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Identification of activators of methionine sulfoxide reductases A and B

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ABSTRACT

The methionine sulfoxide reductase (Msr) family of enzymes has been shown to protect cells against oxidative damage. The two major Msr enzymes, MsrA and MsrB, can repair oxidative damage to proteins due to reactive oxygen species, by reducing the methionine sulfoxide in proteins back to methionine. A role of MsrA in animal aging was first demonstrated in *Drosophila melanogaster* where transgenic flies over-expressing recombinant bovine MsrA had a markedly extended life span. Subsequently, MsrA was also shown to be involved in the life span extension in *Caenorhabditis elegans*. These results supported other studies that indicated up-regulation, or activation, of the normal cellular protective mechanisms that cells use to defend against oxidative damage could be an approach to treat age related diseases and slow the aging process. In this study we have identified, for the first time, compounds structurally related to the natural products fusaricidins that markedly activate recombinant bovine and human MsrA and human MsrB.

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1. Introduction

The free radical or oxidative damage theory of aging in animals was first proposed by Harman who postulated that aging in all animals was due to the accumulation of free radicals resulting in damage to tissues [1]. It is now well established that increased levels of reactive oxygen species (ROS), produced primarily from cellular respiration, are a significant factor in causing tissue damage [2]. However, it is also known that low levels of ROS play an important protective role in cells by initiating mechanisms, such as ischemic preconditioning, that protect cells against oxidative damage [3–5]. Thus, the level of ROS must be balanced carefully, and cells must protect against increased levels of ROS, that occur during aging. It is known that cells have developed mechanisms to specifically guard against excess formation of ROS, and aging appears to be due to an imbalance between the level of ROS production and the ability of the cellular protective mechanisms to prevent oxidative damage. Included in the protective mechanisms are antioxidants such as glutathione, ROS destroying enzymes including superoxide dismutase (SOD) and catalase, and systems to repair oxidative damage, including DNA repair enzymes [6] and the methionine sulfoxide reductase system (Msr) that repairs damage to proteins due to oxidation of methionine (Met) residues in proteins to methionine sulfoxide (Met-(o)) [7]. Our interest has been in the Msr system which was discovered in our laboratory more than 30 years ago [8]. Methionine in proteins is easily oxidized by ROS to methionine sulfoxide (Met(o)), which is present in the protein as two epimers, Met-S-(o) and Met-R-(o). The two major enzymes in the Msr family are MsrA and MsrB which reduce the S and R epimers of Met-(o), respectively, back to Met [7]. Several important enzymes are known to be inactivated by Met oxidation, and their function restored by the Msr system. Those studied in detail, include alpha-1-proteinase inhibitor and calmodulin [9,10]. However, the repair function of the Msr system may not be its only role in protecting cell against oxidative damage. Levine and coworkers [11] were the first to propose that exposed Met residues in proteins could act as catalytic antioxidants through the action of the Msr system and scavenge ROS. The physiological importance of the Msr system, especially MsrA, became most apparent when it was shown that transgenic flies that over-expressed bovine MsrA (bMsrA) had

Abbreviations: Msr, methionine sulfoxide reductases; ROS, reactive oxygen species; Met(o), methionine sulfoxide; DABS, 4-*N*,*N*-dimethylamino-azobenzene-4-sulfonyl chloride; alpha-1-PI, alpha-1 proteinase inhibitor; RPE, retinal pigmented epithelial cells; Myr, myristoylated; Trx, thioredoxin; DMF, dimethylformamide.

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a markedly extended life span, especially when the bMsrA was specifically expressed in their neuronal cells [12]. More recent studies showed that MsrA is also involved in the life extension seen in *Caenorhabditis elegans* mutants in which the *daf* 2 gene was knocked out [13] or in life span extension seen with caloric restriction [14]. It seemed clear that in lower animals increased levels of MsrA activity could extend life span. These results supported other studies showing that flies that over-expressed SOD and mice that were engineered to increase catalase activity in their mitochondria also had extended life spans [15,16]. These previous results suggested that over-expression or activation of known mechanisms that cells use to protect against oxidative damage, such as the Msr system, could serve as a therapeutic approach for the treatment of age related diseases and for the extension of life span of humans. In this study we report, for the first time, the identification of a class of small cyclic peptides that markedly activate recombinant bovine and human MsrA and human MsrB.

2. Materials and methods

The general procedures for the synthesis of fusaricidin analogues combinatorial library, including individual peptides used in this study have been described elsewhere [17,18]. 4-*N*,*N*-dimethylamino-azobenzene-4-sulfonyl chloride, (DABS) and DTT were purchased from Sigma–Aldrich. DABS-R-Met-(o) and DABS-S-Met-(o) were prepared as described previously [19]. Human alpha-1-proteinase inhibitor (alpha-1-PI) and human neutrophil elastase were purchased from Peptide Premiere Peptide Solutions and Athens Research & Technologies, respectively. Succinyl-$(Ala)_3$-*p*-nitroanilide was purchased from Sigma–Aldrich. Clones containing the *E. coli* TrxA and TrxB, *E. coli* and bovine MsrA, human MsrB2 and MsrB3 were overexpressed in *E. coli*, and the respective proteins were purified as described previously [20–23].

For the MsrA and MsrB assays a synthetic substrate, either DABS-Met-S-(o) or DABS-Met-R-(o) was used as described previously [22,23]. Incubations were performed for 30 min. Oxidation of alpha-1-PI and the ability to reduce the chemically oxidized alpha-1-PI with MsrA and determine its effect on elastase activity have been described previously [9,24,25].

The preparation of a calf liver 35–60% ammonium sulfate fraction (liver AS) was as follows: Calf liver was homogenized in 3 volumes of 50 mM Tris pH 7.4. The homogenate was first centrifuged for 20 min at 10,000 × g and the supernatant centrifuged for 2 h at 100,000 × g (S-100). The S-100 was subjected to ammonium sulfate precipitation and the proteins precipitating between 35 and 60% saturation were dissolved in 50 mm Tris-Cl pH 7.4 and dialyzed against this buffer.

Statistical Analysis- The results reported represent the mean of three replicates in each experiment ± SE.

3. Results

As part of our studies aimed at increasing the activity of MsrA in cells we have used several approaches including viral vectors [26] and screening of more than 380,000 small molecules searching for activators of MsrA. Although the initial screening results, using an assay designed for high throughput screening of activators of MsrA were negative [27], recently it was observed that analogues of naturally occurring fusaricidin A, (Fig. 1A) could significantly stimulate the activity of both recombinant MsrA and MsrB. Fusaricidin A, is a bacterial metabolite with antibiotic activity [28]. Its structure contains a hexadepsipeptide with a 15-guanidino-3-hydroxypentadecanoic tail (Fig. 1A). The analogues that we have tested have a basic scaffold that is similar to fusaricidin A, as shown in Fig. 1B. The analogues differ from fusaricidin A in the amino acid

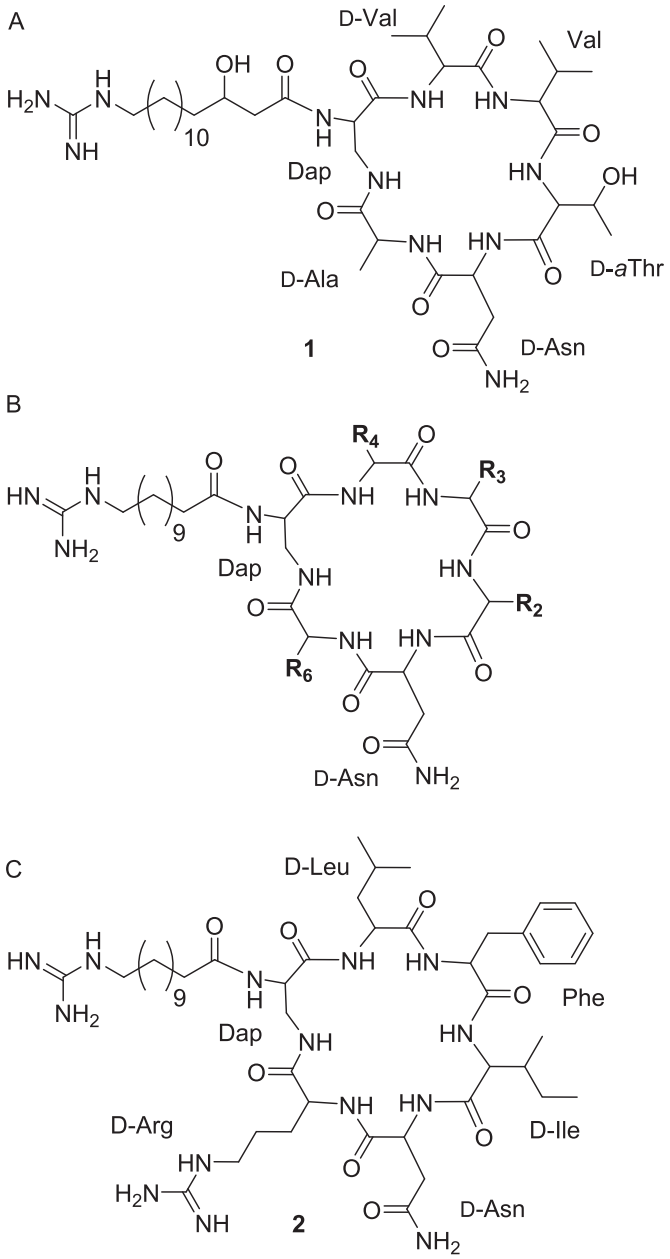

Fig. 1. Structure of fusaricidin A and activators of the Msr enzymes. A-fusaricidin A; B-basic scaffold of activators; C- analogue **2**.

composition of the cyclic hexapeptide and the fatty acid side chain, which is shorter and not hydroxylated. Fig. 1C shows the structure of cyclic lipohexapeptide **2** (analogue **2**), the primary compound used in the present studies. This analogue was obtained from screening of a positional scanning library composed of 130,321 hexapeptides. Table 1 shows the effect of amino acid substitutions in the cyclic hexapeptide moiety on the activation of recombinant bovine MsrA (bMsrA). It can be seen that an arginine or lysine in position R6, present in analogues numbered **2**,**3**,**4**, **8** and **9** listed in Table 1, is able to stimulate MsrA activity between 3.8 and 6 fold. Other structure activity studies showed that the fatty acid tail is required for activation, and the guanidine group is also required for maximal activation (see Fig. S1 and Table S1) The concentration of analogue **2** and other derivatives used in the experiments was 25 μM, but as can be seen in Fig. 2 as little as 10 μM analogue **2** results in >2 fold activation of bMsrA. All of these preliminary

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