[Frontiers in Bioscience E4, 2701-2708, June 1, 2012]

A high-throughput screen to identify inhibitors of SOD1 transcription

Paul D Wright[1], Nicholas Wightman[1], Mickey Huang[2], Alexandra Weiss[1], Peter C Sapp[1,3], Gregory D Cuny[2], Adrian J Ivinson[2], Marcie A Glicksman[2], Robert J Ferrante[4,5], Wayne Matson[5], Samantha Matson[6,7], Robert H Brown Jr[1]

[1]Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01655, United States, [2]Laboratory for Drug Discovery in Neurodegeneration, Harvard NeuroDiscovery Center, Harvard Medical School and Brigham and Women's Hospital, Cambridge, MA 02139, United States, [3]Howard Hughes Medical Institute, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, United States, [4]Neurological Surgery, Neurology, and Neurobiology Departments, University School of Medicine of Pittsburgh, Pittsburgh, PA 15213, United States, [5]Geriatric Research Educational and Clinical Center (00-GR-H), V.A. Pittsburgh Healthcare System, 7180 Highland Drive, Pittsburgh, PA 15206, United States, [6]Neurology Service, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, United States, [7]MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital, Charlestown, MA 02129, United States

TABLE OF CONTENTS

1. ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative motor neuron disease. Approximately 20% of familial ALS cases are caused by mutations in the Cu/Zn superoxide dismutase (SOD1) gene. Rodents expressing mutant SOD1 transgenes develop progressive, fatal motor neuron disease and disease onset and progression is dependent on the level of SOD1. We investigated the possibility that a reduction in SOD1 protein may be of therapeutic benefit in ALS and screened 30,000 compounds for inhibition of SOD1 transcription. The most effective inhibitor identified was N-{4-[4-(4-methylbenzoyl)-1-piperazinyl]phenyl}-2-thiophenecarboxamide (Compound ID 7687685), which in PC12 cells showed an EC50 of 10.6 microM for inhibition of SOD1 expression and an LD50 >30 microM. This compound was subsequently shown to reduce endogenous SOD1 levels in HeLa cells and to exhibit a modest reduction of SOD1 protein levels in mouse spinal cord tissue. These data suggest that the efficacy of compound 7687685 as an inhibitor of SOD1 gene expression is not likely to be clinically useful, although the strategy reported could be applied broadly to screening for small molecule inhibitors of gene expression.

2. INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease or Motor Neuron Disease, is a 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 subsequently become generalized. Death, due to respiratory failure, usually occurs within three to five years. ALS is uniformly fatal. Riluzole is the single FDA-approved treatment for ALS and increases lifespan by only three to six months without substantially alleviating symptoms (1). The majority of ALS cases are sporadic but approximately 10% are familial. Mutations in many genes, such as those encoding cytosolic Cu/Zn superoxide dismutase (SOD1) (2), FUS/TLS (3, 4), TDP-43 (5) and angiogenin (6), have been identified as causes of familial ALS.

The most extensively investigated ALS gene is SOD1, in which nearly 150 ALS-associated mutations have been reported (7). These mutations, which are predominantly missense variants, are found throughout the protein structure but all lead to selective degeneration of

motor neurons. Multiple mechanisms of SOD1 toxicity are hypothesized including protein aggregation, apoptosis, proteasome inhibition and aberrant chemistry. Data overwhelmingly support 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 expressing high levels of mutant SOD1 develop a disease phenotype but those expressing at a lower level do not (9). A similar effect is observed in rats expressing mutant SOD1 (10). There is accumulating evidence that interventions that reduce SOD1 levels improve survival in transgenic rodent models. These include intraventricular administration of SOD1 antisense oligonucleotides (11), lentiviral delivery of SOD1 siRNA (12), immunization with misfolded SOD1 protein (which lowers mutant SOD1 levels in the spinal cord) (13) and the use of Cre/loxP recombinase technology to reduce SOD1 expression in specific neural cell types in the CNS (14, 15). These factors lead us to investigate the possibility that a reduction in SOD1 protein levels using small molecules could attenuate ALS susceptibility and the rate of disease progression. This strategy is precedented in studies documenting that neurodegeneration in mice expressing mutant huntingtin was dramatically reversed at 34 weeks of age by doxycycline-induced inhibition of the mutant transgene (16). As a first step to test the hypothesis a reduction in SOD1 protein could ameliorate the course of ALS, we have developed a cell based screen for small molecules capable of inhibiting the SOD1 promoter (17), with the intention of reducing levels of mutant SOD1 protein. Sequence analysis of the SOD1 promoter has shown the 5' region of SOD1 to contain multiple binding sites for transcription factors. The proximal (400bp) region is known to contain one CF1, one C/EBP, three SP1, three AP2 and GCF binding sites. The distal region has in excess of 50 transcription factor binding sites including AP3, NF-IL6, SP-1, AP2, AP1, SEF-1, HSF, PEA3, ISGF2, MEP-1, NF-kappabeta, TEF2, HiNF-A, CF1 and C/EBP (18). Additionally, ELK-1 and Ying-Yang 1 (19) participate in regulating SOD1 transcription. Hypoxia response elements, peroxisome proliferator response elements, metal response elements, antioxidant response elements and xenobiotic response elements are also present in the SOD1 promoter region (19, 20). Of particular interest was the finding that a 50bp deletion in the SOD1 promoter, observed in an ALS patient, led to an increased age of onset (21). This deletion was associated with reduced levels of SOD1 gene expression.

A library of 30,000 small molecules was initially screened using the fluorescence based assay developed previously in our laboratory (17). In the present study, high throughput screening identified N-{4-[4-(4-methylbenzoyl)-1-piperazinyl]phenyl}-2-thiophenecarboxamide as an inhibitor of the SOD1 promoter. This compound was subsequently shown to lower SOD1 mRNA and protein levels in multiple cell and mouse models. It was also shown to readily pass through the blood brain barrier and high levels were detected in the brain and spinal cord.

3. MATERIALS AND METHODS

3.1. Cell culture

A PC12 cell line stably expressing 2.2Kb of the SOD1 promoter region flanked by GFP (generated in (17)) was maintained in DMEM-F12 (Gibco, USA) supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum (FBS), 1x penicillin, 1x streptomycin and 500 microg/ml G418 (Invitrogen, USA) at 37ºC with 5% CO2. HeLa cells were maintained in DMEM (Gibco, USA) with 10% (v/v) FBS, 1x penicillin and 1x streptomycin at 37ºC with 5% CO2. For growth curves HeLa cells were plated at 100 cells/ microl and typically incubated for one hour to allow attachment. Cells were then exposed to a single 30 microM dose of 7687685 or compound was added every 24 hours – media was aspirated and replaced by fresh media containing 30 microM of 7687685. At 4 hours, 24 hours, 48 hours and 72 hours cells were trypsinized and counted using a hemocytometer. For siRNA experiments cells were plates at 100 cells/ microl then transfected with 20 nM SOD1 siRNA (Avencia, USA) using Lipofectamine 2000, as per manufacturers guidelines. Cells were then counted as above. In each case cells remained in contact with 7687685 or siRNA until trypsinisation.

3.2. Compound screening

Large numbers of PC12 cells were generated using Corning® CellSTACK™ Culture Chambers (Sigma-Aldrich, USA). 200 microl of cells (250 cells/ microl) 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 around one hour to allow attachment. For the Chembridge library (Chembridge, USA) 0.5 microl of 1 mM stock was added to each well giving a final compound concentration of 2.5 microM. This compound library was assembled using small molecules with properties that allow maximum blood brain barrier penetration. For dose response curves compounds were typically added to final concentrations of 30 microM, 10 microM, 3 microM, 1 microM, 0.3 microM, 0.1 microM, 0.03 microM, 0.01 microM, 0.003 microM, 0.001 microM, 0.0003 microM and 0.0001 microM. 0.5 microl of compound solution 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 37ºC, conditions previously shown to give optimal assay conditions (17). For measurements of GFP fluorescence, plates were washed in TBS, and then lysed with 200 microl RIPA buffer (150mM NaCl, 1% Triton X, 0.5% sodium deoxycholate, 0.5% SDS, 40 mM Tris-HCl). Fluorescence levels were determined using 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 Cell Titer-Glo assay (Promega, USA). This assay uses luciferase with luciferin, oxygen and ATP as substrates in a reaction that produces oxyluciferin and releases energy in the form of light. The amount of light produced is directly proportional to cellular ATP levels. Cells were treated and processed as above and luminescence was measured on an EnVision 2102 Multilabel plate reader (Perkin Elmer, USA).

3.3. Western blotting

Western blot analysis was carried out to ascertain compounds' effects on endogenous human SOD1. For *in vitro* work HeLa cells were plated into 6-well plates at a dilution of 200 cells/ microl. Once cells were attached 30 microM of 7687685, 7614572 or 3 microM of 7698016 was added, and cells were then incubated for 72 hours at 37ºC with 5% CO2. Cells were washed with PBS and then lysed in 300 microl 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). 10 microg of protein lysate was then loaded onto the gel. For gel electrophoresis 12% Tris-Glycine gels were used (Invitrogen, USA) and typically run at 125V for 90 minutes using Tris-Glycine SDS running buffer (Invitrogen, USA). Samples were transferred to nitrocellulose using the i-Blot® transfer device (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 then used (LiCor, USA) and blots visualized and analyzed using the Odyssey Imaging System (LiCor, USA)

3.4. Quantitative PCR

HeLa Cells were maintained and treated with drug as for western blotting. After 72 hours RNA was extracted using TRIZOL (Invitrogen, USA) as per manufacturer's guidelines. RNA was quantified using the NanoDrop 1000 (Thermo Scientific, USA). cDNA for qPCR was synthesized using Superscript III Platinum qRT-PCR kit with ROX (Invitrogen, USA). SOD1 target probe hs0096176_m1 (Applied Biosystems, USA) and beta-2-microglobulin (B2M) endogenous control probe 4326319E (Applied Biosystems, USA) were used to quantify SOD1 and B2M mRNA levels using the Taqman 7900HT detection system (Applied Biosystems, USA). For global transcription experiments cells were treated as described here or transfected with SOD1 siRNA as previously described then primers Hs01100224_m1, Hs00606522, Hs01034249_m1, Hs00943178_g1, were used to quantify Fused in Sarcoma (FUS), tumor protein p53 (TP53), TAR DNA-binding protein 43 (TARDBP) and phosphoglycerate kinase 1 (PGK1). All experiments analyzed as described in (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing /documents/generaldocuments/cms_042502.pdf)

3.5. *In vivo* studies

G93A SOD1 transgenic mice of mixed background (Jackson Laboratories, USA) aged 6 weeks were treated by daily intraperitoneal injections for 14 days with 75mg/kg/d of N-{4-[4-(4-methylbenzoyl)-1-piperazinyl]phenyl}-2-thiophenecarboxamide dissolved in DMSO or , as a control, an equivalent volume of DMSO without drug. After treatment, mice were euthanized by CO2 followed by decapitation. Spinal cords and brains were removed and tissues perfused with PBS. Spinal cord samples were homogenized in PBS containing 0.5% Triton X-100 (Sigma Aldrich, USA), 1mM EDTA and 1x protease inhibitors (Roche, Switzerland) using sonication on ice at 20% for 10 seconds (Sonic dismembrator Model 500, Fisher, USA). Protein concentrations were then measured using the BCA protein assay kit (Thermo, USA). 20 microg of total protein was loaded onto gels and electrophoresis and western blotting were carried out as described below. To assess levels of drug present in treated animals' blood, brain and spinal cord, tissues were mixed with extraction buffer (100% ice cold methanol), sonicated on ice for 20 seconds then spun at 15,000 rpm for 30 minutes. 100 microl of supernatant was then analyzed using Ceas 16 channel EC-HPLC. Experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

4. RESULTS

4.1. Screening 30,000 small molecules for inhibitors of the SOD1 promoter

Using a cell line stably expressing the human SOD1 promoter flanked by GFP, the Chembridge small molecule library (30,000 compounds) was screened to identify inhibitors of fluorescence and thus transcription mediated by the SOD1 promoter. Previous work using this cell line (17) had shown that compounds that reduce fluorescence by 30% were to be designated as hits. In this study an average Z' value of 0.35 and an average signal to background ratio of 1:5.22 (Figure 1A) were observed in the initial screen. From this first screen of 30,000 compounds, 20 were confirmed as hits. These were then investigated with fresh compound, using 12 point dose curves (30 microM to 0.0001 microM) to ascertain their effects on SOD1 inhibition and cytotoxicity. For 17 compounds, the dose response curves for SOD1 inhibition and toxicity overlapped, while for three compounds (7614572, 7698016 and 7687685) there was a modestly more pronounced effect on SOD1 inhibition, indicating that the reduction was not merely a result of a decrease in general viability (Figure 1B). The EC50/LD50 values for 7614572, 7698016 and 7687685 were 9.7 microM/>30 microM, 0.8 microM/1.1 microM and 10.6 microM/>30 microM respectively (>30 microM indicates that no toxicity was observed at any of the concentrations tested). To ensure that lead compounds were not acting as a fluorescent quencher and were actively inhibiting SOD1 we carried out qPCR and western blotting to assess endogenous SOD1 mRNA and protein levels using HeLa cells (a human cell line as opposed to the rat-derived PC12 cells). Of the three compounds, only compound 7687685 showed a statistically significant ($p<0.05$) decrease in SOD1 mRNA levels (23% reduction) when normalized to beta2 macroglobulin (Figure 1C). This was associated with a decrease in SOD1 protein levels (western blots normalized to actin, Figure 1C). Similarly 7687685 caused a decrease in endogenous SOD1 protein levels in PC12 cells and SOD1 dismutation activity in HeLa cells (data not shown).

4.2. Structure activity relationship

Structure-activity relationship studies were carried out on the lead compound 7687685. Twenty-two analogues were selected on the basis of modification of selected side groups. When tested across multiple doses, none showed a significant improvement in EC50 without associated toxicity, as compared to compound 7687685

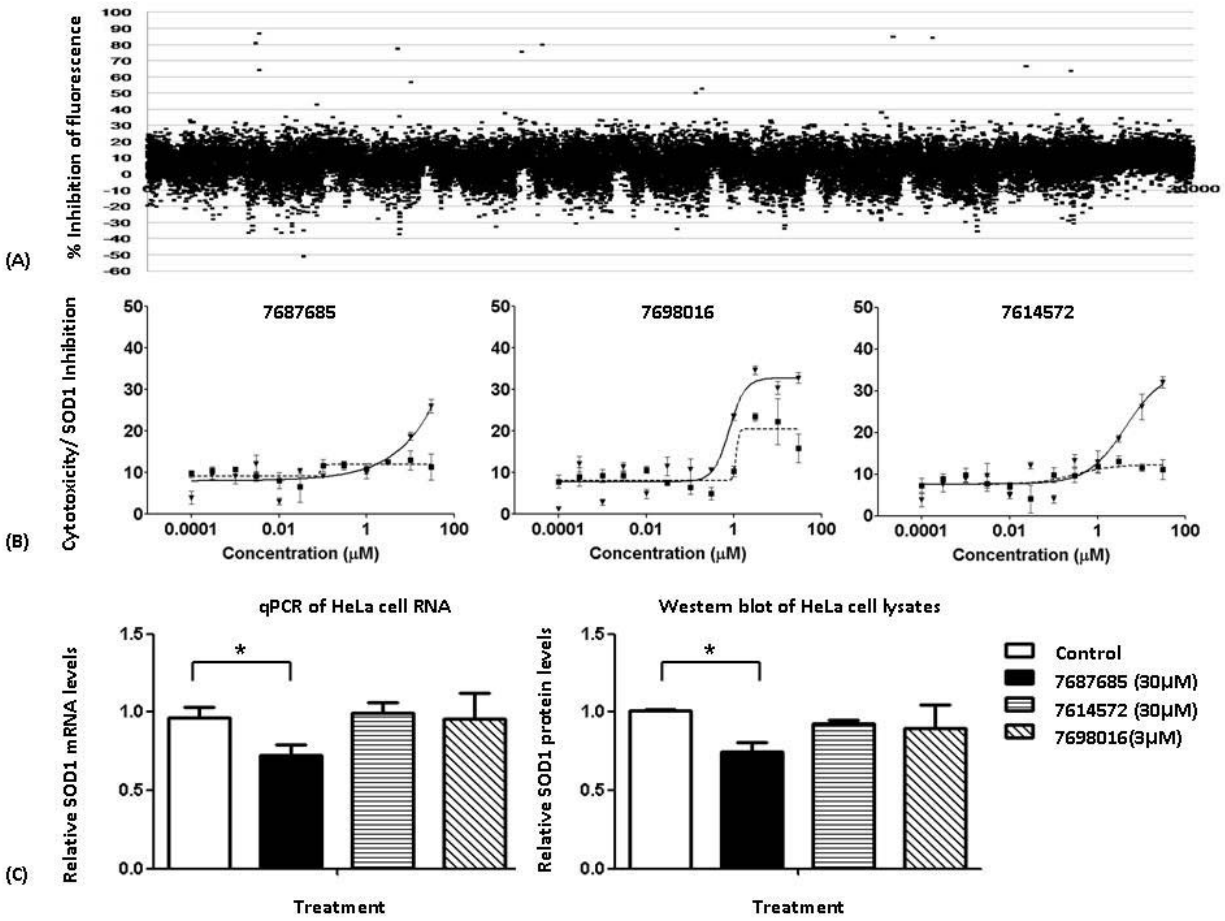


Figure 1. (A) Screening of 30,000 compounds from the Chembridge library for inhibitors of the SOD1 promoter. % Inhibition of fluorescence shows the effects of compounds on the SOD1 promoter. Those demonstrating inhibition of 30% or greater were designated 'hits'. (B) 12 point dose response curves for compounds 7687685, 7698016 and 7614572 illustrate the dose dependence of cytotoxicity (dashed line) and SOD1 inhibition (solid line) relative to DMSO treated controls. Each of these compounds shows a greater inhibition of SOD1 promoter mediated fluorescence than cytotoxicity. (C) qPCR and Western blot analyses document that 30μM 7687685 reduces levels of endogenous SOD1 mRNA and endogenous SOD1 protein in HeLa cells (n=3, $p<0.05$). 30 microM 7614572 and 3 microM 7698016 showed no effect on SOD1 mRNA or endogenous SOD1 protein.

(data not shown). Also several compounds analyzed by western blotting showed no significant differences in SOD1 protein reduction compared to the lead (data not shown). For this reason, further studies focused on compound 7687685 (N-{4-[4-(4-methylbenzoyl)-1-piperazinyl]phenyl}-2-thiophenecarboxamide).

4.3. Effects of SOD1 inhibition on HeLa cell growth rates and general transcription

In studies of the influence of compound 7687685 on mRNA and protein levels in HeLa cells, it became apparent that this molecule slowed the growth rate of the cells. Growth curves (Figure 2A) confirmed that while the doubling time for control HeLA cells was approximately 24 hours, a single treatment with compound 7687685 slowed the doubling time over a three day period to approximately 48 hours. When the compound was added every 24 hours, a substantially greater inhibition of growth was observed (Figure 2A); in this instance, the cells failed to double even after 96 hours of growth. This effect was not due to cell death; HeLa cells plated at high confluency and treated with compound 7687685 did not show significant loss in cell numbers after 24 or 48 hours (data not shown). To validate that this effect on HeLa cells reflected inhibition of SOD1 expression and not a generalized toxicity effect, HeLa cells were transfected with SOD1 siRNA; this completely inhibited cell growth, with cultures showing no significant increase in cell number 72 hours post transfection (Figure 2A). These data are consistent with published data showing that silencing of SOD1 by inhibitory RNA is toxic to numerous cell lines and causes senescence in fibroblasts (22, 23).

To further evaluate the effect of 7687685 on general cellular transcription an additional qPCR profile determined the impact of this compound on expression of several genes other than SOD1 and B2M, including FUS, TP53, TARDBP and PGK1. For each gene, the expression

Table 1. (A) Levels of compound 7687685 in blood, brain, and three regions of the spinal cord after 14 days of once-daily intraperitoneal administration. (B) Levels of SOD1 protein in mouse spinal cord after 14 days of once-daily intraperitoneal administration with compound 7687685

Tissue	**[7687685] ng/mg**	
A		
	Average	Std Dev
Blood	0.66	0.20
Brain	0.96	0.11
Cervical Cord	0.47	0.35
Thoracic Cord	0.28	0.19
Lumbosacral Cord	0.14	0.06
A		
	Relative SOD1 protein level	Std dev
Control	1.000	0.02
Compound 7687685	0.958	0.01

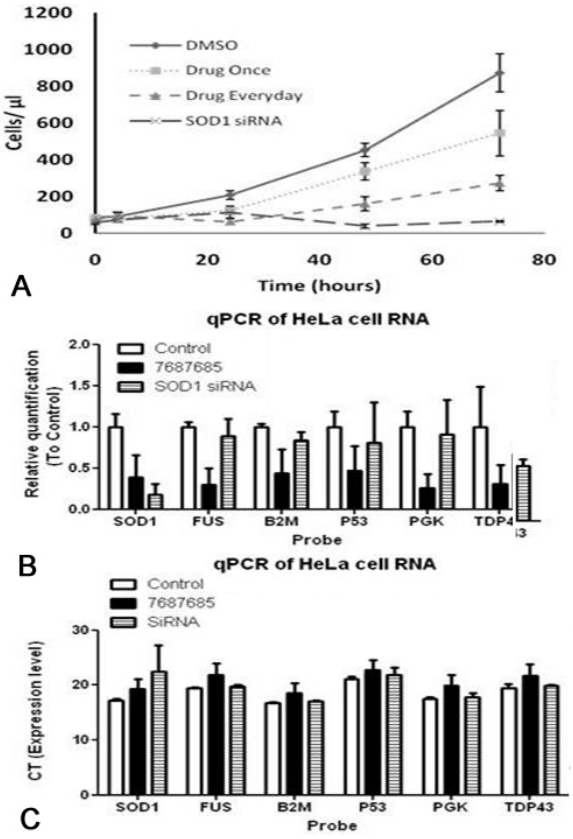


Figure 2. (A) Single or multiple exposures to 30 microM 7687685 reduce the growth rate of HeLa cells. Heavy solid line: DMSO; light solid line: single treatment at start of experiment; short dashed line: once daily treatment for three days; long dashed line: treatment with siRNA directed against SOD1. In each paradigm cells were incubated in the presence of drug or siRNA for 4h, 24h, 48h or 72h. (B) Expression analysis using qPCR documents that there is off target suppression of expression of all six genes tested in HeLa cells treated with either 7687685 or SOD1 siRNA. mRNA levels for each probe are normalised to the control samples (C) Expression analysis shows off target effects of HeLa cells treated with 7687685 and SOD1 siRNA. Higher CT values reflect lower mRNA levels.

level was reduced after exposure to compound 7687685, indicating that this molecule mediates several off target effects, and is likely a weak repressor of global transcription. In parallel studies, we determined that in the same HeLa cell culture system, anti-SOD1 siRNA therapy also reduced expression of these genes, albeit to a lesser extent. With this experimental paradigm we cannot completely distinguish between a direct effect of compound 7687685 on multiple genes and an indirect effect mediated through SOD1 inhibition (Figures 2B and 2C).

4.4. Effects of compound 7687685 on SOD1 transcription *in vivo*

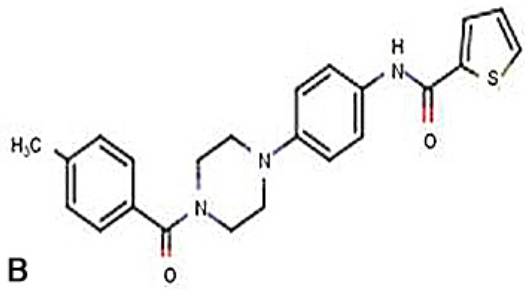
The over-arching goal of this project is to identify compounds that significantly reduce expression of mutant SOD1 *in vivo*, independently of whether the effect is specific or accompanied by off-target effects. We therefore next examined the *in vivo* effects of compound 7687685, to establish whether SOD1 inhibition could be observed *in vivo*, and whether this occurs without significant toxicity. We first conducted an experiment to define the maximum tolerated dose in wild type mice. Animals were initially injected intraperitoneally with 1mg/kg/d of drug; the dose was then doubled each day until toxicity was observed (as defined by behavioral changes or weight loss). From this, we estimated that 75 mg/kg/d is the maximal, non-toxic dose. To determine whether compound 7687685 reduces SOD1 gene expression *in vivo*, we then treated transgenic SOD1G93A mice with this compound at 75 mg/kg/d for 14 days, after which they were sacrificed; blood, brain and spinal cord were analyzed for levels of compound using EC-HPLC. Significant levels of drug were observed in blood, brain and spinal cord (Table 1A); indicating the compound has good transport across the blood brain barrier. Given the chemical properties (molecular weight, hydrogen bond donors/acceptors, polar surface area, logP, rotatable bonds) of this molecule (Figure 3), this was expected (24). Spinal cord tissue from these mice was examined by western blot to ascertain the effects on SOD1 (Table 1B). A small but statistically significant decrease in SOD1 protein levels was observed (5%, p=0.04). While this effect is relatively small, it nonetheless corroborates the cell data, which demonstrate that this compound was only effective at high dosages (>30 microM). It thus appears that despite the effective partition of this molecule between blood and spinal cord, this dose is inadequate to achieve clinically meaningful inhibition of SOD1 expression. Moreover, the toxicity of our lead compound will not permit further increases in the dosage.

5. DISCUSSION

The goal of this study was to identify small molecules that inhibit the human SOD1 promoter. A screen of a library of 30,000 compounds identified N-{4-[4-(4-methylbenzoyl)-1-piperazinyl]phenyl}-2-thiophenecarboxamide (EC50 = 10.6 microM) as a candidate. This compound was subsequently shown to down-regulate levels of SOD1 mRNA and protein and to reduce SOD1 activity in HeLa cells. It also reduced SOD1 protein levels in transgenic SOD1G93A mice. Whilst this compound was shown to cause off target

Property	Value
Molecular Weight	406
logP	2.79
H-bond acceptors	1
H-bond donors	2
tPSA	52.6
Rotatable bonds	4

A



B

Figure 3. (A) Selected properties of compound 7687685 that predict effective penetrance of the blood-brain barrier. (B) Chemical structure of compound 7687685.

inhibition of four other genes, and thus appears not to act specifically on SOD1 alone, it did in fact reduce SOD1 levels in the brain, albeit at a modest, though statistically significant, level. Furthermore it should be noted that a small increase <20% may be of therapeutic benefit in ALS. It is conceivable that chemical modification of the lead compound's structure might increase its efficacy, although this was not evident in twenty-two related compounds so tested. We are therefore doubtful that further structural enhancements, or altered formulation and delivery, of this particular scaffold will allow us to develop this as a clinically relevant inhibitor of SOD1 expression. Our findings do, however, demonstrate that weak general transcription inhibitors when administered at non-toxic doses may represent an interesting class of molecules for SOD1 inhibition in ALS.

We observed that proliferation of immortalized cell lines is inhibited when SOD1 expression is attenuated, in agreement with previous reports (22). Specifically, application of siRNA to HeLa cells at levels that eliminated detection of SOD1 on a Western blot blocked cell proliferation completely. The implication of these data is that, in cell-based models, potent inhibitors of SOD1 are toxic. In this screen, we identified several compounds that clearly inhibited SOD1-mediated GFP fluorescence in the primary assay but which, in the dose response assay, were toxic at LD50's approximately equivalent to the EC50's. In this circumstance, one could not determine whether the toxicity was a consequence of SOD1 inhibition or some more general toxic influence. We therefore assayed these compounds using Western blotting at the EC50 and determined that none inhibited SOD1 expression. Similarly, structure-activity screening of analogues of our lead compound was problematic because small increases in efficacy (i.e. enhanced suppression of SOD1 expression) were associated with increases in toxicity. For all of these reasons, we have now redesigned the screening assay to eliminate toxicity resulting from inhibition of SOD1 by incorporating into test cells a SOD1 transgene driven by an exogenous promoter.

Lowering SOD1 levels represents an attractive target for treatment of ALS mediated by mutant SOD1. To date, no small molecules are known that repress SOD1 expression. Pyrimethamine was recently reported to inhibit SOD1 expression (25), although this result has not been confirmed (26). There has been a single report that activated protein C suppresses SOD1 and extends survival in transgenic ALS mice (27). Most current approaches to silencing SOD1 have explored the use of methods that block translation of RNA, such as anti-sense oligonucleotides (11) and inhibitory RNA (28, 29). Indeed, a current trial of anti-SOD1 anti-sense oligonucleotides is now underway in patients with mutant SOD1-mediated ALS (30). Regardless of the specific modality for SOD1 inhibition, several critical issues arise in application of these therapies. A central problem is pharmacodynamic: do the agents reach the target cells at doses appropriate for silencing? Another issue is the temporal profile of motor neuron death and the time frame within which SOD1 silencing is effective. A third issue is the potential *in vivo* cytotoxicity of suppression of cell proliferation following SOD1 inhibition. Reassuring in this regard are the observations that complete genetic inactivation of SOD1 in mice is not associated with profound pathology (although the SOD1 knock-out mice show subtle impairment of axonal viability following injury) (8). Viewing all of these issues, it remains highly likely that inhibition of SOD1 expression will be therapeutic in ALS cases arising from mutations in the SOD1 gene. Moreover, recent data implicates misfolded wild-type (non-mutant) SOD1 in the pathogenesis of sporadic ALS; strategies to silence this gene may therefore be beneficial in non-familial ALS (31, 32).

6. ACKNOWLEDGEMENTS

This study was supported by the National Institute for Neurological Disease and Stroke, the Angel Fund, the ALS Association, Project ALS, Pierre L. de Bourgknecht ALS Research Foundation, the Al-Athel ALS Foundation and the ALS Therapy Alliance.

7. REFERENCES

1. R. G. Miller, J. D. Mitchell, M. Lyon and D. H. Moore: Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). *Amyotroph Lateral Scler Other Motor Neuron Disord*, 4(3), 191-206 (2003)

2. D. R. Rosen: Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, 364(6435), 362 (1993)

3. C. Vance, B. Rogelj, T. Hortobagyi, K. J. De Vos, A. L. Nishimura, J. Sreedharan, X. Hu, B. Smith, D. Ruddy, P. Wright, J. Ganesalingam, K. L. Williams, V. Tripathi, S. Al-Saraj, A. Al-Chalabi, P. N. Leigh, I. P. Blair, G. Nicholson, J. de Belleroche, J. M. Gallo, C. C. Miller and C. E. Shaw: Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science*, 323(5918), 1208-11 (2009)

4. T. J. Kwiatkowski, Jr., D. A. Bosco, A. L. Leclerc, E. Tamrazian, C. R. Vanderburg, C. Russ, A. Davis, J. Gilchrist, E. J. Kasarskis, T. Munsat, P. Valdmanis, G. A. Rouleau, B. A. Hosler, P. Cortelli, P. J. de Jong, Y. Yoshinaga, J. L. Haines, M. A. Pericak-Vance, J. Yan, N. Ticozzi, T. Siddique, D. McKenna-Yasek, P. C. Sapp, H. R. Horvitz, J. E. Landers and R. H. Brown, Jr.: Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science*, 323(5918), 1205-8 (2009)

5. J. Sreedharan, I. P. Blair, V. B. Tripathi, X. Hu, C. Vance, B. Rogelj, S. Ackerley, J. C. Durnall, K. L. Williams, E. Buratti, F. Baralle, J. de Belleroche, J. D. Mitchell, P. N. Leigh, A. Al-Chalabi, C. C. Miller, G. Nicholson and C. E. Shaw: TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science*, 319(5870), 1668-72 (2008)

6. M. J. Greenway, M. D. Alexander, S. Ennis, B. J. Traynor, B. Corr, E. Frost, A. Green and O. Hardiman: A novel candidate region for ALS on chromosome 14q11.2. *Neurology*, 63(10), 1936-8 (2004)

7. http://alsod.iop.kcl.ac.uk/

8. A. G. Reaume, J. L. Elliott, E. K. Hoffman, N. W. Kowall, R. J. Ferrante, D. F. Siwek, H. M. Wilcox, D. G. Flood, M. F. Beal, R. H. Brown, Jr., R. W. Scott and W. D. Snider: Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet*, 13(1), 43-7 (1996)

9. M. C. Dal Canto and M. E. Gurney: Neuropathological changes in two lines of mice carrying a transgene for mutant human Cu,Zn SOD, and in mice overexpressing wild type human SOD: a model of familial amyotrophic lateral sclerosis (FALS). *Brain Res*, 676(1), 25-40 (1995)

10. M. Nagai, M. Aoki, I. Miyoshi, M. Kato, P. Pasinelli, N. Kasai, R. H. Brown, Jr. and Y. Itoyama: Rats expressing human cytosolic copper-zinc superoxide dismutase transgenes with amyotrophic lateral sclerosis: associated mutations develop motor neuron disease. *J Neurosci*, 21(23), 9246-54 (2001)

11. R. A. Smith, T. M. Miller, K. Yamanaka, B. P. Monia, T. P. Condon, G. Hung, C. S. Lobsiger, C. M. Ward, M. McAlonis-Downes, H. Wei, E. V. Wancewicz, C. F. Bennett and D. W. Cleveland: Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest*, 116(8), 2290-6 (2006)

12. G. S. Ralph, P. A. Radcliffe, D. M. Day, J. M. Carthy, M. A. Leroux, D. C. Lee, L. F. Wong, L. G. Bilsland, L. Greensmith, S. M. Kingsman, K. A. Mitrophanous, N. D. Mazarakis and M. Azzouz: Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med*, 11(4), 429-33 (2005)

13. M. Urushitani, S. A. Ezzi and J. P. Julien: Therapeutic effects of immunization with mutant superoxide dismutase in mice models of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*, 104(7), 2495-500 (2007)

14. S. Boillee, K. Yamanaka, C. S. Lobsiger, N. G. Copeland, N. A. Jenkins, G. Kassiotis, G. Kollias and D. W. Cleveland: Onset and progression in inherited ALS determined by motor neurons and microglia. *Science*, 312(5778), 1389-92 (2006)

15. K. Yamanaka, S. J. Chun, S. Boillee, N. Fujimori-Tonou, H. Yamashita, D. H. Gutmann, R. Takahashi, H. Misawa and D. W. Cleveland: Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci*, 11(3), 251-3 (2008)

16. A. Yamamoto, J. J. Lucas and R. Hen: Reversal of neuropathology and motor dysfunction in a conditional model of Huntington's disease. *Cell*, 101(1), 57-66 (2000)

17. W. J. Broom, K. E. Auwarter, J. Ni, D. E. Russel, L. A. Yeh, M. M. Maxwell, M. Glicksman, A. G. Kazantsev and R. H. Brown, Jr.: Two approaches to drug discovery in SOD1-mediated ALS. *J Biomol Screen*, 11(7), 729-35 (2006)

18. H. T. Kim, Y. H. Kim, J. W. Nam, H. J. Lee, H. M. Rho and G. Jung: Study of 5'-flanking region of human Cu/Zn superoxide dismutase. *Biochem Biophys Res Commun*, 201(3), 1526-33 (1994)

19. M. S. Chang, H. Y. Yoo and H. M. Rho: Positive and negative regulatory elements in the upstream region of the rat Cu/Zn-superoxide dismutase gene. *Biochem J*, 339 (Pt 2), 335-41 (1999)

20. E. Y. Park and H. M. Rho: The transcriptional activation of the human copper/zinc superoxide dismutase gene by 2,3,7,8-tetrachlorodibenzo-p-dioxin through two different regulator sites, the antioxidant responsive element and xenobiotic responsive element. *Mol Cell Biochem*, 240(1-2), 47-55 (2002)

21. W. J. Broom, M. Greenway, G. Sadri-Vakili, C. Russ, K. E. Auwarter, K. E. Glajch, N. Dupre, R. J. Swingler, S. Purcell, C. Hayward, P. C. Sapp, D. McKenna-Yasek, P. N. Valdmanis, J. P. Bouchard, V. Meininger, B. A. Hosler, J. D. Glass, M. Polack, G. A. Rouleau, J. H. Cha, O. Hardiman and R. H. Brown, Jr.: 50bp deletion in the promoter for superoxide dismutase 1 (SOD1) reduces

SOD1 expression *in vitro* and may correlate with increased age of onset of sporadic amyotrophic lateral sclerosis. *Amyotroph Lateral Scler*, 9(4), 229-37 (2008)

22. G. Blander, R. M. de Oliveira, C. M. Conboy, M. Haigis and L. Guarente: Superoxide dismutase 1 knock-down induces senescence in human fibroblasts. *J Biol Chem*, 278(40), 38966-9 (2003)

23. C. M. Troy and M. L. Shelanski: Down-regulation of copper/zinc superoxide dismutase causes apoptotic death in PC12 neuronal cells. *Proc Natl Acad Sci U S A*, 91(14), 6384-7 (1994)

24. H. Pajouhesh and G. R. Lenz: Medicinal chemical properties of successful central nervous system drugs. *NeuroRx*, 2(4), 541-53 (2005)

25. D. Lange: Abstract C46: Pyrimethamine as a therapy for SOD1 associated FALS: Early findings. *Amyotroph Lateral Scler*, 9(Suppl.1), 45-47 (2008)

26. P. D. Wright, M. Huang, A. Weiss, J. Matthews, N. Wightman, M. Glicksman and R. H. Brown Jr: Screening for inhibitors of the SOD1 gene promoter: pyrimethamine does not reduce SOD1 levels in cell and animal models. *Neurosci Lett*. 2010 Oct 4;482(3):188-92. Epub 2010 Jul 16

27. Z. Zhong, H. Ilieva, L. Hallagan, R. Bell, I. Singh, N. Paquette, M. Thiyagarajan, R. Deane, J. A. Fernandez, S. Lane, A. B. Zlokovic, T. Liu, J. H. Griffin, N. Chow, F. J. Castellino, K. Stojanovic, D. W. Cleveland and B. V. Zlokovic: Activated protein C therapy slows ALS-like disease in mice by transcriptionally inhibiting SOD1 in motor neurons and microglia cells. *J Clin Invest*, 119(11), 3437-49 (2009)

28. M. M. Maxwell, P. Pasinelli, A. G. Kazantsev and R. H. Brown, Jr.: RNA interference-mediated silencing of mutant superoxide dismutase rescues cyclosporin A-induced death in cultured neuroblastoma cells. *Proc Natl Acad Sci U S A*, 101(9), 3178-83 (2004)

29. H. Wang, A. Ghosh, H. Baigude, C. S. Yang, L. Qiu, X. Xia, H. Zhou, T. M. Rana and Z. Xu: Therapeutic gene silencing delivered by a chemically modified small interfering RNA against mutant SOD1 slows amyotrophic lateral sclerosis progression. *J Biol Chem*, 283(23), 15845-52 (2008)

30. http://clinicaltrials.gov/ct2/show/NCT01041222: (2010)

31. L. Banci, I. Bertini, A. Durazo, S. Girotto, E. B. Gralla, M. Martinelli, J. S. Valentine, M. Vieru and J. P. Whitelegge: Metal-free superoxide dismutase forms soluble oligomers under physiological conditions: a possible general mechanism for familial ALS. *Proc Natl Acad Sci U S A*, 104(27), 11263-7 (2007)

32. A. Gruzman, W. L. Wood, E. Alpert, M. D. Prasad, R. G. Miller, J. D. Rothstein, R. Bowser, R. Hamilton, T. D. Wood, D. W. Cleveland, V. R. Lingappa and J. Liu: Common molecular signature in SOD1 for both sporadic and familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*, 104(30), 12524-9 (2007)

Abbreviations: ALS: Amyotrophic lateral Sclerosis, SOD1: superoxide dismutase 1, PCR: polymerase chain reaction, HPLC: High performance liquid chromatography, GFP: Green fluorescent protein, DMSO: Dimethyl sulfoxide

Key Words: Amyotrophic lateral sclerosis, SOD1, Cell based assays, Promoter, Therapy

Send correspondence to: Paul D Wright, Department of Neurology, University of Massachusetts Medical School, Worcester, MA 01655, United States, Tel: 15088566688, Fax: 15088566750, E-mail: Paulwright27@ymail.com