Discovery of 1,3,4-oxidiazole scaffold compounds as inhibitors of superoxide dismutase expression

Thomas J. Lukas a,⁎, Gary E. Schiltz b,†, Hasan Arrat d,‡, Karl Scheidt b,c,§, Teepu Siddique d,⁎

a Department of Molecular Pharmacology & Biological Chemistry, Northwestern University, 303 E. Chicago Ave, Chicago, IL 60611, United States
b The Center for Molecular Innovation and Drug Discovery, Silverman Hall, Evanston, IL 60208, United States
c Department of Chemistry, The Center for Molecular Innovation and Drug Discovery, Silverman Hall, Evanston, IL 60208, United States
d Department of Neurology, Northwestern University, Chicago, IL 60611, United States

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A B S T R A C T

The treatment of neurodegenerative diseases is difficult because of multiple etiologies and the interplay of genetics and environment as precipitating factors. In the case of amyotrophic lateral sclerosis (ALS), we have knowledge of a handful of genes that cause disease when mutated. However, drugs to counteract the effect of genetic mutations have not yet been found. One of the causative genes, Cu, Zn-superoxide dismutase (SOD1) is responsible for about 10–15% of the genetically linked autosomal dominant disease. Our rationale was that compounds that reduce expression of the mutant protein would be beneficial to slow onset and/or disease progression. We screened candidate compounds using a cell-based in vitro assay for those that reduce mutant SOD1 (G93A) protein expression. This led to the discovery of 2-[3-iodophenyl)methylsulfanyl]-5-pyridin-4-yl-1,3,4-oxadiazole, a known protein kinase inhibitor that decreases G93A-SOD1 expression in vitro and in the brain and spinal cord in vivo. However, this compound has a biphasic dose response curve and a likely toxophore which limit its therapeutic window for chronic disease such as ALS. Therefore, we designed and tested a focused library of analogs for their ability to decrease SOD1 expression in vitro. This exercise resulted in the identification of a lead compound with improved drug-like characteristics and activity. Development of small molecules that reduce the expression of etiologically relevant toxic proteins is a strategy that may also be extended to familial ALS linked to gain of function mutations in other genes.

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Amyotrophic lateral sclerosis (ALS) is etiologically heterogeneous and in ten percent of cases the disease is inherited as a dominant, recessive or X-linked dominant trait, (familial ALS or FALS). The other 90 percent of cases have no identifiable history of familial disease and are called sporadic ALS or SALS. We and others have identified mutations in five genes that cause ALS. Mutations in these genes, SOD1, TDP-43(TARBP), FUS, optineurin (OPTN), ubiquilin 2 (UBQLN2), and C9ORF72 cause the formation of aggregates in motor neurons of the spinal cord. Of interest is the fact that TDP-43, FUS, optineurin, p-62 (SQSTM1), and ubiquilin2 are also found in inclusions in the motor neurons of some ALS patients.

The treatment of neurodegenerative diseases is difficult because of multiple etiologies and the interplay of genetics and environment as precipitating factors. In the case of amyotrophic lateral sclerosis (ALS), we have knowledge of a handful of genes that cause disease when mutated. However, drugs to counteract the effect of genetic mutations have not yet been found. One of the causative genes, Cu, Zn-superoxide dismutase (SOD1) is responsible for about 10–15% of the genetically linked autosomal dominant disease. Our rationale was that compounds that reduce expression of the mutant protein would be beneficial to slow onset and/or disease progression. We screened candidate compounds using a cell-based in vitro assay for those that reduce mutant SOD1 (G93A) protein expression. This led to the discovery of 2-[3-iodophenyl)methylsulfanyl]-5-pyridin-4-yl-1,3,4-oxadiazole, a known protein kinase inhibitor that decreases G93A-SOD1 expression in vitro and in the brain and spinal cord in vivo. However, this compound has a biphasic dose response curve and a likely toxophore which limit its therapeutic window for chronic disease such as ALS. Therefore, we designed and tested a focused library of analogs for their ability to decrease SOD1 expression in vitro. This exercise resulted in the identification of a lead compound with improved drug-like characteristics and activity. Development of small molecules that reduce the expression of etiologically relevant toxic proteins is a strategy that may also be extended to familial ALS linked to gain of function mutations in other genes.

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Figure 1. Chemical structures of the commercially available compounds tested in the in vitro assays for their ability to inhibit SOD1.

Figure 2. Inhibition of SOD1 protein expression in fibroblasts from G93A-SOD1 mice. Fibroblasts were treated with the title compounds at the indicated concentration for 48 h. Cells were washed, lysed, and SOD1 levels measured by ELISA. SOD1 levels are normalized to the total protein in the lysate.
to CNS distribution. Thus, we have discovered the first non-toxic class of small molecules that may be useful for treating SOD1-FALS and ALS where SOD1 may be involved.

**Small molecule inhibitors of SOD1 expression**

Accumulated evidence suggests that inhibition of glycogen synthase kinase III (GSK3β) might be beneficial to survival of the mutant SOD1 transgenic mouse23–25 however, SOD1 levels were not evaluated in those studies. Thus, we used cultured fibroblasts from the G93A-SOD1 transgenic mouse to screen GSK3β protein kinase inhibitors (Fig. 1) for their effects on SOD1 expression. The transgene construct contains the human SOD1 promoter so that cellular signaling processes that affect transcription of the gene will be fully functional. We chose a structurally diverse group of compounds including some natural products. Figure 2 summarizes the results from six of the commercially available compounds. (Cell preparation and assay methods are in the Supplementary file). Two compounds (Aloisine A and GSK3B-IX) increased SOD1 expression, while several compounds inhibited at lower concentrations but reversed at higher concentrations yielding a biphasic pattern. Among these compounds, GSK3B-II was the most potent at inhibiting G93A-SOD1 expression (Fig. 3A). The IC50 values for GSK3β kinase inhibition from the literature compared to SOD1 expression are shown in Table 1. GSK3β-VI also inhibits SOD1 expression but this compound covalently modifies GSK3β kinase at the active site26 and exhibits cell toxicity at concentrations greater than 5 μM making it a poor therapeutic candidate. Kenpaullone, one of the more potent GSK3β inhibitors (Table 1), only weakly decreased SOD1 expression. Thus, the disconnect between potencies of GSK3β inhibitors and their effect on SOD1 expression suggests that inhibition of GSK3β may not be the primary mechanism for decreasing G93A-SOD1 expression in cultured fibroblasts, although other explanations such as differences in cell permeability cannot be ruled out. We also determined that the decrease of SOD1 expression by GSK3B-II is primarily due to reducing the level of SOD1 mRNA (Fig. 3B). Thus, GSK3B-II affects cellular processes that lead to a decrease in the production of SOD1 mRNA and protein.

While GSK3B-II is a potentially useful molecular probe to study the effects of reducing SOD1 expression, the compound exhibits a biphasic dose–response curve and contains a potential toxophore (Aryl-Iodo) which reduces its therapeutic potential. Hit-to-lead medicinal chemistry was undertaken to quickly expand the SAR and develop more drug-like inhibitors, especially ones without the aryl iodide group. Modifications to previously reported conditions27,28 were used to synthesize new 1,3,4-oxadiazole analogs as shown in Scheme 1. Commercially available ethyl isonicotinate was refluxed with hydrazine hydrate for 1 h at 60 °C and the solvent was evaporated. Trituration of this solid with diethyl ether afforded pure hydrazide 2 in 86% yield. The treatment of hydrazide 2 with Cs2CO3 and KOH in 60 °C for 18 h produced a solid upon concentration in vacuo. It was found that this material could be easily purified by trituration with H2O followed by filtration to give thiol 3 in 78% yield, thus avoiding chromatography over the first two steps. This operationally simple procedure afforded multi-gram quantities of 3 for subsequent focused library synthesis. Finally, diversification of the aryl ring was achieved by nucleophilic displacement reaction of thiol 3 with substituted aryl bromides in room temperature DMF using K2CO3 as base. The final compounds (4) were produced in yields of between 24% and 74% depending on the aromatic ring substituent. All final compounds were judged to be consistent with desired product by 1H NMR spectroscopy and had >95% purity as observed by liquid chromatography–mass spectrometry analysis.

Structure–activity relationships (SAR) were developed using mono aryl substituents on the benzyl ring (Table 2) to probe the

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**Table 1**

<table>
<thead>
<tr>
<th>Cpd Name</th>
<th>GSK3β IC50 (nM)</th>
<th>SOD1 IC50 (nM)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Increases SOD1</td>
<td>Also inhibits CDK5, CDK2, CDK1</td>
</tr>
<tr>
<td>2</td>
<td>10,000</td>
<td>Also inhibits CDK4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10,000</td>
<td>Also inhibits CDK5, CDK2, CDK1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1000</td>
<td>Covalent binding inhibitor</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>650</td>
<td>Increases SOD1</td>
<td>Also inhibits CDK5, CDK1</td>
</tr>
<tr>
<td>6</td>
<td>3000</td>
<td>Active against GSK3B in vivo</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>625</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A Ref. 35.
B Product information datasheet (EMD, Calbiochem).
C Ref. 36.
D Ref. 26.
E Ref. 37.
F Ref. 38.
G Refs. 39, 40.

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**Figure 3.** Inhibition of SOD1 protein and mRNA expression by GSK3B-II in G93A-SOD1 mouse fibroblasts. (A) Dose-dependent changes in G93A-SOD1 protein (protein) or mRNA. Error bars are SEM.

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**Scheme 1.** Synthesis of 1,3,4-oxadiazole compounds. (a) hydrazine–H2O, EtOH, reflux, 1 h; (b) Cs2, KOH, EtOH, 60 °C, 18 h; (c) K2CO3, Aryl-Br, DMF, rt, 2 h.
requirements around this portion of the molecule. Aromatic ring modifications were found to have a significant effect on the compounds’ ability to reduce SOD1 expression. The two best compounds had methoxy substituents at the 3-position (NUCC-433) or 4-position (NUCC-434) and significantly reduced the level of SOD1 expression to 47–50% of control at 10 μM. Some compounds such as NUCC-319 (4-fluoro) and NUCC-435 (4-trifluoromethyl) actually showed a significant increase in SOD1 expression, confirming the importance of the aryl ring in functional activity. The unsubstituted benzyl ring (NUCC-441) activates expression of SOD1 while other substituents (chloro, nitro, bromo, methyl) at the 3 or 4 position of the ring were comparable to control in SOD1 expression. Interestingly, there is a trend where electron withdrawing substituents tended to cause an increase in SOD1 expression (NUCC-319, 435, 432, 440), while electron donating groups tended to produce the largest SOD1 reduction (NUCC-433 and 434). Dose–response relationships were determined for NUCC-433 and 434 as shown in Figure 4. Unlike GSK3B-II (Fig. 3A), both compounds exhibit a flattened rather than upward curvature at higher concentration. A number of other analogs were synthesized and tested which had variations in the pyridine portion of the molecule, including the 3-hydroxyl-, 3-amino-4-pyridyl, 3-pyridyl, and fused ring system derivatives. All of these compounds with the exception of NUCC-322 (3-pyridyl) showed either no effect on SOD1 expression or slight increases.

We sought to quantify compound behavior by Hansch analysis using ClogP and the sigma or sigma+ values for meta and para groups derived by Hammett. Sigma values are based upon the ionization of substituted benzoic acids, while the sigma+ values are based upon the solvolysis of substituted 2-phenyl-2-propyl chlorides. Three equations were tested for correlation of the activity data with compound parameters.

\[
1/(\text{relSOD1}) = \text{ClogP} + \text{sigma} + \text{sigma+} \\
1/(\text{relSOD1}) = \text{ClogP} + \text{sigma} + \text{sigma+} \\
1/(\text{relSOD1}) = \text{ClogP} + \text{sigma} + \text{sigma+} \\
\]

Using the ‘systemfit’ package in R (http://www.R-project.org) the coefficients for the variables were calculated by solving simultaneous equations (Ordinary Least Squares). The best fit (r² = 0.58) was to Eq. 1 and Eq. 2 (r² = .55). The coefficients for Eq. 1 are: 1/relSOD1 = (−0.1667)ClogP + (−0.2331)sigma + (−0.4906)sigma+ + 1.684. A plot of the predicted and experimental values is shown in Figure 5. The sole outlier was NUCC-436 based upon Grubbs test (http://www.graphpad.com). Alternate analysis using Q values (sum of squares of the residuals) gave comparable results.

One test of the specificity of the correlation was from testing of NUCC-322 (Fig. 6), an isomer of the 4-bromo compound (NUCC-320). While NUCC-320 showed no effect on SOD1 expression, NUCC-322 inhibited SOD1 expression to 68.6% of control at 10 μM (p = 0.015). This suggests that both ends of the structure contribute to target binding. Relatedly, NUCC-437 and NUCC-438

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**Table 2**

Inhibition of SOD1 expression by 1,3,4-oxadiazoles

<table>
<thead>
<tr>
<th>ID</th>
<th>R</th>
<th>ClogP</th>
<th>Sigma</th>
<th>Sigma+</th>
<th>Relative SOD1 expression 10 μM compound (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GSK3B-II)</td>
<td>R = 3-Iodo</td>
<td>2.91</td>
<td>0.352</td>
<td>0.359</td>
<td>0.73 (0.022)</td>
</tr>
<tr>
<td>NUCC-433</td>
<td>R = 3-Methoxy</td>
<td>1.71</td>
<td>0.268</td>
<td>0.047</td>
<td>0.50 (0.005)</td>
</tr>
<tr>
<td>NUCC-319</td>
<td>R = 3-Fluoro</td>
<td>1.93</td>
<td>0.337</td>
<td>0.352</td>
<td>1.43 (0.001)</td>
</tr>
<tr>
<td>NUCC-435</td>
<td>R = 4-Trifluoromethyl</td>
<td>2.82</td>
<td>0.54</td>
<td>0.612</td>
<td>1.57 (0.003)</td>
</tr>
<tr>
<td>NUCC-439</td>
<td>R = 3-Chloro</td>
<td>2.51</td>
<td>0.373</td>
<td>0.399</td>
<td>0.96</td>
</tr>
<tr>
<td>NUCC-432</td>
<td>R = 3-Nitro</td>
<td>1.53</td>
<td>0.71</td>
<td>0.674</td>
<td>1.17</td>
</tr>
<tr>
<td>NUCC-318</td>
<td>R = 3-Bromo</td>
<td>2.65</td>
<td>0.391</td>
<td>0.405</td>
<td>0.91</td>
</tr>
<tr>
<td>NUCC-434</td>
<td>R = 4-Methoxy</td>
<td>1.71</td>
<td>0.115</td>
<td>−0.778</td>
<td>0.47 (0.001)</td>
</tr>
<tr>
<td>NUCC-440</td>
<td>R = 4-Chloro</td>
<td>2.51</td>
<td>0.227</td>
<td>0.114</td>
<td>1.23 (0.009)</td>
</tr>
<tr>
<td>NUCC-320</td>
<td>R = 4-Bromo</td>
<td>2.65</td>
<td>0.232</td>
<td>0.15</td>
<td>1.03</td>
</tr>
<tr>
<td>NUCC-321</td>
<td>R = 4-Iodo</td>
<td>2.91</td>
<td>0.18</td>
<td>0.135</td>
<td>0.69 (0.026)</td>
</tr>
<tr>
<td>NUCC-324</td>
<td>R = 4-Fluoro</td>
<td>1.93</td>
<td>0.062</td>
<td>−0.073</td>
<td>0.86</td>
</tr>
<tr>
<td>NUCC-436</td>
<td>R = 4-Methyl</td>
<td>2.29</td>
<td>−0.17</td>
<td>−0.311</td>
<td>1.26</td>
</tr>
<tr>
<td>NUCC-431</td>
<td>R = 4-Nitro</td>
<td>1.53</td>
<td>0.778</td>
<td>0.79</td>
<td>1.02</td>
</tr>
<tr>
<td>NUCC-441</td>
<td>R = H</td>
<td>2.29</td>
<td>NA</td>
<td>0</td>
<td>1.33 (0.07)</td>
</tr>
</tbody>
</table>

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\[ a \text{ClogP was calculated using ChemBioOffice-ChemDraw V13.} \]
\[ b \text{From Ref. 30.} \]
\[ c \text{If not shown.} \]
that have the benzyl group replaced by pyridyl exhibited activity (at 10\(\mu\)M) not significantly different from control indicating a target preference for benzyl groups.

The blood brain barrier permeability of GSK3B-II was determined in nontransgenic mice. At 20 mg/kg (intraperitoneal), the compound rapidly distributes into the brain and spinal cord. This compound is also long-lived in the CNS, with drug still detectable after 24 h in the brain and spinal cord (Table 3). The peak and sustained concentrations found in the spinal cord and brain indicate that the 20 mg/kg dose puts the drug into the inhibitory range found in the cellular assays.

We also tested compound NUCC-434 for distribution in nontransgenic mice. However, the solubility of this compound was poor in the standard 1–2% DMSO vehicle that we used for GSK3B-II. While there are a multitude of methods for improving aqueous solubility of drugs,13 we chose a relatively simple route using a cyclodextrin based carrier (Sulfo-butylether beta-cyclodextrin, Captisol) to solubilize the drug in aqueous solution. Using the cyclodextrin carrier, we were able to solubilize NUCC-434 to 2 mg/mL in water and deliver 10 mg/kg to nontransgenic mice for pharmacokinetics. Like GSK3B-II the compound entered the CNS and persisted for 24 h in the spinal cord. However, the distribution dynamics are different in the presence of the cyclodextrin which appears to slow uptake into the CNS as the peak drug concentration was delayed.

As a proof of concept study, compound GSK3B-II was evaluated in vivo for its ability to reduce levels of SOD1 in vivo. Initial testing was carried out in a small cohort (5–6 animals) of G93A-SOD1 mice (high level of mutant SOD1 expression). The drug was dosed at 20 mg/kg IP every other day for 26 days. Mice were sacrificed and SOD1 was measured by ELISA. In all tissues sampled: spinal cord, cerebellum, brain stem, and cerebral cortex, significant decreases in SOD1 were measured (Fig. 7).

Conclusions

Compounds GSK3B-II, NUCC-433, and NUCC-434 are the first small molecules to exhibit dose dependent reduction of SOD1 expression in vitro and in the G93A-SOD1 mouse (GSK3B-II). Compared to antisense oligonucleotides33 and the APC analog,34 we found a comparable decrease of SOD1 protein in cultured cells with the new 1,3,4-oxadiazole analogs reported here. Thus, these and related compounds may offer an opportunity to develop a small molecule based ALS therapy.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.01.078. These data include MOL files and InChIkeys of the most important compounds described in this article.

References and notes