

Selective Reduction of Methylsulfinyl-Containing Compounds by Mammalian MsrA Suggests a Strategy for Improved Drug Efficacy

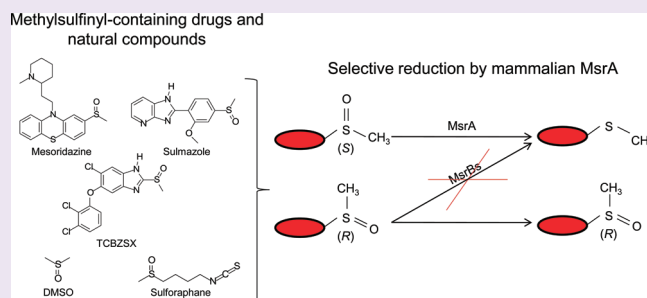
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S Supporting Information

ABSTRACT: Identification of pathways of drug metabolism provides critical information regarding efficacy and safety of these compounds. Particularly challenging cases involve stereospecific processes. We found that broad classes of compounds containing methylsulfinyl groups are reduced to methylsulfides specifically by methionine sulfoxide reductase A, which acts on the *S*-stereoisomers of methionine sulfoxides, whereas the *R*-stereoisomers of these compounds could not be efficiently reduced by any methionine sulfoxide reductase in mammals. The findings of efficient reduction of *S*-methylsulfinyls and deficiency in the reduction of *R*-methylsulfinyls by methionine sulfoxide reductases suggest strategies for improved efficacy and decreased toxicity of drugs and natural compounds containing methylsulfinyls through targeted use of their enantiomers.



Methionine (Met) contains a methylsulfide group in its side chain, which can be easily oxidized by reactive oxygen species (ROS). Met oxidation can disrupt structure and function of proteins and contribute to the development of diseases associated with accumulation of oxidatively damaged proteins.¹ To repair oxidized Met, organisms utilize methionine sulfoxide reductases (Msrs), which reduce methionine sulfoxide (Met-O) back to Met.^{2,3} Msrs are regenerated by thiol oxidoreductases, such as thioredoxin (Trx) and glutaredoxin. Trx, which is powered by NADPH-dependent thioredoxin reductase (TR), is the most common and the best characterized *in vivo* reductant, whereas dithiothreitol (DTT) is often used for the regeneration of Msrs in *in vitro* assays. These reductants were also used in our current study. Met-O is composed of two diastereomers, methionine-*S*-sulfoxide (Met-*S*-SO) and methionine-*R*-sulfoxide (Met-*R*-SO), which are subject to stereospecific reduction by MsrA and MsrB, respectively.^{2,4} An additional Msr type, free methionine-*R*-sulfoxide reductase (fRMsr), is specific for free Met-*R*-SO, although this protein is absent in animals.^{5,6} Mammals have four Msrs: MsrA, MsrB1, MsrB2, MsrB3,⁷ which account for total Msr activity in these organisms.

Like Met, many drugs and natural compounds contain methylsulfide groups, which are oxidized to methylsulfinyls upon exposure to hydrogen peroxide, hypochloric acid, and enzymes such as cytochrome p450 (Cyp450) and flavin monooxygenase (FMO).^{8–10} These methylsulfinyl-containing xenobiotics are also composed of two stereoisomers, represented by *R*- or *S*-sulfoxides.¹⁰ Cyp450, including its many isoforms, is responsible

for oxidation of carbon or sulfur atoms in drug and difference in these enzyme activities in ethnic populations is known to result in variations in drug responses. Numerous enzymes metabolizing drugs have been identified and much research on these proteins focused on the investigation of links between drug metabolism and drug efficacy. Our findings in the current study point to the existence of a stereospecific reductase system for any methylsulfinyl-containing compound in mammals.

We recently reported that mammals reduce free Met-*S*-SO but are unable to reduce free Met-*R*-SO.⁶ This is probably because MsrA reduces both protein-based and free Met-*S*-SO, whereas MsrBs can only reduce protein-based Met-*R*-SO. On the basis of this finding, we hypothesized that mammals may be generally deficient in the reduction of *R*-stereoisomers of methylsulfinyls but could reduce *S*-stereoisomers with MsrA. An indirect support for this idea was provided by the observation that the methylsulfinyl group in the anticancer compound sulindac sulfoxide could be reduced by *E. coli* MsrA, which acted only on the *S*-enantiomer of this compound.¹¹ Our new findings of stereospecific reduction of methylsulfinyls should help improve efficacy of drugs containing this functional group.

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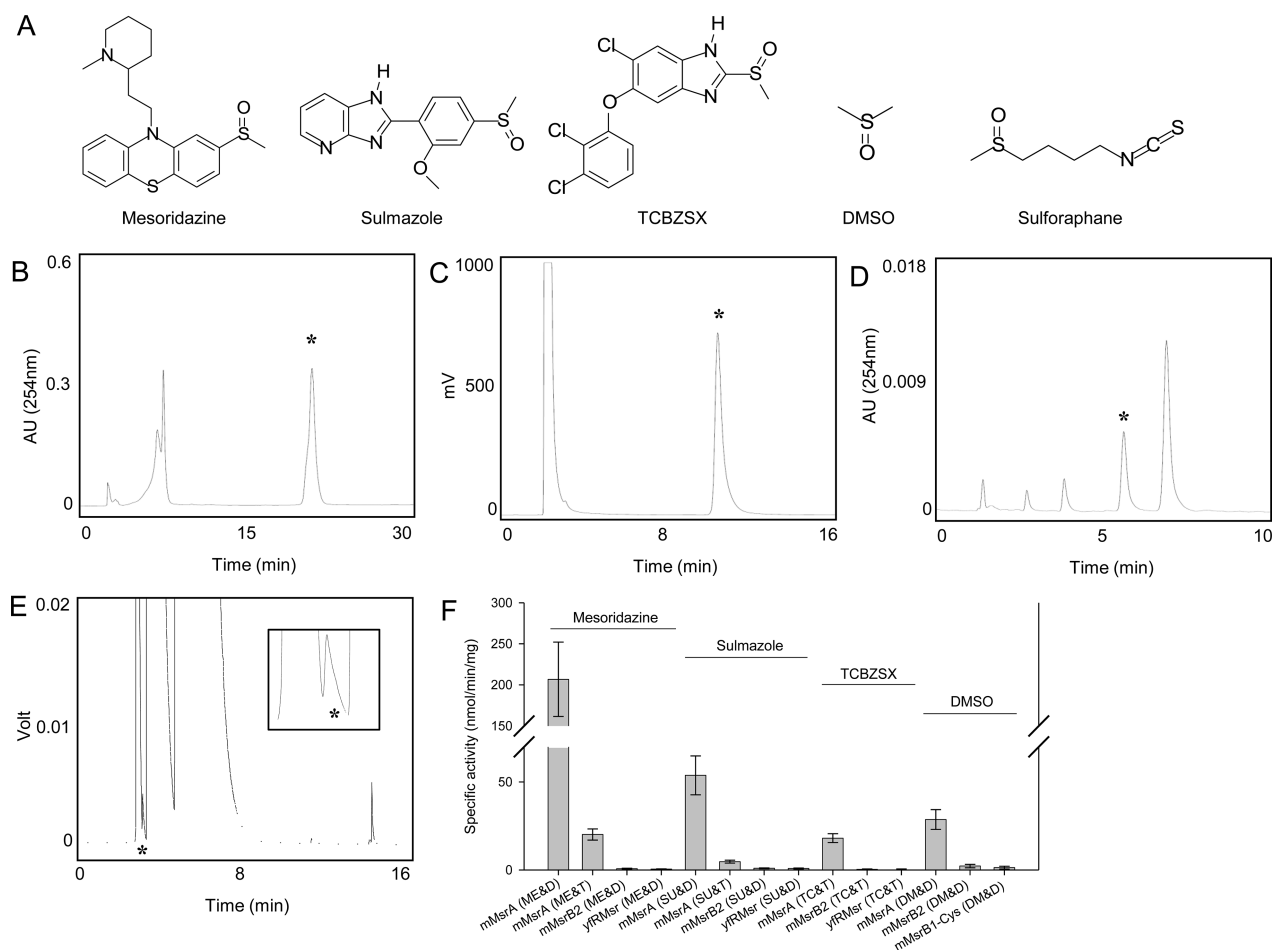


Figure 1. Msrs reduce *S*-stereoisomer but not *R*-stereoisomer forms of methylsulfinyl-containing drugs and natural compounds. (A) Structures of methylsulfinyl-containing compounds used in the study. (B) HPLC analysis of mesoridazine reduction by mMsrA using DTT as a reductant. (C) HPLC analysis of sulmazole reduction by mMsrA with DTT as a reductant. Reduced and oxidized forms were measured by following fluorescence with the excitation at 330 nm and emission at 370 nm. (D) HPLC analysis of TCBZSX reduction by mMsrA with TrxR/Trx/NADPH. (E) GC analysis of DMSO reduction by mMsrA with DTT. The peak of dimethylsulfide, the reduced form of DMSO, is enlarged in the inset. In panels B–E, peaks of original substrates are marked with closed circles, and peaks of reduced forms with stars. (F) Specific activities of mMsrA, mMsrB2, and yfRMsr for reduction of mesoridazine, sulmazole, TCBZSX, and DMSO using either DTT or TrxR/Trx/NADPH as reductants. All measurements were repeated 3 times independently. Abbreviations used are: mesoridazine (ME), sulmazole (SU), TCBZSX (TC), DMSO (DM), DTT (D), TR/Trx/NADPH (T).

RESULTS AND DISCUSSION

To investigate the hypothesis that mammalian Msrs exhibit differences in the reduction of methylsulfinyl-containing drugs and natural chemical compounds, we used mouse MsrA (mMsrA), mouse MsrB2 (mMsrB2), and yeast fRMsr (yfRMsr) (Supplementary Figure S1), which are representative of the three Msr families, for *in vitro* reduction of a diverse set of methylsulfinyl-containing compounds, including an anti-schizophrenia drug mesoridazine, a cardiotoxic drug sulmazole, an anti-fluke drug triclabendazole sulfoxide (TCBZSX), a common solvent and vehicle for application of pharmaceuticals dimethylsulfoxide (DMSO), and a natural anticancer compound sulforaphane (Figure 1A). Mesoridazine, sulmazole, and TCBZSX were used as an approximately 1:1 mixture of *R*- and *S*-enantiomers. As reductants for the regeneration of Msrs, either DTT or TR/Trx/NADPH were used. Although TR/Trx/NADPH is a physiological recycling system used *in vivo*, DTT works efficiently as a reductant in *in vitro* assays.¹²

Reduction of mesoridazine to thioridazine was tested by an HPLC analysis, which revealed that only mMsrA could reduce this compound (Figure 1B), whereas mMsrB2 and yfRMsr were inactive (Supplementary Figure S2). The specific activity of mMsrA for the reduction of mesoridazine was 206.8 ± 45.2 nmol/min/mg in the DTT-dependent assay and 20.2 ± 3.2 nmol/min/mg in the presence of TR/Trx/NADPH (Figure 1F). Sulmazole was also selectively reduced by mMsrA, but we observed no activity with either mMsrB2 or yfRMsr (Figure 1C and Supplementary Figure S3). The MsrA specific activity was 53.8 ± 4.7 nmol/min/mg in the DTT-dependent reaction and 7.1 ± 1.7 nmol/min/mg with TR/Trx/NADPH (Figure 1F). TCBZSX underwent a partial nonenzymatic reduction in the presence of DTT, but the MsrA-dependent activity was well above the background and could be reliably assayed (in the TR/Trx/NADPH-dependent assay, it was 18 ± 2.5 nmol/min/mg) (Figure 1D,F and Supplementary Figure S4), whereas other Msrs were inactive. Reduction of these two drugs by mMsrA was further confirmed by an ion-spray mass spectrometry analysis (Supplementary Figures S7, S8). Since these

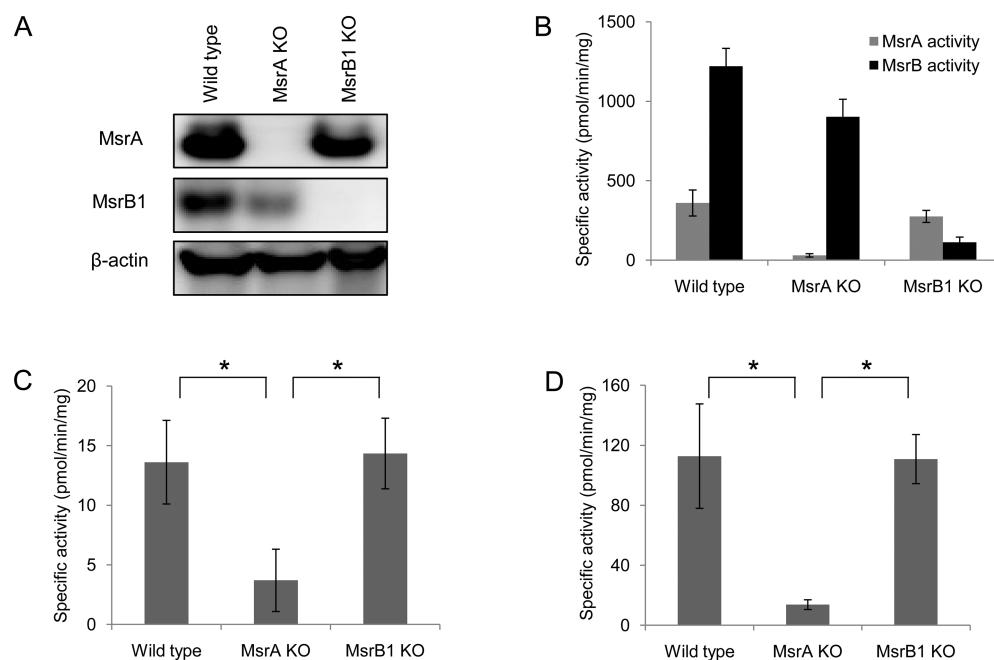


Figure 2. Roles of Msrs in the reduction of methylsulfinyl-containing drugs by liver lysates. (A) Western blot analysis of MsrA, MsrB1, and β -actin in liver lysates from wild type, MsrA KO, and MsrB1 KO mice. (B) Specific MsrA (gray bars) and MsrB (black bars) activities in liver lysates of wild type, MsrA KO, and MsrB1 KO mice. Dabsylated Met-S-SO and dabsylated Met-R-SO were used as substrates for measurement of MsrA and MsrB activities, respectively. (C) Specific activity for the reduction of sulmazole in liver lysates from wild type, MsrA KO, and MsrB1 KO mice. (D) Specific activity for the reduction of mesoridazine in liver lysates from wild type, MsrA KO, and MsrB1 KO mice. All measurements were repeated 3 times independently. Data were analyzed with a Student's *t* test (*: $p < 0.05$).

compounds were used in a mixed *R*- and *S*-sulfoxide form (approximately 1:1 ratio) and MsrA was specific for the *S*-sulfoxide form, our data suggested that only the *S*-sulfoxide forms of three drugs could be reduced.

We further examined DMSO and *R,S*-sulforaphane as substrates. A gas chromatography (GC) analysis revealed DMSO reduction by MsrA (28.6 ± 5.73 nmol/min/mg with DTT as a reductant), but not by other Msrs (Figure 1E,F and Supplementary Figure S5). Since DMSO does not have a chiral group at the sulfur atom, this compound could be quantitatively reduced by MsrA. In the case of sulforaphane, we preincubated the substrate with excess DTT to conjugate its isothiocyanate group prior to the Msr reaction and followed generation of erucin-DTT, which is the reduced form of sulforaphane-DTT, by an HPLC analysis (Supplementary Figure S6). The *R*- and *S*-sulforaphane-DTT forms were partially converted to erucin-DTT in a nonenzymatic reaction; however, addition of MsrA to the reaction mixture increased yield of the product, whereas other Msrs had no effect. Similar results were obtained when mMsrb2 reacted with *R*-sulforaphane and mMsra with *S*-sulforaphane (Supplementary Figure S6). Thus, we found overwhelming evidence that only the *S*-sulfoxide forms of methylsulfinyl-compounds are stereospecifically reduced by MsrA, while other Msrs (*i.e.*, MsrB and yfRMsr) cannot reduce any enantiomeric forms of the drugs.

To extend these *in vitro* findings to the reduction of methylsulfinyls in a more biological setting, we used mouse liver to investigate selectivity of reduction of methylsulfinyl-containing drugs by Msrs. Liver lysates from wild type, MsrA knockout (KO), and MsrB1 KO mice (Figure 2A,B) were used to examine DTT-dependent reduction of mesoridazine and sulmazole. Lysates from wild type and MsrB1 KO mice were equally

effective in reducing mesoridazine and sulmazole, whereas the ability of MsrA KO liver lysates to reduce these compounds was severely compromised (Figure 2C,D) ($p < 0.05$).

Cyp450 and FMO are microsomal proteins capable of oxidizing sulfur atoms in diverse compounds; thus, reoxidation of methylsulfides to methylsulfinyls by these and other enzymes might influence the reduction rate of methylsulfinyls by Msrs. To examine this possibility, we subjected liver cytosolic and microsomal fractions from wild type, MsrA KO, and MsrB1 KO mice to the reduction and oxidation assays using sulmazole, mesoridazine, and thioridazine as substrates. The cytosolic fraction lacking MsrA showed a significant decrease in the reduction of mesoridazine (Supplementary Figures S9A,B, S10B) and sulmazole (Supplementary Figure S10A), whereas this activity was little affected by MsrB1 KO (Supplementary Figures S9C, S10A,B). Using microsomal fractions, we found that generation of mesoridazine was proportional to protein concentration (Supplementary Figure S10C), suggesting that microsomes can oxidize thioridazine; however, the specific oxidase activity was 100 fold lower compared to the specific activity for the reduction of mesoridazine by the cytosol. In addition, no difference in the oxidation activity was detected between wild type, MsrA KO, and MsrB1 KO mice (Supplementary Figure S10D). Thus, although microsomes do oxidize thioridazine to mesoridazine, this activity has little influence on the overall reduction of mesoridazine by mouse liver.

Next, we investigated if the stereospecific reduction of methylsulfinyls influences properties of drugs and natural compounds *in vivo*. To test this possibility, HEK 293 cells, which were transfected with mMsra or mMsrb2 constructs, were treated with various concentrations of mesoridazine. mMsra-expressing

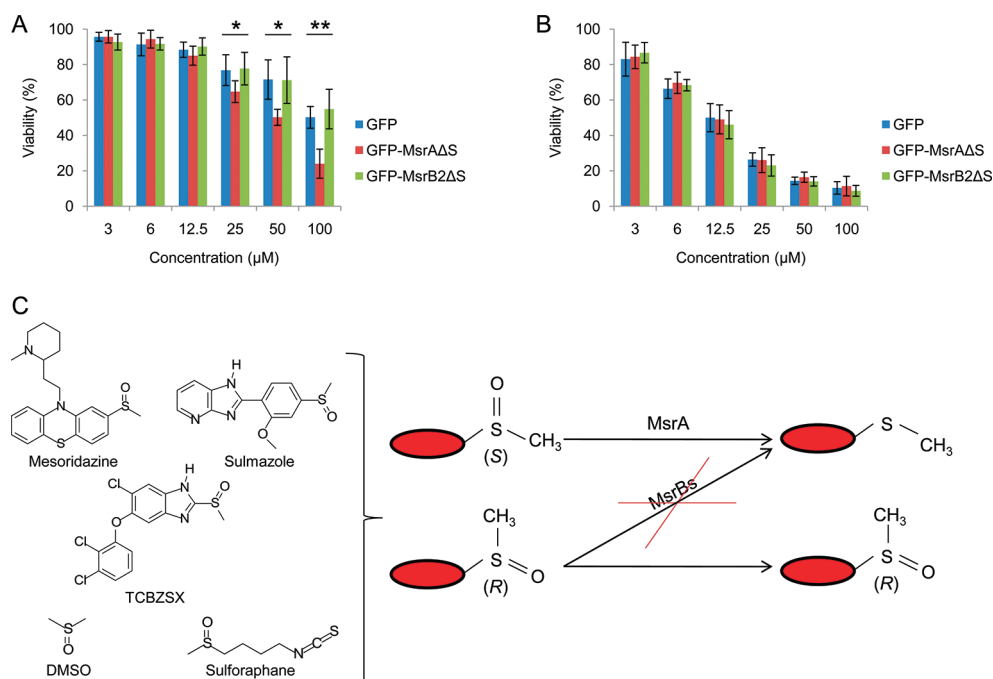


Figure 3. Selective reduction of mesoridazine affects cell viability. (A) Cell viability analysis of HEK 293 cells expressing GFP (control), GFP-fused mMsrAΔS, or GFP-fused mMsrB2ΔS following treatment of cells with mesoridazine (3, 6, 12.5, 25, 50, 100 μM) for 24 h. Constructs were designed to express proteins in the cytosol (where indicated by removing the signal peptide marked as ΔS). (B) Cell viability analysis of HEK 293 cells transfected with GFP (control), GFP-fused mMsrAΔS, or GFP-fused mMsrB2ΔS following treatment with thioridazine (3, 6, 12.5, 25, 50, 100 μM) for 24 h. Constructs were designed to express proteins in the cytosol (ΔS indicates that the signal peptide was removed). All measurements were repeated 6 times independently, and the data were analyzed with a Student's *t* test (**: $p < 0.01$ and *: $p < 0.05$). (C) An overall scheme of stereospecific reduction of methylsulfinyl-containing drugs.

cells showed decreased viability in the presence of mesoridazine compared with control and mMsrB2-expressing cells (25 μM mesoridazine, $p < 0.05$; 50 μM, $p < 0.05$; 100 μM, $p < 0.01$) (Figure 3A), suggesting that reduction of this compound by mMsrA enhanced toxicity of this drug. Consistent with this idea, the reduction product of mesoridazine, thioridazine, was found to be more toxic than mesoridazine (Figure 3B).

Thus, the idea that the two Msr families in mammals, MsrA and MsrB, have completely different catalytic preferences for the reduction of methylsulfinyl-containing compounds is fully supported by our study. Previous research provided some clues. For example, *E. coli* MsrA could efficiently reduce free Met-S-SO, whereas *E. coli* MsrB possessed a 1000-fold lower activity with free Met-R-SO.^{13,14} Also, we previously reported that human SK-Hep1 cells could not use free Met-R-SO as a source of Met for its growth, because this compound was not reduced by MsrBs.⁶ In addition, it was found that mouse plasma had only a single type of free Met sulfoxide, Met-R-SO, whereas Met-S-SO could only be detected in the plasma of MsrA KO mice (it was observed together with Met-R-SO).⁶ One study examined the reduction of the anticancer compound sulindac by *E. coli* Msrs and partially purified cellular fractions.¹¹ In this experiment, purified MsrA and a membranous fraction reduced sulindac, and it was suggested that MsrA was specific for the reduction of the S-sulfoxide form, while a putative membrane-bound Msr, mem-R,S-Msr, reduced the R-sulfoxide form. Whether this protein exists in mammals is not known. Mammals have four Msrs: MsrA, MsrB1, MsrB2, and MsrB3, which appear to account for the reduction of methionine sulfoxides in these organisms. Three MsrBs share a similar function by acting in different cellular compartments, whereas a single MsrA is

targeted to cellular compartments by virtue of its signal peptide and alternative first exon splicing.¹⁵

In support of our hypothesis that mammalian MsrBs cannot reduce R-methylsulfinyls, we found that only mMsrA reduced methylsulfinyls in a broad set of compounds, whereas neither mMsrB nor yfRMsr were active. This selective reduction was further confirmed by examining reduction of DMSO and sulforaphane. DMSO does not have a chiral group; still, only MsrA showed activity toward this compound. Also, only S-sulforaphane was reduced by mMsrA, though a nonenzymatic reduction in the presence of DTT was detected with both stereoisomers. Moreover, liver lysates from MsrA KO mice reduced mesoridazine and sulmazole with low efficiency, whereas liver lysates from MsrB1 KO mice were as efficient as those from wild type mice. Accordingly, stereospecificity for S-sulfoxides and a broad substrate specificity of MsrA, *i.e.*, its reduction of any S-methylsulfinyl, should lead to the stereospecific reduction of mixtures of S-sulfoxide and R-sulfoxide forms of drugs (Figure 3C). This specific MsrA-based reduction of methylsulfinyls is apparently due to the fact that MsrA recognizes methylsulfinyl as a substrate, whereas MsrB and fRMsr require larger functional groups.^{16–19}

Aside from the selective reduction of compounds by mMsrA, MsrA KO mice exhibited a low activity toward methylsulfinyls (Figure 2C,D). This activity is consistent with the occurrence of an unknown low efficiency reductase. A recent study suggested that the R-enantiomer of sulindac may be reduced by mammalian MsrB1.²⁰ Although purified MsrB2, MsrB3, and the Cys mutant of MsrB1 did not reduce this R-enantiomer *in vitro*, the function of wild type MsrB1 could not be tested.²⁰ In the case of

sulmazole, we found that these enzymes also could not reduce this compound, and the MsrB1 KO liver lysate had the same activity toward sulmazole and mesoridazine as the wild type lysate. Nevertheless, it is possible that an unknown reductase is involved in the low activity reduction of methylsulfinyls in sulmazole and mesoridazine. It is unclear if this activity is higher in the case of sulindac,²⁰ or if the sulindac structure (*e.g.*, being less charged and less bulky) is different enough from the compounds tested in our study, so that it is more efficiently reduced by MsrB1. Regardless of the possible low activity of MsrB1 toward some methylsulfinyl-containing xenobiotics, our data strongly support the idea that MsrA is the major reductase for the *S*-stereoisomer reduction and that Msrs lack or exhibit low efficiency in the *R*-stereoisomer reduction.

Potential differences in the metabolism and efficacy of *R*- or *S*-sulfoxide forms of drugs and natural compounds previously received little attention. However, our findings suggest that the differences in metabolizing these forms are an important factor that can be used in the development of drugs with improved efficacy and decreased toxicity. It was previously reported that the affinity of mesoridazine for dopaminergic and α -adrenergic receptors was higher than that of thioridazine, while mesoridazine had a lower affinity for the muscarinic receptor. This differential binding upon oxidation was further supported by clinical data on the side effects that correlated with the use of these drug forms.²¹ For TCBZ, it was suggested that the sulfoxidation status of the methylsulfide group may be associated with the increased resistance of *F. hepatica* to drug treatment.^{22,23} In the case of sulforaphane, studies have shown that the sulfoxide form of this compound was approximately 10 times more effective in inducing NAD(P)H:quinone oxidoreductase (NQO1) activity than the sulfide form.²⁴ Finally, our toxicity assay data showed that the *R*-sulfoxide form of mesoridazine had lower toxicity than the mixture of two enantiomers, whereas the *S*-sulfoxide form was more toxic due to its conversion to the sulfide form by MsrA. Consequently, if the oxidized forms of methylsulfinyl-containing drugs or natural compounds, such as mesoridazine and sulforaphane, are less toxic or show higher efficacy than the reduced forms, the *R*-enantiomers of such compounds can be used for improved drug efficacy. In contrast, if the reduced forms are more active or less toxic, the *S*-enantiomers of these pro-drugs should be utilized. Although our study examined only a limited number of methylsulfinyl-containing compounds, the overall findings are clear and should apply to most other methylsulfinyl/methylsulfide-containing drugs, such as enoximone, nifuratel, albendazole, pergolide, lincomycin, triethylperazine, fensulfothion, clindamycin, captodiamine, flosequinane, and thiocolchicoside. Some newly developed drugs will surely also include this functional group.

METHODS

Cloning, Expression, and Purification of Mouse MsrA, Mouse MsrB2, Yeast fRMs, and Mouse MsrB1-Cys. Previously established expression constructs for mouse MsrB1 (mutant in which Sec is replaced with Cys for increased protein expression),²⁵ mouse MsrB2,²⁵ mouse MsrA,²⁶ and yeast fRMs²⁷ were transformed into BL21 (DE3) *E. coli*. Cells with the plasmid in 500 mL of LB medium containing 50 μ g/mL ampicillin were grown until OD₆₀₀ reached 0.6–0.8, followed by addition of IPTG to 0.3 mM. Protein expression was induced at 30 °C for 4 h, followed by harvesting of cells by centrifugation at 4,000 rpm for 5 min. Cells were washed with PBS and stored at –70 °C until use.

To purify proteins, the cell pellet was dissolved in resuspension buffer (Tris-HCl, pH 7.5, 15 mM imidazole, 300 mM NaCl), and PMSF was added to a final concentration of 0.5 mM. After sonication, supernatant was collected by centrifugation at 8,000 rpm for 30 min. The supernatant was loaded onto a cobalt Talon resin (Clontech) pre-equilibrated with resuspension buffer. Following washing with the same buffer, the protein was eluted with elution buffer (Tris-HCl, pH 7.5, 300 mM imidazole, 300 mM NaCl). Fractions containing the expressed protein were pooled together and dialyzed overnight against PBS in a dialysis cassette (Pierce).

Preparation of Mesoridazine, Triclabendazole Sulfoxide, Sulmazole, and *R*- and *S*-Sulforaphane-DTT. Solutions of mesoridazine (Sigma), triclabendazole sulfoxide (AmXpress), and sulmazole (Sigma) were prepared in either ethanol or distilled water prior to enzyme assays. *R*- and *S*-sulforaphane (LKT Laboratories, Inc.) were incubated with DTT at a 1 to 10 ratio for 1 h at 37 °C and then used in assay mixtures to test enzyme activity.

Activity Assays of Mouse MsrA, Mouse MsrB2, and Yeast fRMs Using DTT. Mouse MsrA, mouse MsrB2, and yeast fRMs were utilized in the reduction assays in the presence of DTT. Dabsylated Met-*R*-SO, dabsylated Met-*S*-SO, and free Met-*R*-SO were prepared as previously described.⁶ In the DTT-dependent reaction, the reaction mixture (100 μ L) contained 50 mM sodium phosphate, pH 7.5, 50 mM NaCl, 20 mM DTT, 500 μ M Met-O (dabsylated Met-*R*-SO, dabsylated Met-*S*-SO, or free Met-*R*-SO), and purified proteins. Reactions were carried out at 37 °C for 30 min and then were stopped by adding 200 μ L of acetonitrile. The reduced product was analyzed by HPLC as described previously.^{6,25}

Reduction Assays for Analyses by Ion-Spray Mass Spectrometry, HPLC, and GC. Mouse MsrA, mouse MsrB2, mouse MsrB1-Cys, and yeast fRMs were used in the reduction assays in the presence of DTT or Trx/TR/NADPH. Mesoridazine, triclabendazole sulfoxide, sulmazole, DMSO, *R*-sulforaphane-DTT, and *S*-sulforaphane-DTT were used as substrates. In the DTT-dependent reaction, a reaction mixture (100 μ L) contained 50 mM sodium phosphate, pH 7.5, 50 mM NaCl, 20 mM DTT, substrates (500 μ M mesoridazine, 500 μ M sulmazole, 500 μ M triclabendazole sulfoxide, 500 μ M DMSO, 5 mM *R*-sulforaphane-DTT or 5 mM *S*-sulforaphane-DTT), and purified proteins. In the Trx-dependent reaction, a reaction mixture (100 μ L) contained 50 mM sodium phosphate, pH 7.5, 50 mM NaCl, 6.8 μ M *E. coli* Trx (Sigma), 0.2 mM NADPH, 0.4 μ M *E. coli* TR, substrates (500 μ M mesoridazine, 500 μ M sulmazole, or 500 μ M triclabendazole sulfoxide), and purified proteins. The reactions were carried out at 37 °C for 30 min and then were stopped by adding 300 μ L of acetonitrile (ACN) in the case of mesoridazine reaction, 900 μ L of ethanol in the case of DMSO reaction, and 10 μ L of trichloroacetic acid (50%) in the case of sulmazole, triclabendazole sulfoxide, and *R*- or *S*-sulforaphane-DTT reactions. After 5 min of incubation at 4 °C, the reaction mixtures were centrifuged at 13,000 rpm for 20 min. Then, supernatants were taken for further ion-spray mass spectrometry, HPLC, or GC analyses. Additionally, each peak from the HPLC analyses was collected and subjected to ion-spray mass spectrometry analyses to confirm identity of the compounds together with the internal standards.

Ion-Spray Mass Spectrometry Analysis. MS and MS/MS spectra were obtained on a Sciex API-III triple-quadrupole mass spectrometer equipped with an atmospheric pressure ionization source. The mass spectrometer was operated in the negative mode. The samples (20 μ L) were introduced in distilled water/acetonitrile/formic acid (50:50:0.3 by volume) (180 μ L). Scanning was done with *m/z* from 100 to 1000.

Animal Studies. Care and treatment of experimental animals were approved by the Animal Care and Use Committee at the University of Nebraska-Lincoln (UNL). Six month old C57BL/6 wild type, MsrA KO, and MsrB1 KO mice were used. MsrA and MsrB1 KO mice were previously described.²⁸

Sample Preparation. Animals were sacrificed, and their livers were dissected and subjected to protein expression and activity assays. Tissues were homogenized in PBS containing protease inhibitors (Roche), and the homogenates were normalized with regard to protein concentration. Western blotting analyses and MsrA and MsrB activity assays were then carried out with these samples.

Protein Expression Analysis. Tissue homogenates were separated on SDS-PAGE gels (40 μ g of protein was loaded). Then, the proteins were transferred onto PVDF membranes and probed with the antibodies indicated (MsrA, MsrB1, and β -actin). Secondary HRP-linked anti-rabbit or anti-mouse antibodies, and ECL substrate detection were from GE HealthCare.

Isolation of Cytosolic and Microsomal Liver Fractions from Wild Type, MsrA KO, and MsrB1 KO Mice and Oxidation of Thioridazine with the Microsomal Fractions. Fresh mouse liver was homogenized in PBS (pH 7.5) containing protease inhibitor cocktail and centrifuged at 13,000 *g* for 30 min. The supernatant was collected and centrifuged again at 100,000 *g* for 2 h. The resulting supernatant was collected as the cytosolic fraction, and the pellet was washed with PBS, recentrifuged for 1 h at 100,000 *g*, and collected as the microsomal fraction. These fractions were resuspended in 2 mL of PBS and subjected to the assays as described below. Microsomal fractions were used to assay for thioridazine oxidation.²⁰ Briefly, 500 μ M thioridazine was incubated with 40, 80, 120, 160 μ g microsomal fractions in PBS (pH 7.5) in the presence of 1.5 mM NADPH, 5 mM glucose-6-phosphate, 300 ng glucose-6-phosphate dehydrogenase, and 5 mM MgCl₂ for 60 min at 37 °C. A total volume of the reaction was 100 μ L. After stopping the reaction by adding 300 μ L of acetonitrile and centrifuging the mixture for 15 min at 13,000 *g*, 50 μ L of the supernatant was injected onto HPLC and analyzed as described below.

Reduction Assays of Mesoridazine and Sulmazole Using Liver Lysates and Liver Cytosolic Fractions. Samples (80–200 μ g) of liver lysates or their cytosolic and microsomal fractions from wild type, MsrA KO, and MsrB1 KO mice were used in the reduction assays in the presence of DTT. Mesoridazine and sulmazole were used as substrates. In the DTT-dependent reaction, the reaction mixture (100 μ L) contained 50 mM sodium phosphate, pH 7.5, 50 mM NaCl, 20 mM DTT, substrates (500 μ M mesoridazine or sulmazole) and liver lysate. Products were also analyzed by using HPLC as described below.

Transfection of Mammalian Cells with Mouse MsrA and Mouse MsrB2 and Drug Toxicity Assays. Constructs expressing GFP fused with mouse MsrA²⁶ or GFP fused with mouse MsrB2 lacking the signal peptide²⁹ were transfected into HEK 293 cells using Lipofectamin 2000 (Invitrogen). After 36 h, indicated concentrations of mesoridazine, thioridazine, sulmazole, DMSO, or triclabendazole sulfoxide were added and the cells were monitored for 24 h. Cell viability was measured with 0.4% trypan blue staining by using a Countess Automated Cell Counter (Invitrogen).

HPLC Analysis of Mesoridazine and Its Reduced form. A 50 μ L portion of the supernatant from the mesoridazine reduction assay was injected onto ZORBAX RX-C18 column (4.6 \times 150 mm) (Agilent). In this assay, solvent A (20 mM sodium acetate, pH 4.0) and solvent B (ACN) were used. Mesoridazine and thioridazine were separated from other compounds at room temperature at a flow rate of 1 mL/min using a linear gradient of 30–50% solvent B from 0 to 30 min, and 50–100% solvent B from 30 to 33 min. Detection was at 254 nm using a Waters 996 PDA detector.

HPLC Analysis of Triclabendazole Sulfoxide and Its Reduced form. A 25 μ L portion of the supernatant from the reduction of triclabendazole sulfoxide was mixed with 25 μ L of distilled water (containing 0.05% trifluoroacetic acid (TFA)) prior to injection onto a ZORBAX RX-C18 column (4.6 \times 150 mm) (Agilent). For this analysis, solvent A (distilled water with 0.1% TFA) and solvent B (ACN containing 0.1% TFA) were used. Triclabendazole and triclabendazole

sulfoxide were separated from other compounds at RT at a flow rate of 1.5 mL/min using an isocratic of 45% solvent B until 8 min and then a linear gradient of 45–100% solvent B from 8 to 10 min. Detection was at 254 nm using a Waters 996 PDA detector.

HPLC Analysis of Sulmazole and Its Reduced form. A 2.5 μ L sample of the supernatant from the reduction reaction of sulmazole was mixed with 47.5 μ L of 20 mM sodium phosphate (pH 7.4)/ACN/distilled water (2:1:3, v/v) prior to injection onto a ZORBAX RX-C18 column (4.6 \times 150 mm) (Agilent). For this analysis, solvent A (20 mM sodium phosphate, pH 7.4/ACN, 2:1 ratio in v/v) and solvent B (ACN) were used. Sulmazole and its reduced form were separated from other compounds at RT at a flow rate of 1.5 mL/min using an isocratic of 100% solvent A until 15 min. Detection was by fluorescence using a Waters 474 scanning fluorescence detector with excitation at 330 nm and emission at 370 nm.

HPLC Analysis of Sulforaphane-DTT and Its Reduced form. A 10 μ L sample of the supernatant from the reduction reaction of *R*- or *S*-sulforaphane-DTT was mixed with 40 μ L of distilled water (0.06% TFA) prior to injection onto a ZORBAX RX-C18 column (4.6 \times 150 mm) (Agilent). For this analysis, solvent A (0.06% TFA) and solvent B (ACN containing 0.06% TFA) were used. *R*- or *S*-sulforaphane-DTT conjugate and its reduced form were separated from other compounds at RT at a flow rate of 1.0 mL/min using a linear gradient of 0–40% solvent B from 0 to 40 min, and 40–100% from 40 to 45 min. Detection was at 214 nm using a Waters 996 PDA detector.

GC Analysis of DMSO and Its Reduced form. A 20 μ L sample of the supernatant from the reduction of DMSO was mixed with 980 μ L of ethanol, and then this mixture was analyzed by GC. For this analysis, a 6890N gas chromatograph (Agilent Technologies) was used, hydrogen gas was used as a carrier, and detection was in the range from 40 to 250 °C by using a FID detector.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ REFERENCES

- (1) Stadtman, E. R., Moskovitz, J., and Levine, R. L. (2003) Oxidation of Methionine Residues of Proteins: Biological Consequences. *Antioxid. Redox Signaling* 5, 577–582.
- (2) Weissbach, H., Resnick, L., and Brot, N. (2005) Methionine Sulfoxide Reductases: History and Cellular Role in Protecting against Oxidative Damage. *Biochim. Biophys. Acta* 1703, 203–212.
- (3) Imlay, J. A. (2008) Cellular Defenses against Superoxide and Hydrogen Peroxide. *Annu. Rev. Biochem.* 77, 755–776.
- (4) Stadtman, E. R. (2004) Cyclic Oxidation and Reduction of Methionine Residues of Proteins in Antioxidant Defense and Cellular Regulation. *Arch. Biochem. Biophys.* 423, 2–5.
- (5) Lin, Z., Johnson, L. C., Weissbach, H., Brot, N., Lively, M. O., and Lowther, W. T. (2007) Free Methionine-(R)-Sulfoxide Reductase from

Escherichia coli Reveals a New GAF Domain Function. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9597–9602.

(6) Lee, B. C., Le, D. T., and Gladyshev, V. N. (2008) Mammals Reduce Methionine-S-Sulfoxide with MsrA and Are Unable to Reduce Methionine-R-Sulfoxide, and This Function can be Restored with a Yeast Reductase. *J. Biol. Chem.* 283, 28361–28369.

(7) Kim, H. Y., and Gladyshev, V. N. (2007) Methionine Sulfoxide Reductases: Selenoprotein Forms and Roles in Antioxidant Protein Repair in Mammals. *Biochem. J.* 407, 321–329.

(8) Lynch, T., and Price, A. (2007) The Effect of Cytochrome P450 Metabolism on Drug Response, Interactions, and Adverse Effects. *Am. Fam. Physician* 76, 391–396.

(9) Beedham, C. (1997) The Role of Non-P450 Enzymes in Drug Oxidation. *Pharm. World Sci.* 19, 255–263.

(10) Bentley, R. (2005) Role of Sulfur Chirality in the Chemical Processes of Biology. *Chem. Soc. Rev.* 34, 609–624.

(11) Etienne, F., Resnick, L., Sagher, D., Brot, N., and Weissbach, H. (2003) Reduction of Sulindac to its Active Metabolite, Sulindac Sulfide: Assay and Role of the Methionine Sulfoxide Reductase System. *Biochem. Biophys. Res. Commun.* 312, 1005–1010.

(12) Sagher, D., Brunell, D., Hejtmancik, J. F., Kantorow, M., Brot, N., and Weissbach, H. (2006) Thionein Can Serve as a Reducing Agent for the Methionine Sulfoxide Reductases. *Proc. Natl. Acad. Sci. U.S.A.* 103, 8656–8661.

(13) Grimaud, R., Ezraty, B., Mitchell, J. K., Lafitte, D., Briand, C., Derrick, P. J., and Barras, F. (2001) Repair of Oxidized Proteins. Identification of a New Methionine Sulfoxide Reductase. *J. Biol. Chem.* 276, 48915–48920.

(14) Lee, B. C., and Gladyshev, V. N. (2011) The Biological Significance of Methionine Sulfoxide Stereochemistry. *Free Radical Biol. Med.* 50, 221–227.

(15) Lee, B. C., Dikiy, A., Kim, H. Y., and Gladyshev, V. N. (2009) Functions and Evolution of Selenoprotein Methionine Sulfoxide Reductases. *Biochim. Biophys. Acta* 1790, 1471–1477.

(16) Gruez, A., Libiad, M., Boschi-Muller, S., and Branlant, G. (2010) Structural and Biochemical Characterization of Free Methionine-R-Sulfoxide Reductase from *Neisseria meningitidis*. *J. Biol. Chem.* 285, 25033–25043.

(17) Boschi-Muller, S., Gand, A., and Branlant, G. (2008) The Methionine Sulfoxide Reductases: Catalysis and Substrate Specificities. *Arch. Biochem. Biophys.* 474, 266–273.

(18) Neiers, F., Sonkaria, S., Olry, A., Boschi-Muller, S., and Branlant, G. (2007) Characterization of the Amino Acids from *Neisseria meningitidis* Methionine Sulfoxide Reductase B Involved in the Chemical Catalysis and Substrate Specificity of the Reductase Step. *J. Biol. Chem.* 282, 32397–32405.

(19) Ranaivoson, F. M., Antoine, M., Kauffmann, B., Boschi-Muller, S., Aubry, A., Branlant, G., and Favier, F. (2008) A Structural Analysis of the Catalytic Mechanism of Methionine Sulfoxide Reductase A from *Neisseria meningitidis*. *J. Mol. Biol.* 377, 268–280.

(20) Brunell, D., Sagher, D., Kesaraju, S., Brot, N., and Weissbach, H. (2011) Studies on the Metabolism and Biological Activity of the Epimers of Sulindac. *Drug Metab. Dispos.* 39, 1014–1021.

(21) Bylund, D. B. (1981) Interactions of Neuroleptic Metabolites with Dopaminergic, Alpha Adrenergic and Muscarinic Cholinergic Receptors. *J. Pharmacol. Exp. Ther.* 217, 81–86.

(22) Alvarez, L. I., Solana, H. D., Mottier, M. L., Virkel, G. L., Fairweather, I., and Lanusse, C. E. (2005) Altered Drug influx/efflux and Enhanced Metabolic Activity in Triclabendazole-Resistant Liver Flukes. *Parasitology* 131, 501–510.

(23) Brennan, G. P., Fairweather, I., Trudgett, A., Hoey, E., McCoy, McConville, M., Meaney, M., Robinson, M., McFerran, N., Ryan, L., Lanusse, C., Mottier, L., Alvarez, L., Solana, H., Virkel, G., and Brophy, P. M. (2007) Understanding Triclabendazole Resistance. *Exp. Mol. Pathol.* 82, 104–109.

(24) Ahn, Y. H., Hwang, Y., Liu, H., Wang, X. J., Zhang, Y., Stephenson, K. K., Boronina, T. N., Cole, R. N., Dinkova-Kostova, A. T., Talalay, P., and Cole, P. A. (2010) Electrophilic Tuning of the

Chemoprotective Natural Product Sulforaphane. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9590–9595.

(25) Kim, H. Y., and Gladyshev, V. N. (2005) Different Catalytic Mechanisms in Mammalian Selenocysteine- and Cysteine-Containing Methionine-R-Sulfoxide Reductases. *PLoS Biol.* 3, e375.

(26) Kim, H. Y., and Gladyshev, V. N. (2005) Role of Structural and Functional Elements of Mouse Methionine-S-Sulfoxide Reductase in its Subcellular Distribution. *Biochemistry* 44, 8059–8067.

(27) Le, D. T., Lee, B. C., Marino, S. M., Zhang, Y., Fomenko, D. E., Kaya, A., Hacıoglu, E., Kwak, G. H., Koc, A., Kim, H. Y., and Gladyshev, V. N. (2009) Functional Analysis of Free Methionine-R-Sulfoxide Reductase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 284, 4354–4364.

(28) Novoselov, S. V., Kim, H. Y., Hua, D., Lee, B. C., Astle, C. M., Harrison, D. E., Friguet, B., Moustafa, M. E., Carlson, B. A., Hatfield, D. L., and Gladyshev, V. N. (2010) Regulation of Selenoproteins and Methionine Sulfoxide Reductases A and B1 by Age, Calorie Restriction, and Dietary Selenium in Mice. *Antioxid. Redox Signal.* 12, 829–838.

(29) Kim, H. Y., and Gladyshev, V. N. (2004) Methionine Sulfoxide Reduction in Mammals: Characterization of Methionine-R-Sulfoxide Reductases. *Mol. Biol. Cell* 15, 1055–1064.