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Screening for inhibitors of the *SOD1* gene promoter: pyrimethamine does not reduce SOD1 levels in cell and animal models

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Abstract

Mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene are detected in 20% of familial and 3% of sporadic amyotrophic lateral sclerosis (ALS) cases. Although mutant *SOD1* is known to induce motor neuron death via multiple adverse acquired functions, its exact pathogenic mechanism is not well defined. *SOD1* toxicity is dose dependent; levels of mutant *SOD1* protein in transgenic mice determine disease susceptibility, onset and rate of progression. We therefore sought to identify small molecules that reduce *SOD1* levels by inhibiting the *SOD1* promoter. We tested pyrimethamine (previously reported to suppress *SOD1* expression), several compounds currently in trials in human and murine ALS, and a set of 1,040 FDA-approved compounds. In a PC12 cell-based assay, no compounds reduced *SOD1* promoter activity without concomitant cytotoxicity. Additionally, pyrimethamine failed to repress levels of *SOD1* protein in HeLa cells or homogenates of liver, spinal cord and brain of wild-type mice. 34 compounds (including riluzole, ceftriaxone, minocyclin, PBA, lithium, acetylcysteine) in human and mouse ALS trials and an additional set of 1,040 FDA approved compounds also showed no effect on *SOD1* promoter activity. This present study thus failed to identify small molecule inhibitors of *SOD1* gene expression.

Keywords

Amyotrophic lateral Sclerosis; *SOD1*; superoxide dismutase; assay; compound screening; Pyrimethamine

Introduction

Amyotrophic lateral sclerosis (ALS) is an age-dependent, neurodegenerative disease characterized pathologically by the selective loss of motor neurons in the brain and spinal cord. Symptoms of muscle weakness, cramp or spasticity begin in a single limb but become

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generalized, and death, due to respiratory failure, occurs within three to five years. ALS is uniformly fatal. The single FDA-approved treatment for ALS (Riluzole) increases lifespan by only three to six months and does not substantially alleviate symptoms [1]. The majority of ALS cases are sporadic but approximately 10% are familial. Mutations in the genes encoding FUS/TLS [2], [3], TDP-43 [4], angiogenin [5] and cytosolic Cu/Zn superoxide dismutase (SOD1) [6] have been shown to cause ALS. The most extensively studied of these genes is *SOD1*.

To date nearly 150 ALS-associated mutations have been reported in *SOD1* [7]. Most are missense mutations, which occur throughout the protein. Through multiple mechanisms that remain fully to be defined, *SOD1* mutations are pathogenic; data overwhelmingly supports the view that mutant SOD1 protein has acquired adverse cytotoxic properties. *SOD1* knockout mice show no overt phenotype [8], whereas mice over-expressing mutant *SOD1* develop progressive paralysis and death due to motor neuron loss [9]. Importantly, transgenic mice and rats expressing high levels of mutant *SOD1* develop a disease phenotype but those expressing at a lower level do not [9] [10]. This evidence, along with the findings that siRNA directed against SOD1 prolong survival in mice [11] lead us to investigate the possibility that a reduction in SOD1 levels could attenuate ALS susceptibility and the rate of disease progression. To test this hypothesis, we developed a cell based screen for small molecules capable of inhibiting the *SOD1* promoter [12], thereby reducing levels of mutant SOD1 protein. Mutant SOD1 is thought to act in both a cell autonomous and a non-cell autonomous manner [13], [14], [15]. Reduction of levels of mutant SOD1 in motor neurons delays onset of paralysis in transgenic ALS mice [16] while diminished levels of mutant SOD1 in astrocytes and microglial cells delays microglial activation and slows disease progression after onset [17]. Thus, the potential benefits of compounds that suppress *SOD1* expression may be mediated by motor neurons and surrounding non-neuronal cells. We note that there is a precedent for a beneficial influence of induced gene repression in a transgenic model of Huntington's disease [18].

For these reasons, we have developed screening assays to identify compounds that inhibit expression of the *SOD1* gene. Our studies focused initially on pyrimethamine, several compounds currently in trials in human and murine ALS and a set of 1,040 FDA-approved compounds. We elected to study pyrimethamine in detail because this compound has previously been reported to reduce SOD1 protein levels in lymphocytes of ALS patients by up to 60% [19]. Pyrimethamine (5-(4-chlorophenyl)-6-ethyl-2,4-pyrimidinediamine) is an anti-protozoal drug whose primary mode of action involves the preferential inhibition of protozoal dihydrofolate reductase [20]. It also induces peripheral blood lymphocyte apoptosis via activation of caspase 8- and caspase 10-dependent cascades, leading to mitochondrial depolarization [21]. How pyrimethamine might reduce activity of the *SOD1* gene is not clear.

Materials and Methods

Cell Culture

A PC12 cell line, stably expressing 2.2Kb of the *SOD1* promoter region flanked by the gene encoding green fluorescent protein (GFP) was maintained in DMEM-F12 (Gibco, USA) with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum (FBS), 1x penicillin, 1x streptomycin and 500µg/mL G418 (Invitrogen, USA) at 37°C with 5% CO₂ [12]. HeLa cells were maintained in DMEM (Gibco, USA) with 10% (v/v) FBS, 1x penicillin and 1x streptomycin at 37°C with 5% CO₂.

Animal experiments

C57BL/6J mice (Jackson Laboratories, USA) aged 8–10 weeks were treated with 10mg/kg/d pyrimethamine (based on [22]) or PBS. Treatments were administered by Intraperitoneal (IP) injections for 14 days. After this mice were euthanized by CO₂ followed by decapitation. Brain, spinal cord and liver were removed. Samples were homogenized using 20mM Tris-HCl pH 7.5, 2mM DTT, 1mM EGTA, 1mM EDTA and 1x protease inhibitors (Roche, Switzerland). Homogenates were sonicated on ice at 20% for 10 seconds using a Sonic-dismembrator Model 500 (Fisher, USA). Protein concentrations were measured using the BCA protein assay kit (Thermo, USA). 20µg of total protein was loaded onto gels and electrophoresis and western blotting were carried out as described below. Experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Compound Screening

Large numbers of PC12 cells were generated using Corning® CellSTACK™ Culture Chambers (Sigma, USA). 200µL of cells (250 cells/µL) were plated into 96 well black-well/clear bottom plates (Perkin Elmer, USA) using the Multidrop 384 (ThermoScientific, USA) automated cell plater. Cells were typically incubated for one hour to allow attachment. For the US Drug Collection Library (MicroSource, USA) 0.5µL of 1mM stock was added to each well giving a final concentration of 2.5µM. For dose response curves compounds were added to final concentrations of 25µM, 10µM, 2.5µM, 1µM, 0.25µM, 0.1µM, 0.025µM, 0.01µM, 0.0025µM, 0.001µM, 0.00025µM and 0.0001µM. In each case 0.5µL was added to each well of a 96 well plate using Biomek FX liquid handling platform (Beckman Coulter, USA).

Cells were incubated for 72 hours at 37°C, conditions previously shown to give optimal assay conditions [12]. For measurements of GFP fluorescence, plates were washed in TBS, and then lysed with 200µl RIPA buffer (150mM NaCl, 1% Triton X, 0.5% sodium deoxycholate, 0.5% SDS, 40mM Tris-HCl). Fluorescence levels were determined using the EnVision 2102 Multilabel plate reader (Perkin Elmer, USA), excitation 485nm, emission 535nm. Compound toxicity analysis was gauged by determining levels of ATP, as a measure of cell viability, using the luminescence based Cell Titre-Glo assay (Promega, USA). Cells were treated and processed as above and luminescence was measured on the EnVision 2102 Multilabel plate reader (Perkin Elmer, USA).

Western Blotting

HeLa cells were plated into 6-Well plates at a dilution of 200 cells/µL. Once cells were attached, pyrimethamine (Sigma-Aldrich, USA) was added to a final concentration of 25µM, 2.5µM or 0.25µM. Cells were incubated for 72 hours at 37°C with 5% CO₂. Cells were washed with PBS, then lysed in 300µL of RIPA buffer. Lysates were sonicated on ice at 20% for 10 seconds. Protein concentrations were measured using the BCA protein assay kit (Thermo, USA) and 10µg of protein lysate was loaded onto gels. For gel electrophoresis 12% Tris-Glycine gels were used (Invitrogen, USA) and run at 125V for 90 minutes using Tris-Glycine SDS running buffer (Invitrogen, USA). Samples were transferred to nitrocellulose using the i-Blot® (Invitrogen, USA). Nitrocellulose membranes were blocked using blocking solution (LiCor, USA) and probed for SOD1 using a sheep anti-SOD1 antibody (Binding Site, UK) and a rabbit anti-actin antibody (Sigma, USA). IR labeled secondaries were used (LiCor, USA) and blots visualized/analyzed using the Odyssey Imaging System (LiCor, USA).

Statistics

Dose response curves were fitted using GraphPad Prism (GraphPad, USA). For each data set 'log (agonist) vs response - variable slope' was used, which uses the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC}_{50} - X) * \text{HillSlope}})$. For T-test analysis Microsoft Excel (Microsoft, USA) was used. For ANOVA analysis GraphPad Prism was used. In each case a p-value of 0.05 was assigned to ascertain a statistically significant difference.

Results

Effects of pyrimethamine on *SOD1*-promoter and *SOD1* protein levels

To investigate the effect of pyrimethamine on the *SOD1* promoter, this drug was added to our stable PC12 cell lines that express GFP under the control of the human *SOD1* promoter. In these cells, fluorescence levels directly indicate promoter activity. Using a 12 point dose curve (25 μM - 0.0001 μM) we observed a 42% reduction in the level of GFP relative to the DMSO treated controls at the highest dose tested. This was associated with a 68% reduction in general cell viability, as indicated by a decrement in ATP. We thus conclude that the pyrimethamine-induced reduction in *SOD1* promoter activity was a consequence of non-specific toxicity (Figure 1). When dose-response curves are fitted (GraphPad Prism, USA) the respective EC_{50} and LD_{50} values obtained using PC12 cells are 9.2 μM and 2.8 μM , confirming inhibition of *SOD1* by pyrimethamine is likely to be a non-specific consequence of cytotoxicity. In addition, we also analyzed the effect of pyrimethamine on endogenous *SOD1* in HeLa cells. When normalized to the DMSO controls, the pyrimethamine-treated cells, at all concentrations tested, showed no differences in *SOD1* protein levels (Figure 2). One way analysis of variance between the DMSO and each concentration tested produced a p value of 0.95, indicating no significant difference between the means. Addition of pyrimethamine to HeLa cells also produced a significant level of toxicity; exposures to 2.5 and 25 μM lead respectively to 41% and 72% reductions in viability.

We next examined the effect of pyrimethamine on endogenous *SOD1* levels in WT mice. Pyrimethamine is widely reported to cross the blood brain barrier [23] [24], and it is routinely used to treat CNS toxoplasmosis. Moreover, the chemical structure adheres to quantitative structure activity relationship (QSAR) parameters [25] that are characteristic of compounds that are likely to show CNS penetrance. Mice were treated daily for 14 days with pyrimethamine at 10mg/kg/d by IP. *SOD1* protein levels were the tested in brain, spinal cord and liver homogenates. No significant difference was observed in *SOD1* levels in treated and untreated mice (Figure 3). T-test analysis gave p values of 0.55, 0.25 and 0.54 for brain, spinal cord and liver respectively. We therefore conclude that in these cell models and wild-type mice, pyrimethamine does not affect levels of *SOD1*.

Effects on *SOD1* promoter of drugs currently investigated in ALS

To test whether any drugs currently in clinical trials in human or murine ALS inhibit expression of *SOD1* protein, we determined dose response curves for the test compounds using our PC12 model. 34 drugs in total were tested (Table 1), 17 were in clinical trials in ALS patients; the others are being investigated in the *SOD1* ALS mouse model. 23 compounds had no effect on the *SOD1* promoter (labeled no effect in Table 1). Eleven compounds were shown to inhibit fluorescence, but in each case the LD_{50} was lower than the EC_{50} , indicating non-specific, cytotoxic activity (labeled toxic in Table 1). Three showed an inhibitory effect that was greater than the observed toxicity (labeled re-screened in Table 1). The effect of these three compounds (fluorouracil, progesterone and cyclosporine) was marginal. The $\text{EC}_{50}/\text{LD}_{50}$ for these three compounds was 2.1 $\mu\text{M}/6.6 \mu\text{M}$ for fluorouracil, 9.8 $\mu\text{M}/14.0 \mu\text{M}$ for progesterone and 9.8 $\mu\text{M}/30.6 \mu\text{M}$ for cyclosporine. In

addition, when re-screened at single concentrations near the EC₅₀ all three showed no significant difference between SOD1 inhibition and cytotoxicity (data not shown).

Screening of the 1,040 US Drug collection Library

We next screened for potential inhibitors of the SOD1 promoter among 1,040, FDA approved, compounds in the US Drug Collection (MicroSource, USA). The PC12 SOD1-promoter cell line has previously been used to screen several compound libraries [12], testing compounds at a concentration of 1 μ M. In those earlier screens, no hits were identified. In the present study we used a 96 well plate format and tested each compound at 2.5 μ M. An average Z' of 0.3 and an average Signal to background ratio of 3.2:1 was calculated for this assay. Nine compounds (dactinomycin, mitomycin C, cytarabine, daunorubicin, pyriithione zinc, phenylmercuric acetate, cycloheximide, doxorubicin and epirubicin hydrochloride) reduced the level of fluorescence by 30% or more and thus were designated hits. When re-screened and analyzed for cytotoxicity, all were cytotoxic; the reduction in *SOD1* promoter activity being non-specific. These data demonstrate that none of the FDA-approved compounds currently available in the collection significantly reduce *SOD1* promoter activity.

Discussion

The goal of this study was to identify compounds that reduce levels of SOD1 protein without altering cellular viability; such compounds might be beneficial in mutant-*SOD1*-mediated ALS. We elected to examine pyrimethamine, several compounds currently being tested in trials in human and murine ALS and a set of 1,040 FDA approved compounds. Of these, only pyrimethamine has previously been reported to lower levels of SOD1 without any associated toxicity [19], [22]. In our PC12 SOD1-reporter assay, pyrimethamine reduced fluorescence but simultaneously induced cytotoxicity, findings that strongly suggest that the down-regulation of expression of the *SOD1* gene is non-specific. To further pursue this result, we also tested the effect of pyrimethamine on endogenous SOD1 levels in HeLa cells and wild type mice and were again unable to discern any SOD1 protein reduction after treatment. We therefore cannot conclude that pyrimethamine attenuates *SOD1* expression.

Additional screening of 34 drugs presently in human or mouse ALS trials, as well as a set of 1,040 FDA approved drugs, also failed to identify any inhibitors of *SOD1* transcription. It should be noted that some of these drugs might act to reduce SOD1 levels via mechanisms of gene silencing other than promoter inhibition; which would not be detected in our assay, which directly assessed *SOD1* promoter activity. Such examples include the aminoglycosides, erythromycin [26] and PTC 124, which influence post transcriptional events (e.g. modulating mRNA stability and efficiency during protein synthesis), and are candidate therapies for disorders like Duchenne muscular dystrophy and cystic fibrosis [27].

Silencing of the mutant SOD1 gene is an attractive target for future ALS therapies. Small molecules represent just one experimental paradigm. Approaches using RNAi techniques are being developed; siRNA has been shown to slow disease progression in mice [11] [28], as have lentiviral constructs [29]. Intrathecally delivered anti-sense oligonucleotides reduced SOD1 protein levels in rat spinal cord, and produce a corresponding increase in survival in transgenic ALS rats [30]. This particular therapy is now currently in trial in ALS patients. Activated Protein C (APC), an endogenous plasma protease which modulates gene expression by activation of several transcription factors, down-regulates SOD1 expression, leading to delayed progression and extended survival in ALS mice [31]. These studies establish the general principle that suppression of SOD1 expression can prolong survival in rodent SOD1 ALS models. For this reason, we are currently extending our studies using larger, more diverse small molecule libraries to identify novel lead compounds. In our view,

it is likely that even minimal suppression of SOD1 levels will be beneficial in ALS mediated by mutant SOD1 protein and that small molecule-repressors of SOD1 will prove clinically efficient.

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Figure 1.

Dose response curves illustrate the effect of pyrimethamine on SOD1 inhibition ($EC_{50} = 9.2$) and toxicity ($LD_{50} = 2.8$) in PC12 cells. *SOD1* promoter inhibition and cytotoxicity are shown as a percentage decrease relative to the DMSO treated control cells.

Figure 2.

Pyrimethamine does not affect endogenous SOD1 levels in HeLa cells. (A) Western blot analysis of lysates from HeLa cells exposed to DMSO (0.25%) or pyrimethamine (25 μM , 2.5 μM or 0.25 μM). Pyrimethamine does not alter levels of Actin or SOD1 levels, as compared to DMSO. (B) Densitometry shows no significant difference in SOD1 levels is observed at any of the concentrations tested.

Figure 3.

Pyrimethamine does not affect SOD1 levels in mice. (A) As assessed by Western blotting, neither pyrimethamine (+) nor PBS control (-) treatment depresses levels of SOD1 or Actin. (B) Densitometry shows no significant difference in control (-) or treated groups (+), n=5.

Figure 4. summarizes results of screening of the 1,040 compound US Drug Collection Library for inhibition of *SOD1* promoter activity (fluorescence) as a percentage of decrease relative to DMSO cells. Compounds that showed a 30% inhibition of the *SOD1* promoter were designated hits.

Table 1

List of candidate ALS compounds analyzed using the PC12-SOD1 promoter cells assay. 34 compounds in animal (Table 1A) or clinical (Table 1B) studies were analyzed for their ability to inhibit the *SOD1* promoter. Compounds that showed less than 30% inhibition were designated 'no effect'. Those in which the LD₅₀ was lower than the ED₅₀ were designated toxic. Three compounds had an EC₅₀ lower than LD₅₀; these were re-screened but showed no significant difference between inhibition and cytotoxicity at the EC₅₀.

Table 1A		
Compound	Trial Status	Effect
Adenosine	Animal	No effect
Chlorpromazine	Animal	No effect
Colchicine	Animal	No effect
Cycloheximide	Animal	Toxic
Cycloserine	Animal	No effect
Desipramine	Animal	Toxic
Fluoruracil	Animal	Re-screened
Ginseng	Animal	No effect
Hydroxyurea	Animal	No effect
Indomethacin	Animal	No effect
Lithium Citrate	Animal	No effect
Methotrexate	Animal	Toxic
Penicillamine	Animal	No effect
Progesterone	Animal	Re-screened
Puromycin	Animal	Toxic
Quinacrine	Animal	Toxic
Tamoxifen	Animal	Toxic
Vincristine	Animal	No effect

Table 1B		
Compound	Trial Status	Effect
Acetyl cysteine	Clinical	No effect
Ceftriaxone	Clinical	No effect
Celecoxib	Clinical	No effect
Creatine	Clinical	No effect
Cyclosporine	Clinical	Re-screened
Gabapentin	Clinical	No effect
Memantine Hydrochloride	Clinical	No effect
Minocyclin	Clinical	Toxic
PBA(Sodium phenyl butyrate)	Clinical	No effect
Pimozide	Clinical	No effect
Pramipexole dihydrochloride	Clinical	No effect
prednisolone	Clinical	No effect

Table 1B		
Compound	Trial Status	Effect
Riluzole	Clinical	No effect
Sodium Valproate	Clinical	No effect
Sulindac	Clinical	No effect
Thalidomide	Clinical	No effect