and Pachauri 2010).

DMPS and DMSA mercury binding agents are negatively charged which prevents effective cell penetration keeping the agents primarily in the blood. This allows for a general attraction for all positively charged metals, even essential metals such as Ca^{2+} and Mg^{2+}, and clearance by the kidney into the urine. This can facilitate the non-specific depletion of essential metals by the relatively rapid kidney filtration process (Risher and Amier 2005). DMPS and DMSA preferentially clear mercury through the kidneys (urine) whereas NBMI preferentially clears through the liver (feces) (Table 6).

Two of the overall objectives of this research were to develop an improved method for treating mercury toxicity that reduced the problems of the lack of cell and brain permeation and the co-excretion of specific essential metals. NBMI, which is a non-charged, lipophilic, dithiol-based chelator specific for heavy metals that are attracted to –SH groups, but with no general ionic attraction to essential metals, seemed a good candidate. Especially, since the freedom of rotation and orientation of the two thiol groups allows binding of mercury one to one by NBMI in the favored $180°$ coordination geometry which results in a very stable NBMI–Hg complex that does not release the mercury in *in vitro* testing even under extremely rigorous chemical condition (George et al. 2004; Zaman et al. 2007).

Testing reported herein has shown that NBMI, given in one dose, appears to effectively reduce toxicity associated with acute exposure to Hg^{2+}. No other chelator has been reported to be able to prevent acute mercury toxicity with just one exposure to chelator. In this regard, NBMI appears to show promise for acute mercury toxicity treatment. This is most likely due to its longer plasma half-life, its ability to penetrate cell membranes, and to cross the blood brain barrier and chelate the Hg^{2+} into a complex that eliminates the bioavailability of the Hg^{2+} and essentially eliminates the toxic effects. Additionally, the antioxidant properties of NBMI would also be able to reduce the hydroxyl free radical toxicity levels immediately on entering cells suffering from oxidative stress. It is possible that the combined chelation of Hg^{2+} and the scavenging of the hydroxyl free radicals both contribute significantly to the protective effects observed with NBMI.

The contribution of the free radical scavenging to the protective effects of NBMI were not determined in this study. However, the pharmacokinetics indicates that NBMI clears the body by about 90% from peak values within 24 h ingestion (Table 4). It is unlikely that enough NBMI exists at day three or later of injection to prevent Hg-based toxicity by just scavenging hydroxyl free radicals alone. The high level of tissue retention of Hg, at known toxic levels, without detectable toxicity after NBMI treatment indicates that an NBMI–Hg complex is being formed that is of very low to no toxicity and is excreted from the body at a much slower rate than NBMI without metal chelated.

Table 7 shows that increasing the dosage of $HgCl_2$ decreased the survival time of the rats as expected. At the two lower levels of $HgCl_2$, the subsequent treatment of the

rats with excess NBMI totally prevented any death or noticeable toxic effects such as ataxia, weight loss, or loss of appetite. However, at the highest dosage of $HgCl_2$, all animals, including the NBMI-treated animals, showed immediate toxic effects. The rats given the highest dose of $HgCl_2$ and not given NBMI treatment all succumbed to death within the first half day. NBMI treatment lead to about a 67% survival in this group, and the surviving rats were kept for 3 months to ascertain their apparent total recovery.

The NBMI-treated rats that did die at the highest $HgCl_2$ dose did lose their ataxia and tremors and expired after 2 days due to undetermined causes. We do not know if multiple daily doses of NBMI would be more effective in removing mercury or preventing the deaths observed at the high $14\,mg\,kg^{-1}$ dose of $HgCl_2$. Nor have we determined the pre-treatment effects of NBMI on subsequent mercury exposure. Additional testing and different formulations for delivery of NBMI is definitely needed.

The mechanism of NBMI protection also likely involves prevention or reactivation of the enzymes known to be mercury inhibited. For example, the enzymes that are involved in the recovery of GSH from GSSG and in the initial synthesis of GSH are in this category (Ercal, Gurer-Orhan, and Aykin-Burns 2001). Also, mercury inhibition of the enzyme that removes hydrogen peroxide (glutathione peroxidase) could be reversed. This would lead to decreasing H_2O_2 levels and thereby decreasing any Fenton catalyzed production of hydroxyl free radicals by removing this precursor (Hirota et al. 1980). Further, any Fe^{2+} or Fe^{3+} freed by displacement by Hg^{2+} could also be chelated by the NBMI reducing iron cation participation in the Fenton reaction production of hydroxyl free radicals. Additional studies would be needed to confirm this.

There are many other critical enzymes that are inhibited by Hg^{2+} whose activity could be protected or recovered by NBMI thereby increasing survival. For example, the sodium-potassium transport ATPase (Anner, Moosmayer, and Imesch 1992) and the thioredoxin system (Carvalho et al. 2008) are critical to survival and are known to be inhibited by low levels of Hg^{2+}. Mercury has also been proposed to bind to the thiol group of S-adenosylmethionine, which is a cofactor for catecholoamine-O-methyl transferase, the enzyme that converts norepinephrine, epinephrine, and dopamine by methoxylation (Torres, Rai, and Hardiek 2000). This results in a clinical syndrome that resembles a pheochromocytoma crisis with malignant hypertension in acute mercury toxicity accompanied by increase in urinary catecholamines (Houston 2011). The data presented herein show that NBMI was effective in assuring the long-term survival of rats treated with lethal doses of $HgCl_2$. This strongly supports the conclusion that NBMI either implements a protection or reactivation of most of these enzymes or proteins or allows survival until they can be replaced by new synthesis. However, it will take additional experiments with purified enzymes to actually demonstrate NBMI's ability to reactivate an enzyme inhibited by hydroxyl free radicals or bound Hg^{2+}. There are other biomarkers that can be used to determine if NBMI would be effective at treating chronic mercury toxicity. First, mercury is known to lower blood glutathione levels and to cause an abnormal urinary porphyrin profile (Woods and Southern 1989; Geier et al. 2011) and the work by others demonstrates that Hg^{2+} can either activate or inhibit enzymes thought to be involved in some disease processes (Houston 2011; Secor et al. 2011)

Another mechanism for NBMI protection may be its ability to scavenge any hydroxyl free radicals produced salvaging the GSH levels and preventing GSSG production. GSSG is known to be a molecular precursor of apoptosis and decreasing its cellular level could contribute to cell survival (Franco and Cidlowski 2009). Also, many enzymes are inhibited by reactive oxygen species which may be reversed by GSH recovery. Therefore, the

elevation of GSH and the decrease in GSSG by NBMI could play a major role in NBMI protection against mercury exposure.

Finally, the results of this study support that conclusion that NBMI primarily mitigates Hg^{2+} toxicity by reducing the bioavailability of Hg^{2+} by binding it irreversibly in a stable, non-reactive NBMI–Hg complex. However, the determination of the contribution of the antioxidant effects of NBMI cannot be discounted and the relative contributions will require further studies. Also, these studies were done with single oral or subcutaneous applications of NBMI without significant research into determining the optimal formulations or multiple treatments to increase effectiveness. This research initially characterizes the effects of a single NBMI dose for the treatment of acute, high-dose exposures to inorganic Hg, and showed that it was very effective in reducing or eliminating the toxicity. However, in this study NBMI was given within 20 min of mercury chloride exposure and it may not be as effective if given at later times. Also, this study was done only on $HgCl_2$ which releases Hg^{2+}. The treatment of inhaled mercury vapor (Hg^0) or ingested or injected organic mercury compounds with NBMI may not work as well. However, NBMI has been shown to prevent the toxic effects of methylmercury in cell culture experiments (Secor et al. 2011). Based on these results, further studies to characterize the effects of NBMI treatment on chronic, low-dose exposures to inorganic and other Hg species needs to be verified by further studies.

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