Interaction of Half Oxa-/Half *cis*-Platin Complex with Human Superoxide Dismutase and Induced Reduction of Neurotoxicity

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Supporting Information

ABSTRACT: The formation of amorphous protein aggregates containing human superoxide dismutase (hSOD1) is thought to be involved in amyotrophic lateral sclerosis onset. *cis*-Platin inhibits the oligomerization of apo hSOD1, but its toxicity precludes any possible use in therapy. Herein, we propose a less toxic platinum complex, namely oxa/*cis*-platin, as hSOD1 antiaggregation lead compound. Oxa/*cis*-platin is able to interact with hSOD1 in the disulfide oxidized apo form by binding cysteine 111 (Cys111). The mild neurotoxic phenomena induced in vitro and in vivo by oxa/*cis*-platin can be successfully reverted by using lypoyl derivatives, which do not interfere with the antiaggregation properties of the platin derivative.

KEYWORDS: Human superoxide dismutase, chemotherapy drugs, lypoyl derivatives, SOD1 oligomerization, neuropathic pain

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by a progressive loss of motor neurons in the central nervous system, which results in devastating disorders of the neuromuscular apparatus.^{1,2} Although several therapies have been tried, the only FDA-approved drugs for the treatment of ALS, riluzole (a glutamate antagonist) and edaravone, extend the lifespan of ALS patients by only few months.^{3,4} So far, there is no effective remedy for this progressive, fatal disorder as a consequence of the fact that the mechanisms involved in ALS pathogenesis remain unclear and are thought to be multiple.¹ More than 90% of ALS incidences are of unknown cause and are termed sporadic (sALS). The remaining ALS cases show familial inheritance, possibly related to mutations in a number of genes in a dominant manner (fALS). It has been shown that human superoxide dismutase 1 (hSOD1) is implicated in a sizable fraction of fALS.⁵ Several missense mutations in the *sod1* gene, scattered throughout the primary protein sequence, have been linked with the onset of fALS.⁶

hSOD1 is ubiquitously expressed in human cells where it is mainly localized in the cytosol and in the mitochondria intermembrane space at micromolar concentrations.^{7,8} It is also present in the nucleus and in the peroxisomes at lower levels. hSOD1 is a homodimeric metalloprotein harboring in each subunit a catalytic copper ion and a structural zinc ion, an intrasubunit disulfide bond between a highly conserved pair of cysteines (Cys-57 and Cys-146), and two free cysteines (Cys-6 and Cys-111). Functionally hSOD1 is responsible for protecting cells from oxidative damage by eliminating superoxide ions through disproportionation.⁹

Although still under debate,^{10,11} a prominent hypothesis for the onset of ALS involves the formation of amorphous protein aggregates containing misfolded hSOD1 as the main component in amyloid-like deposits, which have been widely found both in the spinal cords and glia of ALS patients^{12,13} and in transgenic mice developing ALS.^{14–16} The precursors of these protein aggregates are believed to be soluble oligomeric intermediates of the hSOD1 aggregation process. hSOD1 aggregation has been proposed to be related to the toxic gain of function, similar to what has been proposed for other neurodegenerative diseases.¹⁷

The early species of the maturation process (i.e., SOD1 lacking metal ions) have been shown to have high tendency to oligomerize in vitro, while the mature form of hSOD1 is not prone to aggregate.^{18,19} Previous studies have shown that, in the apo hSOD1, the two free cysteines (Cys-6 and Cys-111) become solvent accessible and form intermolecular disulfide bonds, which cross-link the molecules into high-molecular-weight oligomers under physiological conditions (37 °C, pH 7, and 100 μ M protein concentration).^{19,20} These oligomers of high molecular weight (over MDa) are remarkably stable, persisting in the soluble state for months, and exhibit positive ThT binding response.¹⁹

A recent study has shown that *cis*-diaminedichloroplatinum(II) (*cis*-platin) (Figure 1) can be selected as potential lead compound for blocking hSOD1 oxidative oligomerization.²¹

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Figure 1. Structure of *cis*-platin, oxaliplatin (OXA), [PtCl₂(1R,2R-1,4-DACH)], ADM_09, and ADM_12.

cis-Platin is a small chemotherapeutic drug molecule with very high binding affinity toward thiol groups in proteins.²² It has been proved that *cis*-platin binds apo hSOD1 with a dissociation constant of $37 \pm 3 \mu\text{M}$ (at 37 °C) and inhibits its oligomerization in vitro and in vivo by covalently binding to the solvent exposed Cys-111.²¹ Of note, it also leads to the dissociation of already formed apo hSOD1 oligomers without affecting the normal hSOD1 enzymatic activity.²¹ Nevertheless, clinical use of this chemotherapeutic agent is severely limited by the development of serious side effects including nephrotoxicity, emetogenesis, ototoxicity, and neurotoxicity.²² The peripheral neuropathy associated with these neurotoxic effects can be prolonged, persistent, and often responsible for therapy interruption. Recent data have suggested that neurotoxicity induced by chronic administration of *cis*-platin and oxaliplatin (OXA, another widely used chemotherapy drug) (Figure 1) is related to the formation of reactive oxygen species (ROS) that trigger changes in the expression and sensitivity of TRPs nociceptor sensor²³ and cell death.²⁴ It is thus clear that the availability of safe and effective analgesic drugs, able additionally to reverse or counterbalance the toxic effects of *cis*-platin related to the formation of ROS, is of wide interest. Two candidates successfully tested in the treatment of neuropathic pain are the lipophilic-containing derivatives ADM_09 and ADM_12.^{25,26} They are structurally simple (Figure 1), obtained by reacting the commercially available ($\pm$) α -lipoic acid, whose antioxidative and analgesic properties are well documented,²⁷ with L-carnosine (ADM_09) or homotaurine (ADM_12). L-Carnosine has been proven to scavenge reactive oxygen species (ROS),²⁸ while homotaurine is largely employed in ROS-mediated cognitive diseases.²⁹ Noteworthy, in ADM_09 and ADM_12, a synergic combination of features of, respectively, lipoic acid and carnosine or homotaurine was observed.²⁹ In addition, it has been proven that these compounds are able to effectively revert in vivo neuropathic pain induced by OXA, without eliciting negative side effects. In vitro tests, in turn, clearly showed the absence of any toxic or cardiotoxic effect for both ADM_09 and ADM_12.^{25,26,30}

Considering the ability of *cis*-platin to inhibit the oligomerization of hSOD1 and the properties of ADM_09 and ADM_12 as radical scavengers, we investigated the propensity of these two lipophilic derivatives to revert in vivo the neuropathic pain induced by *cis*-platinum and evaluated in vitro the lack of possible interference when used with *cis*-platin in hSOD1 antiaggregation tests. As reported,³¹ however, the

high toxicity of *cis*-platin caused mice to die before inducing the development of neuropathic pain. So, even though the antiaggregation ability of *cis*-platin is preserved also when used in combination with ADM_09 or ADM_12 (Figure S1), we replaced *cis*-platin with the less toxic OXA. The treatment of hSOD1 with OXA, however, was completely ineffective, and oligomerization was observed as with the control (hSOD1 alone). Thus, reasoning that the two chlorine ligands should be preserved, we used [PtCl₂(1R,2R-1,4-DACH)] (Figure 1), another platinum complex less toxic than *cis*-platin and featuring the required structural elements to rationally inhibit the oligomerization of hSOD1.

We found that [PtCl₂(1R,2R-1,4-DACH)] complex (herein referred as oxa/*cis*-platin) is indeed able to interact with hSOD1, inhibiting its aggregation also in the presence of ADM_09 or ADM_12, and that these two latter molecules are effective in reverting oxa/*cis*-platin-induced neuropathy in vivo without eliciting side-effects. These results might open a door toward a novel, less aggressive, therapeutic strategy to address fALS.

Synthesis of ADM_09 and ADM_12. Activated ($\pm$) α -lipoic acid was reacted with L-carnosine or homotaurine to form, after purification, ADM_09 (81%) or ADM_12 (52%), respectively^{25,26} (see SI). The two lipophilic derivatives were used to study the oligomerization of hSOD1 in the presence of oxa/*cis*-platin and to evaluate their ability to revert oxa/*cis*-platin-induced neuropathic pain *in vivo*.

SOD Aggregation Tests. Apo hSOD1 was incubated with ADM_09 and ADM_12 (1 mM) under physiologically like conditions (pH 7.0, 37 °C, $\sim 100 \mu\text{M}$ protein concentration) in the presence of the oxa/*cis*-platin complex (0.4 mM). In the absence of oxa/*cis*-platin, apo hSOD1 showed a progressive enhancement of ThT fluorescence signal, consistent with a previous finding.¹⁹ As hypothesized, oxa/*cis*-platin efficiently inhibits the oligomerization of apo hSOD1, showing a slower increase in ThT fluorescence over time, similar to that observed with the neurotoxic *cis*-platin.³¹ Oligomerization of hSOD1 in the presence of oxa/*cis*-platin together with ADM_09 or ADM_12 was still inhibited, meaning that the two lipophilic derivatives do not modify the behavior of the platinum complex with respect to hSOD1 oligomerization (Figure 2). The dissociation constant of oxa/*cis*-platin to apo hSOD1, as

Figure 2. Fluorescence due to ThT binding to hSOD1. Plot of intensity of fluorescence versus time (hours) for disulfide oxidized form of apo hSOD1 incubated with small molecules: ■ hSOD1 control; ● hSOD1 + oxa/*cis* platin complex, 0.4 mM; ▲ hSOD1 + ADM_12, 1 mM, + oxa/*cis*-platin complex, 0.4 mM; ► hSOD1 + ADM_09, 1 mM, + oxa/*cis* platin complex, 0.4 mM.

determined from equilibrium dialysis (Figure S2), was $176 \pm 12 \mu\text{M}$, allowed a conversion of about 70% of apo hSOD1 into oxa/cis-platin-bound form.

Cell Viability (cis-Platin, OXA, and Oxa/cis-Platin). The neurotoxicity of the anticancer compound oxa/cis-platin was studied in SH-SY5Y neuron-like cells after differentiation with retinoic acid (see SI for details). Cells were treated with increasing concentrations of oxa/cis-platin (1, 3, 10, 30, and $100 \mu\text{M}$, see Table S1) and tested after 24 and 48 h. After 24 h, the viability of the cells treated with the highest concentration of oxa/cis-platin was significantly decreased. In this case, the cells survived by about 50% (Figure 3). After 48 h, treatment

Figure 3. Effect of ADM_12 against oxa/cis-platin-induced toxicity in SH-SY5Y neuron-like differentiated cells, treated with increasing amount of oxa/cis-platin and tested after 24 h in the absence or in the presence of $100 \mu\text{M}$ ADM_12. Cell viability was quantified by MTT assay. Absorbance was measured at 550 nm, and values were expressed in percentage of control ($0 \mu\text{M}$ treatment) as mean $\pm$ SEM of six experiments. $^{\wedge}P < 0.05$ and $^{\wedge\wedge\wedge}P < 0.001$ versus control; $^{\#\#}P < 0.05$ versus oxa/cis-platin.

with $30 \mu\text{M}$ oxa/cis-platin decreased cell viability by about 86% (Table S1). As reference compounds, OXA and cis-platin were tested in the same conditions. cis-Platin showed the highest potency in evoking toxicity at both incubation times (Table S1). To evaluate the protective profile of ADM_12 against the neuronal toxicity of oxa/cis-platin, cell viability was measured after a 48 h cotreatment with $100 \mu\text{M}$ ADM_12 and increasing concentration of oxa/cis-platin. As shown in Figure 3, ADM_12 significantly prevented cell death, as 25% of cells maintained viability also in the presence of the highest concentration ($100 \mu\text{M}$) of oxa/cis-platin. ADM_09 in a similar test provided a less interesting protective profile.

In Vivo Tests. As known, repeated administration of OXA (2.4 mg kg^{-1} , daily, intraperitoneally (i.p.)) in mice induces the progressive development of a painful neuropathy.³² In Figure 4, the evaluation of the pain threshold as response to a non-noxious thermal stimulus (cold plate test, allodynia-like measure) is shown. OXA induced hypersensitivity starting from day 7, and the effect increased on days 14 and 21. On this basis, we studied the neurotoxicity of oxa/cis-platin setting up a novel model of neuropathic pain. Because of the higher neurotoxicity of cisplatin in comparison to oxaliplatin,²³ we chose to daily treat animals i.p. with 1.5 mg kg^{-1} oxa/cis-platin. This compound induced a moderate neuropathy on day 21 only, confirming the lower neurotoxicity of this still underestimated platinum derivative. However, neuropathic pain evoked by oxa/cis-platin was sensibly reduced by ADM_12 (Figure 5). A single per os (p.o., starting from 10 mg kg^{-1}) administration on day 21, dose-dependently, relieved the cold

Figure 4. Effect of oxa/cis-platin on pain threshold in mice. Oxa/cis-platin (1.5 mg kg^{-1}) was injected daily i.p. In comparison, different groups were daily treated i.p. with OXA (2.4 mg kg^{-1}). The response to a non-noxious thermal stimulus (allodynia-like) was measured by the cold plate test on days 0, 7, 14, and 21 before the daily treatment. Each treatment group consists of 10 mice analyzed in two different experimental sets. Data were shown as mean $\pm$ SEM. $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ versus vehicle-treated.

Figure 5. Effect of ADM_12 against oxa/cis-platin-induced neuropathic pain. Oxa/cis-platin (1.5 mg kg^{-1}) was daily i.p. injected. On day 21, the pain threshold was measured before and after, over time, with the administration p.o. of increasing doses of ADM_12 (10 – 100 mg kg^{-1}). The cold plate test was used to measure the response to a non-noxious thermal stimulus (allodynia-like). Each treatment group consists of 10 mice analyzed in two different experimental sets. Data were shown as mean $\pm$ SEM. $^{\wedge}P < 0.05$ and $^{\wedge\wedge}P < 0.01$ versus vehicle-treated animals; $^*P < 0.05$ versus oxa/cis-platin + vehicle.

hypersensitivity induced by oxa/cis-platin. ADM_12 *per se* did not alter pain threshold of control animals (Table S2), and it did not possess analgesic effects but was able to counteract neuropathic hypersensitivity.

X-ray Analysis of Oxa/cis-Platin with SOD1. To demonstrate how oxa/cis-platin interacts with the protein to prevent apo hSOD1 aggregation, we solved the structure of apo hSOD1 where crystals were soaked with the platinum complex, which was added as a powder directly into the drop and left to incubate for 24 h. The elementary crystal cell of apo hSOD1 was clearly superimposable with that of the previously solved one in complex with cis-platin²⁵ (3RE0), and no major differences were present. The crystal structure contains two dimers: one of the two shows a clear electron density for all residues from the N-terminal to the C-terminal,

whereas in the second dimer two loops lack any kind of density due to the mobility caused by the absence of zinc. As a main goal, crystal structure verified the presence of the platinum compound and visualized its binding mode to Cys111, which is the residue where it was expected to bind. Therefore, the data set was collected at a wavelength (0.972 Å) that was slightly above the LIII platinum absorption edge (1.072 Å) where Pt has a strong fluorescence. The existence of this fluorescence (known as anomalous scattering) at binding distance from the sulfur atom of Cys111 confirms the presence of platinum. The latter is showed by large peaks of standard and anomalous density (blue and red respectively) at each Cys111 (Figure 6).

Figure 6. Electron density map contoured at 1σ level (blue) showing the two adjacent Cys111 residues and anomalous difference map (red) contoured at 10σ level clearly showing the presence of platinum in the expected positions.

As expected, platinum is bound to each of the four Cys111 present in the asymmetric unit, but in all cases, it has a partial occupancy with values ranging from 0.2 to 0.4, indicating that Pt is not bound to all Cys111 simultaneously. Indeed, as in the case of the *cis*-platin adduct (3RE0), the partial occupancy is mainly due to steric hindrance, which makes it impossible for the two platinum ions to bind to the two adjacent Cys111 of each dimer at the same time. It results that each oxa/*cis*-platin molecule loses a labile chlorine atom upon binding to the sulfur of the cysteine, also in agreement with previous observation.^{19,21} Each Pt atom shows a quite distorted square planar coordination where the four donor atoms are the two nitrogens, one chlorine, and the cysteine sulfur (Figure 7).

Figure 7. Electron density map contoured at 0.8σ level around Cys111 of one of the four monomers in the asymmetric unit showing the faint density of the six-term ring of the ligand and the distorted square planar coordination on platinum.

The absence of a neat electron density accounting for the six-term ring of the ligand might reasonably be due to several factors: (i) the low occupancy of the ligand itself, (ii) a certain degree of mobility of the ligand, which could swing about the platinum pivot point, and (iii) the overwhelming platinum electron density, which could somehow hide that of the much lighter atoms on the ring. The well-documented chemical

stability of nitrogen–platinum chelating complexes³³ and a patch of density appearing in the putative position of the ring when lowering the contour value of the electron density from 1.0 to 0.7σ (including thus weaker signals) strongly support the hypothesis that the platinum moiety is bound to Cys111 although with partial occupancy.

In conclusion, we showed the ability of oxa/*cis*-platin to interfere with hSOD1 aggregation, a pathological event and object of controversial scientific debates but supposed to be involved in ALS onset. Structurally, oxa/*cis*-platin is related to both *cis*-platin and OXA, but the chlorine ligands reasonably account for the capability of oxa/*cis*-platin of hampering apo hSOD1 oligomerization, as known for *cis*-platin but lacking in the case of OXA. Our data confirmed the lower toxicity of oxa/*cis*-platin in vitro and in vivo with respect to *cis*-platin. Nonetheless, some neurotoxic effects were still observed that deserve further investigations. Of note, in vivo neurotoxicity induced by oxa/*cis*-platin can be relieved by the lipoyl derivative ADM_12. Altogether, the data herein reported show that oxa/*cis*-platin could be a good lead compound for the design of new drug entities.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.8b00199.

Materials and methods; synthesis and characterization of ADM_09 and ADM_12; Tables S1–S3; Figures S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; hSOD, human superoxide dismutase; sALS, sporadic ALS; fALS, familial ALS; OXA, oxaliplatin; ROS, reactive oxygen species

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