**Rational discovery of a SOD1 tryptophan oxidation inhibitor with therapeutic potential for amyotrophic lateral sclerosis**

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**ABSTRACT**

Formation of Cu, Zn superoxide dismutase 1 (SOD1) protein inclusions within motor neurons is one of the principal characteristics of SOD1-related amyotrophic lateral sclerosis (ALS). A hypothesis as to the nature of SOD1 aggregation implicates oxidative damage to a solvent-exposed tryptophan as causative. Here, we chart discovery of a phenanthridinone based compound (Lig9) from the NCI Diversity Set III by rational methods by *in silico* screening and crystallographic validation. The crystal structure of the complex with SOD1, refined to 2.5 Å, revealed that Lig9 binds the SOD1 β-barrel in the β-strand 2 and 3 region which is known to scaffold SOD1 fibrillation. The phenanthridinone moiety makes a substantial  –  interaction with Trp32 of SOD1. The compound possesses significant binding affinity for SOD1 and inhibits oxidation of Trp32; a critical residue for SOD1 aggregation. Thus, Lig9 is a good candidate from which to develop a new library of SOD1 aggregation inhibitors through protection of Trp32 oxidation.

**Keywords:** ALS, SOD1; Trp oxidation; Modulators; NCI diversity set, Virtual screening, Phenanthridinone derivative, Crystal structure; Binding assay

**1. Introduction**

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder involving motor neuron degeneration that leads to progressive muscle weakness and paralysis. Ultimately, respiratory failure leads to death (Brown and Al-Chalabi, 2017). The timing of disease onset is variable; however, patients usually die within 3-5 years of symptom onset. A familial form of ALS can caused by mutations in the superoxide dismutase-1 (SOD1) protein. Mutant SOD1 protein induces the formation of abundant neuronal cytoplasmic protein aggregates in human patients and transgenic mouse model (Bruijn et al., 1998; Kato et al., 1996). Proteomic analysis of aggregates from spinal cord sections in ALS mouse models shows they contain mutant SOD1 proteins in addition to unmodified, full length, wild-type SOD1 polypeptides (Shaw et al., 2008). Over 150 different disease mutations have been reported in the *SOD1* gene. Some of these mutations have been shown to negatively affect native SOD1 homodimer stability. Structural instability of SOD1 potentially results in homologous (Lang et al., 2015; Deng et al., 1993; Banci et al., 2008; Kim et al., 2015) or heterologous (Fujisawa et al., 2012; Nishitoh et al., 2008) protein aggregation. Mutations are also associated with a decreased metal binding (Lindberg et al., 2002; Johnston et al., 2000) and misplaced disulfide formation (Tiwari and Hayward 2003; Niwa et al., 2007; Cozzolino et al., 2008). Metal loss increases the conformational flexibility of both electrostatic and zinc-binding loops exposing the β-barrel core, possibly causing non-native interactions leading to aggregation (Strange et al., 2007).

SOD1 bears only one tryptophan (Trp32) which is found in β-strand 2 exposed to solvent on the surface of the protein. This region is pathophysiologically important as it forms repeating anti-parallel β-sheet structures and is found at the core of SOD1 aggregates (Sangwan et al., 2017). Mutational studies have revealed that Trp32 oxidation is a crucial factor in the bicarbonate-dependent, covalent aggregation of SOD1 (Zhang et al., 2003). Intriguingly, oxidative post-translational modification of Trp32 to kynurenine and N-formyl kynurenine results in the covalent crosslinking of oxidized SOD1 monomers (Zhang et al., 2003). The proclivity of SOD1 Trp32 to undergo oxidation has gained significance due to its detection in ALS mouse models with mutant SOD1 (Johnston et al., 2000). Compared to any other position 32 variants, oxidation profoundly increases SOD1 aggregation (Taylor et al., 2007; Grad et al., 2011) indicating that inhibition of Trp oxidation could be a potential therapeutic target to prevent aggregation and slow disease progression in ALS.

Considerable effort has been expended to discover a successful drug against ALS. The only two FDA approved drugs to slow down the disease progression in ALS are Rilutek (riluzole) and Radicava (edaravone) (Traynor, 2017). However, the effect of riluzole is modest with only 9% gain in survival for a year after the initiation of treatment (Messori et al., 1999). There is, therefore, an urgent need for new SOD1 modulators for the treatment of ALS. Clioquinol and PBT2 are two of the many kynurenine pathway inhibitors designed as to treat ALS based on their structural similarity with kynurenine and quinolinic acid respectively (Chen & Guillemin, 2012). The latter products are catabolites involved in tryptophan oxidation to form N-formyl kynurenine. Tryptophan oxidation protection conferred by various natural antioxidant phenolics has also been studied (Salminen & Heinonen, 2008) but not implemented as a therapy. In addition, a Trp32 targeted drug molecule, 5-fluorouridine, has been shown to reduce SOD1 aggregation in human cell culture (Pokrishevsky et al., 2017) despite low binding affinities (Wright et al., 2013). This study addressed the demand for quantifiable protein-ligand interactions targeted to the Trp32 site with therapeutic applications in ALS. However, small molecules with optimal inhibitory effect on SOD1 and Trp32 oxidation and the structural basis of such inhibitors have not been extensively studied.

To address this and find structurally diverse molecules targeted to SOD1, we carried out structure-based virtual screening of the NCI Diversity Set III library followed by X-ray crystallographic validation to obtain novel SOD1 modulators. This search identified a small molecule phenanthridinone derivative SOD1 modulator (NSC127133; hereafter, Lig9).

**Materials and methods**

***Virtual screening of NCI database***

The crystal structure of a previously determined SOD1-inhibitor complex (PDB Id: 5YTO) was used for docking studies (reference). Protein preparation for computational studies included removal of the ligand from the binding site, addition of protein hydrogens, bond optimization and energy minimization. The grid box for the docking process was created using the Autodock/Vina plugin for PyMol (Seeliger & de Groot, 2010). The docking grid-box was centered at the Trp32 residue with a box size of 22.50 x 22.50 x 22.50 Å that covers the entire binding site around Trp32. The database of 1620 compounds of the NCI diversity set III were retrieved and prepared using AutoDock utilities. The screened molecules, subjected to the Lipinski Rule of Five, were docked against the receptor site using AutoDock Vina (Trott & Olson, 2010). The resultant poses were analyzed based on cut off docking score values and subsequent visual inspection for an accurate understanding of intermolecular interactions between SOD1 and ligands. The shortlisted ligands from the *in-silico* analysis were procured from the National Cancer Research Institute at Bethesda, USA.

*Biological section*

*Reagents*

A SOD1 expression plasmid encoding a tobacco etch virus (TEV) protease cleavable His tag-SOD1 fusion protein was generated by ligation of the SOD1 DNA coding sequence in frame between the NcoI and XhoI sites in the pETM-11 vector. All the chemicals, unless specified, were purchased from Sigma, India.

*Gene expression and protein purification*

SOD1 was expressed in *E. coli* strain BL21 star (DE3) (Stratagene). *E. coli* cells were grown at 37 °C until its optical density at 600 nm reached 0.6 and then SOD1 expression was induced by addition of 0.4 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 20 °C overnight. Cells were harvested by centrifugation and the pellet was stored at -80° C. Cells were lysed in 20mM Tris HCl, 300mM NaCl, 5mM -mercaptoethanol, pH 7.5 by sonication (Vibra cell) at 30 % amplitude with 3 second pulse and 5 second rest. The supernatant was loaded onto a Ni-NTA column (HisTrap FF, GE Healthcare, USA) for metal affinity purification and eluted with 200 mM of Imidazole. TEV protease was used to remove the His-tag by incubation with the former at 4 °C on a rocker overnight then concentrated and separated from remaining impurities by size exclusion chromatography (Superdex 75G 16/60 from GE Healthcare, USA) on an AKTA Prime Plus (GE Healthcare, USA).

***Ligand soaking and X-ray crystallography***

The SOD1 protein, with a concentration of 8-9 mg/ml in 20 mM Tris pH 8.0, was crystallized by the hanging-drop method. Drops consisting of 2 µl protein mixed with 2 µl reservoir solution in 24-well plates were equilibrated against a 500 µl reservoir solution of sodium citrate pH 6.0.

Crystals were then soaked separately with the five compounds (50 mM stock) dissolved in 1.5 M sodium citrate pH 6.0 for 3 hours, 6 hours and overnight at 20 °C. X-ray crystallographic data sets were collected on a RAXIS IV++ diffractometer (Rigaku Corporation, Japan) at NCBS, Bangalore. Diffraction data were integrated and scaled using HKL2000 (Minor and Otwinowski, 1997). The crystals belong to the space group *C*2221, with cell dimensions of a = 164.36 Å, b = 203.69 Å and c = 144.21 Å. The crystal structures were solved by molecular replacement using Molrep (Vagin & Teplyakov, 1997) in the CCP4 software package (Collaborative Computational Project, Number 4, 1994). The intact dimer structure of SOD1 (PDB Id: 5YTO) (Manjula et al., 2018) was used as the search model. The structure solution yielded five SOD1 dimers in the asymmetric unit, for all the five crystals tested. The 2|Fo| - |Fc| and the difference Fourier |Fo| - |Fc| maps were carefully analyzed. No electron density was found corresponding to any of the compounds except for compound, Lig9. Energy minimized coordinates and crystal information files (CIF) for Lig9 were produced using Jligand (Lebedev et al., 2012).

The structure of the SOD1 complex was refined using the *Phenix.refine* (Terwilliger et al., 2008) then *REFMAC5* (Murshudov et al., 1997. A few rounds of refinement cycles and model building using Coot (Emsley et al., 2004) were performed to obtain the final structure. The final refined structure of the SOD1 complex in the asymmetric unit contains 11081 protein atoms, nine Zn2+ ions, four glycerol molecules, three polysulfane molecules and 1324 water molecules, with a final Rwork of 16.6 % and an Rfree of 23.7 % at 2.5 Å resolution. The stereochemistry of these crystal structures is good, as assessed with *MOLPROBITY* (Chen et al., 2010). The X-ray data collection, scaling and refinement statistics are summarized in Table 2. The structural coordinates of the SOD1 – Lig9 complex has been deposited in the RCSB (PDB Id: 6A9O).

***Molecular dynamic (MD) simulations***

The crystal structure of the SOD1-Lig9 complex, determined in the present study, was used to perform MD simulations to check ligand interaction stability at the binding site in the presence or absence of SOD1-Trp32. The Desmond program from the D. E. Shaw group was used to perform the MD simulation. A tryptophan-32 to alanine mutant SOD1 protein structure was generated in Maestro. The wild-type and W32A SOD1 structures were minimized and checked for other penalties including the addition of hydrogens. The simulation box set up was cuboidal in nature with the minimum number of solvent molecules added to the protein. The solvated water box was generated and minimized using the steepest descent method until a gradient threshold of 1.0 kcal/mol/A was reached with a minimum step size of 10. The Coulombic interactions were cutoff at 9.0 Å. Default force constant was applied and no other restraints on the protein and solvent molecules were applied during the minimization process. The production run consisted of 8 stages including relaxation of the complete system before the simulation run and was performed at 300 K and 1.01325 bar pressure. The production run was carried out for a period of 50ns. The trajectory was saved at a step size of 4.8 ps/frame which generated about 10,417 frames. The trajectory was analyzed using both the Desmond and Maestro programs from the Schrodinger software package (Desmond, 2015).

***Surface plasmon resonance (SPR) assay***

SPR assay was performed to check the binding efficiency of the selected 20 compounds (Supplementary Table 1) against SOD1 using a Biacore T200 or Biacore 3000 (GE Healthcare, USA) instruments. The immobilization was performed using purified SOD1. Reference surfaces were prepared by blocking carboxyls on the sensor chip, and no protein was added. Ligand molecule (analyte) was allowed to flow over the immobilized-protein surface, and the binding response of analyte to protein was recorded. The chip surface was regenerated by the removal of the analyte with regeneration buffer. Initially, a single concentration was used to check binding response and different compounds were taken in 100 µg/ml concentration. The maximum response with each analyte reflects relative binding affinity. To analyze the dose dependency of the Lig9 compound, different concentrations were used. The final response of bound protein, expressed in response units (RU), was calculated by subtraction of the reference RU. PBS with 5% DMSO was used as both running and analyte binding buffer. 250 µM and 125 µM concentrations of Lig9 were used to compare the binding efficiency between wild-type SOD1 and W32A mutant.

***Microscale thermophoresis (MST) assay***

SOD1 was labeled with a fluorescent dye, NT-647. The dye NT-647 labels the primary amine groups on lysine residues of the protein. The dye was dissolved in DMSO and diluted with 1X MST buffer with 0.05 % Tween-20. 100 μl of 20 µM protein was then mixed with 100 μl of diluted dye, and the reaction mixtures were incubated for 30 min at room temperature in the dark. Unreacted dye was removed using PD10 desalting columns, and the capillary scan was performed for labeled protein to check its fluorescence.

A stock solution of Lig9 with a concentration of 20 mM was prepared in 100 % DMSO.

5 mM of Lig9 was prepared in TBS buffer in 10l volume, and it was serially diluted (two-fold each time) 16 fold in TBS containing 10% DMSO, followed by adding an equal amount of labeled SOD1 (100 nM) to each titration. The mixed samples were loaded into Monolith NT.115 capillaries, and subjected to MST analysis using a Monolith NT.115 (NanoTemper Technologies), performed at MST power of 40 % and LED power of 80 %, at 22 °C temperature. Same protocol was followed for the W32A mutant as well, to assess the binding efficiency of Lig9. The fluorescence data was normalized to fraction bound (0 = unbound, 1 = bound), and the binding constands (Kd) were determined using the equation derived from the law of mass action.

***Fluorimetric study of ligands on tryptophan oxidation***

The kinetic profile of protein tryptophan (Trp) residue consumption induced by AAPH-derived radicals was assessed by fluorescence spectroscopy (Fuentes-Lemus et al., 2016; Manjula et al., 2018). A reaction mixture containing protein (5 µM) with 6 mM AAPH (2, 20-azobis (2-amidinopropane) dihydrochloride) was incubated in phosphate buffer 100 mM, pH 7.4 at 45 °C. Trp consumption was evaluated by the progressive decrease of the fluorescence intensity at 360 nm (excitement wavelength 295 nm). The fluorescence intensity was measured for 90 min with 2 min of time intervals. Fluorescence measurements were carried out using a Perkin Elmer LS-55 spectrofluorimeter.

**Results and discussion**

***Virtual screening***

The objective of the virtual screen was to precisely find compounds that can camouflage the exposed SOD1 tryptophan (Trp32) binding site and the nearby region. AutoDock Vina (Vina, 2010) was used to obtain hit compounds from the NCI diversity set III containing 1620 molecules. The virtual screening results were ranked based on the predicted binding free energies (ΔGvina). The PyMOL program plugged with AutoDock Vina (Seeliger & de Groot, 2010) was used to visually check the predicted binding conformations for the selected conformations from the sorted list. Post-docking visual analysis and cutoff ΔG values filtered out compounds with weak or distal binding to the SOD1 Trp32 surface site. The top 20 compounds were selected based on their orientation and levels of interaction with Trp32 and its neighbouring residues (Supplementary Table 1). The analysis revealed that the compounds with simpler heterocyclic composition yielded a better probability of stacking orientation over Trp32 residue to form π-π stacking interactions and acceptable energy values. The shortlisted compounds, intriguingly, contain unique heterocyclic scaffolds like naphthalene, acridine, oxadiazole, etc. throughout except for a few like quinolones (Table 1). The docked poses show an overlay of the heterocyclic ring compounds over Trp32. The presence of charged residues Ser98, Lys30, Asn19 and Glu100 around Trp32 at the protein binding site establishes ligand binding through hydrogen bonding and π-cation interactions with the carboxyl and amide moieties from either the amino acid residues or ligand chemical groups. These five compounds exhibited robust docking interactions with SOD1 (Figure 1) and relatively high binding affinities as determined by SPR assay, described below.

***Compound Lig6 (NCI 326182)***

The core moiety of Lig6 does not contribute to the -  interaction with Trp32, instead the benzodioxol component does (Fig 1A). The possibility of  - cation interaction of Lig6 core with Lys30 also plays an important role. The Ser98 exerts ionic interactions with the amino groups from the core moiety, benzodioxol. The dimethyl benzene extension does not interact with the surface residues and is more solvent exposed.

***Compound Lig9 (NCI 127133)***

The major interaction of the compound 9 (Lig9) with SOD1 involves the establishment of face to face  -  stacking interactions between the phenanthridine moiety of Lig9 and the indole ring in Trp32 of SOD1 (Figure 1(B)). The amino group of the phenanthridine moiety forms a strong hydrogen bond with Ser98 (1.9 Å). Lys30 shows a higher probability of the formation of  - cation interactions with the phenanthridine ring. The biphenyl component of Lig9 remains solvent exposed and does not form any significant interactions with the surface of the protein.

***Compound Lig11 (NCI 135168)***

The fluorene moiety of Lig11 overlays tryptophan 32 forming significant  - stacking interactions (Figure 1(C)). The  - cationic interactions are found to be exhibited by Lys30 on the fluorene moiety. The nitro group attached to the core contributes in the establishment of ionic interactions with Ser98. No other hydrophobic interactions were found to exist because of the burial of hydrophobic side chains of the protein sheets.

***Compound Lig13 (NCI 295300)***

The benzazepine moiety compound Lig13 contributes to the  -  stacking interactions with Trp32 (Figure 1(D)). The nitrogen atom bearing pyrrole part of the indole moiety in Trp32 exerts  - cation interactions on the benzyl ring of Lig12. The foresaid interactions orient the compound in a concave overlay on the protein surface. The amino group of the core moiety contributes to weak hydrogen bonding with Ser98. Additionally, significant ionic interactions are also observed between the amino group of benzazepin in Lig13 and Ser98 residue on SOD1 surface.

***Compound Lig15 (NCI 117987)***

The best pose of compound Lig15 is oriented on the Trp32 residue through  -  stacking interactions (Figure 1(E)). The anthracene ring of the compound plays a pivotal role in the formation of the interactions with the protein surface. The nitrogen atoms on Lys30 and the indole moiety of Trp32 contribute to the formation of  - cation interactions with the anthracene and benzyl moiety of Lig15, respectively. The carboxyl group and one of the oxygen atoms from the dioxo group attached to the anthracene ring contributes to weak electrostatic interactions with Ser98 and Asn96, respectively.

***Surface plasmon resonance assay***

The 20 compounds short-listed by analysis of docking were procured from National Cancer Institute (NCI) at Bethesda, USA. Five out of 20 compounds exhibited a robust interaction with SOD1 (Figure 2(A)). Compound Lig9 presented the best binding interaction. Analysis of the binding efficiency of Lig9 with SOD1 was performed through surface plasmon resonance (SPR) assays. For SPR analysis, different concentrations of Lig9 were used which yielded concentration-dependent binding as expected (not shown). The binding efficiency was compared between wild SOD1 and W32A mutant of SOD1 (Figure 2(B)). To check the W32 interaction with Lig9 both SOD1 and W32A were immobilized to a sensor chip and the on and off rates for ligand binding were recorded on a BIAcore 3000. The recorded sensorgrams show that Lig9 interaction efficiency with the wild-type SOD1 protein is high compared to the W32A mutant (Figure 2(B)).

***Crystal structure of the SOD1–Lig9 complex***

The five compounds, short-listed from the SPR assay, were used for X-ray studies. High-resolution X-ray diffraction data from crystals soaked with these five compounds were collected and their structures determined by molecular replacement. The SOD1-Lig9 complex, diffracted to 2.5 Å resolution, was crystallized in the C2221 space group with five SOD1 dimers and one ligand in the asymmetric unit. Chain G of this crystal structure has no visible electron density corresponding to the loop IV (aa: 67-79) and loop VII (aa: 126-141) regions. This may be due to the absence of Zn ion in this chain. Although the Cu ion is missing in the complex, the overall tertiary structure of the complex is very similar to the known typical SOD1 homodimer (Parge et al., 1992). The crystallographic and refinement data are summarized in Table 2.

The Lig9 molecule binds on the interface formed by chain F (of EF dimer) and chain I (of IJ dimer) (Figure 3(A) and (B)). Electron density for the ligand, Lig9 (NSC127133) was clearly visible in the difference Fourier map (Figure 3(C)). The phenanthridinone moiety and the indole ring of Trp32 (chain F) creates  interactions between them. It successfully camouflages the tryptophan moiety of SOD1. In addition, intermolecular hydrophilic interactions are also observed between O29 atom of Lig9 and the backbone N atom of Ile99 (Figure 3(D)). Glu100 residue contributes a  – cationic interaction with phenyl-benzoic acid group of Lig9. Water molecules bridge the hydroxy atom of the phenanthridinone moiety and Asp96. The carboxyl oxygen near the phenyl moiety of Lig9 interacts very closely to form a hydrogen bond with Ser988 of SOD1. The phenyl group lays sandwiched between the residues Lys30, Glu100 and Ser98. The crystallographic structure, where we can find significant ligand and protein interactions, correlates well with our SPR analysis. When comparing the docked pose of Lig9 and its crystal structure, the position of the phenanthridinone moiety is very similar. However, the position of carbamoyl linker and phenyl-benzoic acid groups are quite different between the docked and crystal structures (Suppl. Figure S1).

*MD analysis of the wild-type and W32A SOD1 in complex with Lig9*

The trajectories generated by molecular dynamic simulations were analyzed for the stability of Lig9 binding near the tryptophan binding site. MD simulations were performed for 50ns. Root mean square deviation (RMSD) analysis (Figure 4(A)) and root mean square per residue fluctuation (RMSF) analysis (Suppl. Figure 2) of the protein backbone dictate the absence of any conformational effects in both the control protein (wild-type SOD1) as well the W32A mutant. But in the presence of Lig9, the RMSD analysis of the ligand position with respect to the protein backbone highlights the loss of binding stability of Lig9 in the W32A complex compared to the wild-type complex (Figure 4B). Notably, a significant loss of intermolecular interactions between the ligand and the W32A-SOD1 protein in the post 20ns simulation time suggesting that W32 is indeed important to make a stable complex with the ligand through  –  interactions. The RMSF contribution of the individual atoms of the ligand also showed increased fluctuations throughout the simulation time in the mutant dimer compared to the wild-type complex (not shown). Analysis of the radius of gyration (Rg) of the ligand backbone showed the negligible difference between the wild-type and W32A mutant.

Visual analysis of the trajectory run revealed the loss of ligand interactions with the binding site when the W32A mutation was incorporated. Towards the end of the simulation, there was a change in the Lig9 binding region as it moved closer to the protein dimer interface. The loss of interaction in the Trp32 site correlates well with the RMSD of the ligand binding with respect to the protein backbone which shows drastic increases in deviation post 20ns simulation time (Figure 4B). A similar fluctuation is also observed in the RMSF graph of the ligand position relative to the protein (not shown). Each ligand atom is found to be fluctuating twice or more with respect to protein position in the mutated W32A SOD1-Lig9 complex.

Trajectory cluster analysis provides perspective on the difference in the ligand positioning in wild-type and mutant SOD1. A total of 1042 frames at a step size of 10 from the original set was considered for the cluster analysis. A total of 8 clusters and 24 clusters were obtained in the cluster analysis of the wild-type and W32A complex, respectively (Fig. 5; Suppl. Table 2 and 3). The increase in the number of clusters in the mutated species indicates the instability of the ligand interaction with a particular region, allowing it to explore a wider RMSD matrix from the original reference position. The overlap of the interaction regions in both the cases defines drifting of ligand binding from the W32 binding site in the absence of tryptophan residue, stating its pivotal role in the binding of Lig9 (Figure 5(C)). A close ligand residue analysis throughout the 50ns simulation highlighted the loss of contact with Trp32 by the complete abolishment of  –  interactions. Taken together, the MD simulations highlight the importance of  –  interaction is important to obtain a stable complex with SOD1 at the Trp32 binding site. This inference correlates well with both crystallographic and affinity studies.

***Microscale thermophoresis (MST) assay***

As a quantitative binding assay could not be performed by SPR due to the presence of high DMSO in the Lig9 buffer, MST was carried to evaluate the binding efficiency of Lig9 against SOD1 and the W32A mutant. In MST, the binding affinity (Kd) is calculated based on the directional movement of molecules along a temperature gradient, and the protein-ligand interactions are measured using thermophoretic properties of the interacting molecules. The Lig9 compound binding to the native SOD1 results in the binding affinity of 5.7 ± 1.3 µM, suggesting that Lig9 significantly binds to SOD1 (Figure 6(A)). As expected, the W32A mutant yielded low binding with a Kd value of 1.2 ± 0.3 mM which suggest the importance of the Trp32 for making strong interaction with the ligand.

***Tryptophan oxidation assay***

Next, we assessed the effect of Lig9 binding on the oxidation of SOD1Trp32 using AAPH (2, 20-azobis (2-amidinopropane) dihydrochloride), a well-established pro-oxidant that selectively oxidizes Trp residues. The AAPH derived free radicals induce Trp oxidation thereby quenching its fluorescence. The kinetic profile of Trp oxidation was assessed by fluorescence spectroscopy. In the absence of ligands, SOD1 exhibited time-dependent increase in oxidation of Trp residues in the presence of AAPH, as indicated by reduced fluorescence. However, Lig9 showed significant inhibition of Trp oxidation (Figure 6(B)). The  –  stacking interaction of Lig9 with the Trp32 residue of SOD1 is indeed contributed more to the oxidation inhibition, in addition to the electrostatic interaction due to dihydroxy phenyl moiety of Lig9.

***Comparison between the Lig9 and SBL1 complexes***

We have recently reported the crystal structure of a naphthalene-derived, designed compound (SBL1) in complex with SOD1 (Kershaw et al., 2013; Manjula et al., 2018) (PDB Id: 5YTO). The SBL1 molecule also binds near the Trp32 site. The naphthalene moiety exhibits  –  interactions with the indole ring of Trp32 (site 1), while the dihydroxy phenyl moiety is positioned in a small hydrophilic shallow pocket formed by the loop II connection of -strands 2 and 3 (site 2). Notably, the present Lig9 complex structure suggests a new secondary hydrophilic site (site 2’) (described below). Comparison of Lig9 and SBL1 complexes indicates the binding modes of these compounds are similar; however, the interaction exerted by Trp32 with the naphthalene and phenanthridine moiety of SBL1 and Lig9 respectively, do not overlap atom for atom (Figure 7(A)). Intriguingly, the positioning of the dihydroxy phenyl moiety and the phenyl benzoic acid moiety of SBL1 and Lig9, respectively, are different; but, both recognize hydrophilic regions. The side chain conformations of the binding site residues, including Trp32, also differ because of the changes in the interactions of both compounds. The side-chain conformations of Lys23 and Lys30 are quite different in each case. The common boundary of the site 2 and site 2’ is defined by the orientation of the side-chain of Lys30. Minor rotational variations were found on the carboxylate heads of both Glu21 and Glu100. Glu100 is an important residue for hydrophilic interactions with the moiety whether it is in site 2 or site 2’. Overall, our recently reported structure and the present structure revealed an alternative hydrophilic site (site 2’) which may need to be considered, besides site 1 and site 2, when developing small molecules by computational studies.

***Comparison between the Lig9, aniline and Quinazoline complexes***

The crystal structures of aniline in complex with L38V SOD1 (PDB Id: 2WZ0) and a quinazoline derivative with G93A SOD1 (PDB Id: 2WZ6) have been reported (Antonyuk et al., 2010). Superposition of these complexes revealed that the phenanthridinone moiety of Lig9, aniline and quinazoline bind to SOD1 in a similar manner (Figure 7(B) and (C)). The main difference between them in the ‘site 1’ region is that the indole ring of Trp32 is flipped to form proper  –  interactions with the respective cyclic ring moiety of these ligands. The side chain conformation of Glu21 is also different in the hydrophilic binding pocket. These conformational changes are required to accommodate the ligand in the Trp32 site and to avoid a steric clash between Trp32 and Glu21.

**Conclusion**

Despite decades of effort there is still no cure for ALS. Drug molecules designed and tested for application in familial ALS should have the capacity to stabilize the SOD1 dimer and prevent aggregation. Our studies infer that, in addition to these properties, ligands that prevent SOD1 Trp32 oxidation have therapeutic potential. Virtual screening against the NCI Diversity set III dataset and the SPR binding assay yielded five potential compounds that bind to SOD1. From those five compounds, we have shown that a phenanthridinone derivative (Lig9) binds *in crystallo* to SOD1 at the Trp32 site. In addition, a 50ns MD study showed that ligand binding is and suggested the importance of  –  interactions with Trp32. A binding study using SPR and MST correlate well with the crystal structure of SOD1-ligand complex. Moreover, a Trp-oxidation assay confirmed that the Lig9 molecule significantly inhibits Trp32 oxidation in SOD1. We propose, based on the crystal structures of the Lig9 and SBL1 complexes, that (i) site 1 is pivotal to protect the oxidation of Trp by making  –  interactions with ligand, and (ii) the hydrophilic sites (site 2 and site 2’), containing critical interacting residues Glu21, Lys30, Ser98 and Glu100, play an important role in making stable SOD1-ligand binding interactions. Chemical modifications of Lig9 in the terminal phenyl-benzoic acid ring portion may help to improve the binding of phenanthridinone derived compounds with SOD1. Thus, the discovered compound, Lig9, can serve as an excellent template, to identify novel phenanthridinone based SOD1 modulators.

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**Figure legends**

Figure 1. Binding of the compounds (A) Lig6, (B) Lig9, (C) Lig11, (D) Lig13 and (E) Lig15 with the SOD1. The respective ligands and the interacting residues are shown using stick representation. The –  or  – ionic interactions are indicated by dots on the respective ring structures. Oxygen and nitrogen atoms are colored in red and blue, respectively.

Figure 2. Surface plasmon resonance (SPR) assay of SOD1-ligand interactions. (A) SPR sensorgram for the binding of screened molecules on SOD1 immobilized on a CM5 chip was obtained at 250 µM ligand concentration. SPR sensorgrams were established at association and dissociation time intervals of 60 sec and 180 sec, respectively. Lig9 showed maximum response compared to other ligands. (B) Sensorgram for the binding of Lig9 on wild and W32A mutant of SOD1 immobilized on a CM5 chip detecting 125 µM and 250 µM of Lig9 with association and dissociation time intervals of 120 and 180 s, respectively. The dose dependency of Lig9 binding indicates the binding efficiency of the compound is higher for wild-type compared to W32A mutant protein.

Figure 3. The tertiary structure of the SOD1–Lig9 complex. (A) Lig9 binds between chain F of the EF dimer and chain I of the IJ dimer (arbitrary view). (B) Rotation 90o of (A) about its horizontal axis. (C, D) A close-up view of SOD1–Lig9 interaction. 2|Fo| - |Fc| map contoured at 1.0s in the binding site region of Lig9. The benzoate moiety of Lig9 is not shown as the corresponding electron density is absent. The ligand and interacting residues are shown by sticks. Hydrogen bonds are indicated by a dotted line.  –  interactions are shown by dots on the ring structures. Water molecules are shown as spheres. The Zn ion is shown by grey spheres.

Figure 4. Molecular dynamics simulations of the wild-type SOD1 dimer, mutant W32A-SOD1, and their complex with Lig9. (A) The root-mean square deviation (RMSD) of the wild-type SOD1 dimer (black), and the mutant W32A-SOD1 dimer (grey), (B) RMSD of Lig9 in complex with wild-type SOD1 (black), and mutant W32A-SOD1 (grey).

Figure 5. molecular dynamics simulations of Lig9 in complex with the wild-type SOD1 dimer, and the W32A-SOD1 dimer. (A) Trajectory cluster analysis of the wild-type complex (green mesh), (B) Trajectory cluster analysis of the W32A mutant complex (red mesh), and (C) Overlap view of the clusters shown in (A) and (B). Respective ligands clusters are shown as sticks.

Figure 6. Microscale thermophoresis (MST) and Trp-oxidation assay. (A) MST interaction analysis of the wild-type and W32A SOD1 proteins with Lig9. Dose-response curves for the binding interaction between Lig9 and native or W32A SOD1. Plots show the fraction bound against the ligand concentration (0 = unbound, 1 = bound). Lig9 shows substantial binding with the wild-type SOD1 (brown) (Kd of 5.7 ± 1.3 µM), whereas binding is very weak against the W32A mutant (green) (Kd:1.2 ± 0.3 mM) demonstrating the importance of Trp32 for ligand binding. (B) Kinetic profiles of SOD1 Trp32 consumption mediated by AAPH free radicals in the presence and absence of Lig9. The mutant protein W32A was used as a control to check significant deviations in fluorescence emission at 360nm (excitation at 295nm) with time. The kinetic profile of SOD1 in presence of Lig9 was almost the same as the normalized data of W32A.

Figure 7. Comparison of the SOD1-Lig9 complex and SBL-1. Superposition of the SOD1– Lig9 complex (A) on to the SOD1 – SBL-1 complex (PDB Id: 5YTO). The naphthalene moiety of SBL-1 makes  –  interactions with SOD1 Trp32 (Site 1). The dihydroxy moiety of SBL-1 binds in the hydrophilic region of Site 2, whereas the benzene group of Lig9 binds in the Site 2’ hydrophilic region, (B) on to the SOD1-Aniline complex (PDB Id: 2W20). The aniline group contributes  –  interactions with Trp32 of SOD1, and (C) on to the quinazoline complex (PDB Id: 2WZ6). The quinazoline group contributes  –  interactions with Trp32 of SOD1. The ligands and interacting residues are shown by sticks. The nitrogen and oxygen atoms are colored in blue and red, respectively. Dotted circles represent Site 1, Site 2, and Site 2’. The Lig9, SBL-1, aniline and quinazoline molecules are colored in olive green, brown, yellow and pink, respectively.

**Table 1: A short-listed compounds based on SPR assay**

|  |  |  |
| --- | --- | --- |
| **Ligand ID** | **NCI Compound** | **2D Structure** |
| Lig6 | NCI 326182 |  |
| Lig9 | NCI 127133 |  |
| Lig11 | NCI 135168 |  |
| Lig13 | NCI 295300 |  |
| Lig15 | NCI 117987 |  |

**Table 2: Crystallographic data collection and refinement statistics.**

|  |  |
| --- | --- |
| **Data collection parameters** | **SOD1–Lig9 Complex** |
| Space group | C2221 |
| Cell dimensions a b c (Å) | 164.36, 203.69, 144.21 |
| Wavelength (Å) | 1.5814 |
| Resolution (Å)# | 38.43 - 2.5 |
| Unique reflections | 83750 |
| Rmerge † | 0.079 (0.548) |
| ⟨I/σ⟩ | 19.7 (2.0) |
| Completeness (%) | 100 (100) |
| Redundancy | 6.5 (5.7) |
| CC1/2## | 0.824 |
| Wilson plot B-factor (Å2) | 39.1 |
| Refinement | |
| Resolution (Å) | 20.0 – 2.50 |
| No. of reflections | 79255 |
| ǂRwork  / +Rfree  (%) | 16.6 / 23.7 |
| Total no. of atoms  Protein atoms  Water molecules  DMSO  Glycerol molecules  Ligand molecules  Polysulfane molecules  Zn-ion | 12350  11005  1234  2  4  1  3  9 |
| Average B-factor (Å2) | 42.05 |
| RMSD Bonds (Å) | 0.032 |
| RMSD Angles (°) | 2.77 |

#Numbers in parentheses are values in the highest resolution shell.

†, where *Ii(hkl)* is the intensity of the *ith* measurement and ⟨*I(hkl)*⟩ is the mean intensity for that reflection.

ǂ, where |*Fobs*| and |*Fcalc*| are the observed and calculated structure-factor amplitudes, respectively.

+*Rfree* was calculated with 5.0% of reflections in the test set.

##Values correspond to the highest resolution shell

**Supplementary Information:**

**Supplementary Table 1: Docking scores for the 20 compounds from the NCI diversity set III.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Ligand ID** | **NCI Compound** | **Dock Score (kcal/mol)** | **Ligand efficiency**  **(kcal/heavy atom)** |
| Lig1 | NCI 84100 | -7.5 | -0.25 |
| Lig2 | NCI 116702 | -7.4 | -0.2643 |
| Lig3 | NCI 55862 | -6.9 | -0.3 |
| Lig4 | NCI 43998 | -6.8 | -0.2833 |
| Lig5 | NCI 60785 | -6.7 | -0.2481 |
| Lig6 | NCI 326182 | -6.6 | -0.287 |
| Lig7 | NCI 156516 | -6.5 | -0.2167 |
| Lig8 | NCI 214009 | -6.5 | -0.2407 |
| Lig9 | NCI 127133 | -6.4 | -0.1939 |
| Lig10 | NCI 13051 | -6.3 | -0.2172 |
| Lig11 | NCI 135168 | -6.3 | -0.2625 |
| Lig12 | NCI 283845 | -6.1 | -0.2905 |
| Lig13 | NCI 295300 | -6 | -0.24 |
| Lig14 | NCI 295486 | -6 | -0.2308 |
| Lig15 | NCI 117987 | -5.9 | -0.2269 |
| Lig16 | NCI 121868 | -5.9 | -0.1844 |
| Lig17 | NCI 170955 | -5.9 | -0.2682 |
| Lig18 | NCI 211490 | -5.8 | -0.1657 |
| Lig19 | NCI 116709 | -5.5 | -0.2037 |
| Lig20 | NCI 163443 | -5.1 | -0.17 |

**Supplementary Table 2: Trajectory cluster analysis – SOD1-#10 complex**

|  |  |
| --- | --- |
| Cluster no. | No. of members |
| 1 | 412 |
| 2 | 217 |
| 3 | 152 |
| 4 | 91 |
| 5 | 79 |
| 6 | 50 |
| 7 | 28 |
| 8 | 13 |

**Supplementary Table 3: Trajectory cluster analysis – W32A-#10 complex**

|  |  |
| --- | --- |
| Cluster no. | No. of members |
| 1 | 109 |
| 2 | 93 |
| 3 | 86 |
| 4 | 74 |
| 5 | 55 |
| 6 | 47 |
| 7 | 46 |
| 8 | 44 |
| 9 | 42 |
| 10 | 41 |
| 11 | 40 |
| 12 | 40 |
| 13 | 38 |
| 14 | 35 |
| 15 | 35 |
| 16 | 35 |
| 17 | 33 |
| 18 | 31 |
| 19 | 22 |
| 20 | 21 |
| 21 | 20 |
| 22 | 19 |
| 23 | 19 |
| 24 | 17 |



**Supplementary Figure S1**

Superposition of the crystal structure of the SOD1 – Lig9 complex on to the docked structure. The  –  interactions with Trp32 of SOD1 are conserved both in the crystal and docked structures.



**Supplementary Figure S2**

The molecular dynamics simulations of the wild-type SOD1 dimer, mutant W32A-SOD1, and their complex with Lig9. The root-mean square fluctuations (RMSF) of the wild-type SOD1 dimer (black), and the mutant W32A-SOD1 dimer (grey).