A High-Throughput Screening Compatible Assay for Activators and Inhibitors of Methionine Sulfoxide Reductase A

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ABSTRACT

The methionine sulfoxide reductase (Msr) system has been shown to play an important role in protecting cells against oxidative damage. This family of enzymes can repair damage to proteins resulting from the oxidation of methionine residues to methionine sulfoxide, caused by reactive oxygen species. Previous genetic studies in animals have shown that increased levels of methionine sulfoxide reductase enzyme A (MsrA), an important member of the Msr family, can protect cells against oxidative damage and increase life span. A high-throughput screening (HTS) compatible assay has been developed to search for both activators and inhibitors of MsrA. The assay involves a coupled reaction in which the oxidation of NADPH is measured by either spectrophotometric or fluorometric analysis. Previous studies had shown that MsrA has a broad substrate specificity and can reduce a variety of methyl sulfoxide compounds, including dimethylsulfoxide (DMSO). Since the chemicals in the screening library are dissolved in DMSO, which would compete with any of the standard substrates used for the determination of MsrA activity, an assay has been developed that uses the DMSO that is the solvent for the compounds in the library as the substrate for the MsrA enzyme. A specific activator of MsrA could have important therapeutic value

for diseases that involve oxidative damage, especially age-related diseases, whereas a specific inhibitor of MsrA would have value for a variety of research studies.

INTRODUCTION

Cells protect against oxidative damage by 2 general mechanisms, that is, both by destroying the reactive oxygen species (ROS) before damage can occur and by repairing the damage to the macromolecules after it occurs. Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase can destroy the ROS, and their role in protecting cells against oxidative damage is well established. With regard to the repair of oxidative damage to macromolecules, repair systems for DNA have been extensively studied,¹ but recently there has been considerable interest directed toward repair of protein damage due to oxidation. One of the systems that has been extensively studied is the repair of methionine (Met) oxidation in proteins by the methionine sulfoxide reductase (Msr) system.² Met is one of the most easily oxidized amino acids by ROS, being converted to methionine sulfoxide (Met(o)) as seen in *Figure 1*. Because the sulfur in Met(o) is a chiral center, chemical oxidation of Met to Met(o) yields a mixture of the 2 epimers of Met(o) called Met-R-(o) and Met-S-(o). The Msr enzymes, MsrA and MsrB, can reduce the S and R epimers of Met(o), respectively, in proteins in the presence of a reducing system such as thioredoxin (*Fig. 1*). Further oxidation of Met(o) results in the formation of methionine sulfone. Although MsrA was identified in our laboratory >25 years ago,³ it has only been in the past 10 years that the importance of the Msr system in protecting cells against oxidative damage has

ABBREVIATIONS: DABS, dimethylaminoazo-benzenesulfonyl; DMSO, dimethylsulfoxide; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; HTS, high-throughput screening; Met(o), methionine sulfoxide; MLSCN, Molecular Libraries Screening Centers Network; Msr, methionine sulfoxide reductase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NEM, *N*-ethylmaleimide; ROS, reactive oxygen species; SeCm, selenocystamine; Trx, thioredoxin; TrxB, thioredoxin reductase.

Fig. 1. Oxidation of methionine in proteins (or free methionine) by reactive oxygen species (ROS) and reduction catalyzed by the methionine sulfoxide reductase (Msr) system. The primary oxidation products are the R and S epimers of Met(o). Further oxidation yields methionine sulfone (taken from Weissbach *et al.* ²).

Fig. 2. The Msr system can repair damage to proteins resulting from Met oxidation and can also function as part of a scavenger system in which Met residues in proteins act as catalytic antioxidants.

become of major interest. Most of the genetic studies have been performed with MsrA where it was shown that deletion of the gene in *Escherichia coli*,^{4,5} yeast,⁶ and animal cells⁷ rendered the cells more sensitive to oxidative stress. *In vivo*, mice lacking MsrA have also been shown to be more sensitive to oxidative stress.^{8,9} On the other hand, overexpression of MsrA in animal cells makes them more resistant to oxidative stress.^{10–13} A recent example of the *in vitro* effect of overexpression has been reported using cardiac myocytes. In that study,¹³ cardiac myocytes were subjected to hypoxia and reoxygenation that caused cell death due to oxidative damage. When these cells were transfected with adenovirus containing the *MsrA* gene, significant protection of the cells from death was observed. What has attracted considerable attention was the *in vivo* finding that when MsrA was overexpressed in *Drosophila*,¹⁴ the organism became resistant to paraquat treatment and, in addition, showed a 70% increase in life span with a much slower age-related decline in physical and sexual activities than the wild-type control.¹⁴ Overexpression of MsrA in yeast grown aerobically has also resulted in an extension of life span.¹⁵ In addition to the genetic studies and the involvement of MsrA in aging, Met oxidation in proteins and MsrA have also been implicated in several diseases including emphysema, acute respiratory distress syndrome, cardiovascular disease, and cataracts.^{16–20}

Although it was thought originally that the Msr system was only protecting cells by repairing damage to proteins in which critical Met residues were oxidized, it appears that the Msr enzymes are also involved in an ROS scavenger system. As first postulated by Levine *et al.*,²¹ the Msr system may also function by allowing all the exposed Met residues in proteins to act as catalytic antioxidants.

This is summarized in Figure 2. Each round of Met oxidation and reduction by the Msr system will destroy one equivalent of ROS. Support for this catalytic antioxidant role of Met in proteins has come from cell-culture experiments. Overexpression of MsrA in PC12 cells causes the level of ROS to go down,¹¹ whereas knocking out of MsrA in lens cells results in an increase in the ROS levels in the cells.⁷ Thus, the unique feature of the Msr system is that it can protect cells against oxidative damage in 2 ways, by repairing damage to proteins in which critical Met residues have been oxidized and by an ROS-scavenging mechanism in which Met residues in proteins function as catalytic antioxidants due to the action of MsrA.

At present there are no known direct activators of MsrA, although the activity of the enzyme can be increased by agents such as selenium compounds that increase the activity of the required reducing system. It appears from the different systems in which MsrA levels have been increased through genetic manipulations that this enzyme affords significant protection against oxidative damage and cell death, resulting in increased longevity. The finding of a small molecule that could increase the activity of this enzyme could have therapeutic uses.

MATERIALS AND METHODS

High-expression vectors for *E. coli* thioredoxin (Trx) and thioredoxin reductase (TrxB) were obtained from Dr. Todd Lowther, Wake Forest University School of Medicine. The recombinant proteins and bovine MsrA were overexpressed and purified from *E. coli*, as previously described.²² Nicotinamide adenine dinucleotide phosphate (NADPH), selenocystamine (SeCm), dimethylsulfoxide (DMSO), and *N*-ethylmaleimide (NEM) were purchased from Sigma. Each incubation contained, in a final volume of 80 μL , 50 mM Tris-Cl pH 7.4, 25 mM DMSO, 4 μg MsrA, 4 μg Trx, 0.5 μg

TrxB, 40 nmol NADPH, and 20 μ M SeCm or 30 μ M NEM. In the incubations containing SeCm, the SeCm was preincubated with all of the components in the reaction mixture (except MsrA) for 5 min to adjust for the rapid initial reduction of SeCm by the Trx system in the absence of MsrA. The oxidation of NADPH was then measured after the addition of MsrA. A correction was also made for the slow reduction of SeCm observed during the 30-min incubation in the absence of MsrA. For the reactions containing NEM, the MsrA was first pretreated with NEM for 5 min and then the NEM was destroyed by adding 1 mM mercaptoethanol. An aliquot of the NEM-treated MsrA containing 4 μ g of MsrA was removed and added to the standard reaction mixture lacking MsrA. The reactions were carried out at room temperature in black, 384-well clear bottom plates and read in a Molecular Devices Spectramax™ spectrophotometer at 340 nm for the absorbance assay. For the fluorescence assay, the same instrument was used, measuring emission at 450 nm after excitation at 365 nm.

RESULTS

The initial methods used to assay for MsrA activity were based on the reduction of Met(o) in an *E. coli* ribosomal protein³ or the reduction of free Met(o) using nitroprusside as a colorimetric reagent.²³ The former assay is cumbersome, and the colorimetric assay is not very sensitive. However, once it was apparent that the enzyme had a broad substrate profile and could reduce any compound containing a methyl sulfoxide group, other assays were developed. A sensitive radioactive method was developed using *N*-acetyl-Met(o), which is converted to *N*-acetyl-Met.²⁴ More recently, dimethylaminoazo-benzenesulfonyl-Met(o) [DABS-Met(o)] has been used as a substrate, with the product, DABS-Met, measured colorimetrically²⁵ or by HPLC.²⁶ The radioactive assay

Fig. 3. Coupled reaction for the reduction of methyl sulfoxide compounds catalyzed by methionine sulfoxide reductase enzyme A (MsrA). Nicotinamide adenine dinucleotide phosphate (NADPH) is the hydrogen donor for the reduction of oxidized Trx (Trx(o)) by thioredoxin reductase (TrxB). The reduced Trx reduces methyl sulfoxide compounds catalyzed by MsrA with the formation of a methyl sulfide and regeneration of Trx(o). In the assay described in this article, the methyl sulfoxide compound is dimethylsulfoxide (DMSO). The reduction of DMSO by the Trx system and MsrA would result in the oxidation of NADPH, which can be measured at 340 nm (see text for more details).

and the colorimetric DABS assay both involve extraction procedures that make them impractical for high-throughput screening (HTS) application. However, for all MsrA substrates, it is possible to follow the oxidation of NADPH at 340 nm, in a coupled reaction, when Trx is used as the reducing system.²² As pictured in Figure 3, any methyl sulfoxide substrate can be used in this coupled reaction. In the first reaction, the NADPH will reduce oxidized Trx in a reaction catalyzed by Trx reductase. In the second reaction, the reduced Trx will supply the hydrogens to permit the MsrA to reduce the methyl sulfoxide substrate to the sulfide. This coupled reaction, resulting in NADPH oxidation, appears to be a reasonable approach to develop an HTS assay for MsrA activity.

An initial problem in developing an HTS assay based on NADPH oxidation was the realization that the compounds in the screening libraries are all dissolved in DMSO, a known substrate for MsrA.²² Given a compound concentration of 10^{-3} M in the library and 10^{-5} M in the assay, the DMSO concentration would be ~100 mM (100% DMSO is 14 M, and the compounds are typically in 80% DMSO). At this concentration, DMSO would be an overwhelming competitive substrate for all of the substrates that have been routinely used to assay MsrA activity. However, this problem was easily solved, since there was no reason that the DMSO in which the chemicals are dissolved could not be used as a substrate for the enzyme. In this case, the activity is assayed by measuring the oxidation of NADPH using Trx as the reducing system, as described in Figure 3. Because of the high concentration of DMSO used, one would not expect to see competitive inhibitors showing up in the HTS screen. Furthermore, since the K_m for DMSO under the reaction conditions used in this

Fig. 4. Effect of selenocystamine (SeCm) and *N*-ethylmaleimide (NEM) on the reduction of DMSO by MsrA. The oxidation of NADPH is measured using an absorbance assay for reactions containing MsrA, DMSO, and the thioredoxin reducing system (control, ♦) plus either SeCm (▼) or NEM (■).

study is 500 μM , with maximum reaction velocity maintained at 5 mM and above (data not shown), the DMSO concentration would not be rate-limiting at compound concentrations at or above 10^{-6} M.

Figure 4 illustrates the results (mean of 5 replicate experiments) using DMSO as substrate and 4 μg of bovine MsrA. As shown in Figure 4, there is about a 50% decrease in NADPH concentration after 30 min of incubation (control experiment, Fig. 4). To demonstrate that the assay could be used to identify both activators and inhibitors of the enzyme, we tested a known indirect activator, SeCm, and a known inhibitor, NEM, at previously determined optimum concentrations (data not shown). It was previously demonstrated that selenium compounds, such as SeCm (20 μM), could stimulate the activity of MsrA indirectly by increasing the efficiency of the Trx reducing system required for the catalytic reaction.²⁷ As shown in Figure 4, SeCm increases the rate of oxidation of NADPH catalyzed by MsrA by about two-fold since SeCm is known to be reduced by the Trx system²⁷ (see Materials and Methods for details of the incubation).

Although the primary goal of this study is to develop an HTS method to identify activators of Msr, the assay as described could be modified to detect inhibitors of MsrA. As an example, in Figure 4, NEM (30 μM) was used because this compound inhibits the enzyme by reacting with the SH groups on the enzyme (see Materials and Methods for details of the incubation). As also shown in Figure 4, there was complete inhibition of the reaction at the NEM concentration used. The calculated Z factors for the SeCm and NEM experiments are 0.95 (SD 0.003) and 0.92 (SD 0.007), respectively. These values were determined at the 20-min time point, although there was little variation over the course of the experiment. These Z factors indicate that the assay is reproducible.

In addition to the absorbance assay described earlier, there is also a fluorescence assay for NADPH. The fluorescence assay has been successfully used in an HTS format to screen for inhibitors of the *Schistosoma mansoni* redox cascade.²⁸ Because NADPH is naturally fluorescent, emitting at 450 nm, while NADP is not, it would be relatively easy to switch to this type of assay. At present, we do not anticipate problems with the absorbance assay that cannot be controlled for, but if that should occur, we have also optimized conditions for a fluorescence-based NADPH assay (see Materials and Methods). Figure 5 shows the results of experiments using fluorescence to assay for the change in NADPH concentration dependent on MsrA, as well as the stimulation of the reaction by SeCm and the inhibition by NEM. As can be seen, there is a significant stimulation by SeCm and inhibition by NEM, which closely parallels the results seen in the absorbance assay. The calculated Z factor for this assay at 20 min of incubation is 0.90. We plan to use either of the above described assays to screen

Fig. 5. Effect of SeCm and NEM on the reduction of DMSO using a fluorescence assay. The oxidation of NADPH is measured using a fluorescence assay for reactions containing MsrA, DMSO, and the thioredoxin reducing system (control, $\blacklozenge$) plus either SeCm ($\blacktriangledown$) or NEM ($\blacksquare$).

the MLSCN compound library at the Scripps Florida Research Institute HTS facility. A summary of the experimental protocol is given in Table 1.

DISCUSSION

We have developed an HTS-compatible assay to look for both activators and inhibitors of MsrA. The unique feature of this coupled assay, in which the oxidation of NADPH is measured, is the use of DMSO as a substrate for MsrA. DMSO is the solvent that is routinely used to dissolve the compounds in most chemical

Table 1. Summary of High-Throughput Screening Assay Protocol

Step	Parameter	Value	Description
1	Reaction mixture	79 μL	Reaction mixture freshly prepared
2	Library compounds	1 μL	Standard compounds dissolved in DMSO
3	Incubation time	20 min	Ambient temperature
4	Assay readout	340 or 450 nm	Absorbance or fluorescence

Step Notes

- 50 mM Tris-Cl pH 7.4, 4 μg MsrA, 4 μg Trx, 0.5 μg TrxB, 40 nmol NADPH.
- 1 μL of library compound will establish a DMSO concentration of ~ 100 mM and a compound concentration of $\sim 10^{-5}$ M.
- Absorbance is measured at 340 nm; fluorescence is measured with an excitation at 365 nm and an emission at 450 nm.

libraries, but because DMSO contains a methyl sulfoxide group, it is an excellent substrate for MsrA. Although DMSO is not generally used as a substrate for the Msr enzymes, it would be impossible to use a standard substrate (a Met(o) derivative), since the amount of DMSO added to the incubations would effectively out-compete any other substrate. The assay as described is very reproducible, and the oxidation of NADPH can be measured using either absorbance or fluorescence. Although some studies have shown that miniaturized UV absorbance assays may lack sufficient signal strength,^{28,29} one of the authors has successfully utilized absorbance assays in 1,536-well format.³⁰ With regard to the fluorescence assay, it has been reported that several percent of the compounds in a typical compound library may fluoresce at excitation and emission wavelengths similar to NADPH.³¹ However, this would be reflected as a change in the readings at time 0, for which a correction could easily be made.

It should be stressed that although we are using saturating amounts of both Trx and Trx reductase, the assay involves a coupled system in which reduced Trx is generated using NADPH and Trx reductase. Thus, any active compounds that appear in the screen will have to be tested to make sure they are not affecting the activity of the Trx system. This is easily done by setting up a Trx reductase assay without MsrA, in which substrate levels of oxidized Trx are used, so that the oxidation of NADPH is dependent only on the reduction of the Trx. Any increase or decrease in NADPH oxidation under these conditions would indicate that the compound was affecting the Trx reaction and not the MsrA activity. Alternatively, any active compound could be obtained in a DMSO-free solution and assayed using a different substrate with dithiothreitol (DTT) as the reductant.

The assay can detect both activators and inhibitors of MsrA, although there is more interest in obtaining an activator of the enzyme. However, previous studies in eukaryotic systems have shown that a knockout of MsrA results in increased ROS levels^{7,32} and shortened life span,⁸ and in the case of some bacteria decreased adherence and virulence.^{33,34} Thus, instead of relying on genetic mutations to eliminate the enzyme activity, it would also be of great value to obtain a chemical inhibitor of the enzyme. Clearly a specific inhibitor would eliminate the need for generating knockouts and permit one to modulate the level of enzyme activity, which would be especially useful in cell-culture systems. One might expect that a relative large number of inhibitors would be detected in a library screen, since the enzyme has essential cysteine residues, and any compound that reacted with thiol groups would inhibit the MsrA activity.

As mentioned earlier, the main goal of this project is to find novel activators of MsrA. Since oxidative damage is involved in so many diseases, especially age-related diseases and aging,

any compound that would increase the activity of MsrA in tissues could have important therapeutic value. It is clear from the many studies showing that the Msr system plays an important role in protecting cells against oxidative damage that an activator of MsrA could provide a novel therapeutic approach to diseases caused by oxidative damage resulting from increased ROS production.

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