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A new platinum complex of triazine demonstrates G1 arrest with novel biological profile in human breast cancer cell line, MDA-MB-468

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Abstract—A novel class of platinum(II) complexes of pyridine sulfide derivatives of triazine was synthesized, characterized, and investigated using the human breast cancer cell line, MDA-MB-468. S-30 was one of the most potent derivatives of its class (IC_{50}, 0.39 μM) eliciting the greatest biological response. S-30 induced arrest in the G1 phase and apoptosis (TUNEL assay) in a $p53/p21^{WAF1/CIP1}$-consistent manner. Modeling and docking experiments were performed for three known targets for cisplatin, d(GpG), d(ApG), and a protein (Cu/Zn superoxide dismutase, SOD) from bovine origin. A Blast search of bovine SOD was performed to identify analogous human protein targets resulting in about 22 human proteins. A multi-sequence alignment of those targets showed >80% sequence identity and >88% similarity. One of them is SOD1 that is differentially expressed (based on global gene expression pattern) in various forms of cancer and other diseases. SOD1 controls apoptosis via p53/BAD/BAX/BCL2 in the amyotrophic lateral sclerosis (ALS) pathway and is also involved in various other KEGG's pathways. Results suggest that the S-30 is a potential cytotoxic agent.

Breast cancer is one of the most common cancers in women and the second most lethal cancer in Western countries.[1] Despite therapeutic advances, breast cancer still has a high mortality rate.[2] Cisplatin is one of the most effective antitumor agents used against a variety of cancers and has been studied extensively.[3–11] Unfortunately, the use of cisplatin is limited in some cancers, such as breast cancer, due to the development of drug resistance, as well as its side effects. There has been an increasing interest in the development of cisplatin analogues designed to overcome drug resistance, reduce toxicity, and increase efficacy in combination with other therapies.[12]

We have proposed our rationale in designing simple platinum complexes to improve specificity and potency.[13–16] Here, we report the synthesis of new pyridinesulfide derivatives of triazine and their platinum(II) complexes (Scheme 1). Ligands **3**–**5** were synthesized in a two-step procedure and used for preparing platinum(II) complexes, **6**–**8**. All compounds were characterized by NMR, mass, and elemental analysis. These data indicate that S-30 contained one molecule of water in its crystals. The structure of one of the platinum(II) complexes, S-19 ($PtC_{12}H_{16}N_6Cl_2S$), was confirmed by X-ray analysis (Fig. 1). In S-19, the platinum is coordinated by two nitrogen atoms, and two chlorine atoms completing a PtN_2Cl_2 core analogous to cisplatin.

The estrogen receptor negative human breast cancer cell line, MDA-MB-468, was used to examine the biological properties of this class of anticancer agents. The rationale behind using MDA-MB-468 cell line for this study was that this cell line represents an aggressive ER

Keywords: Cytotoxic agent; MDA-MB-468; Platinum compound; Cell cycle; TUNEL; Electron micrographs; Apoptosis; Immunohistochemistry; Subcellular localization; p53; $p21^{WAF1/CIP1}$; Molecular modeling; Triazine.
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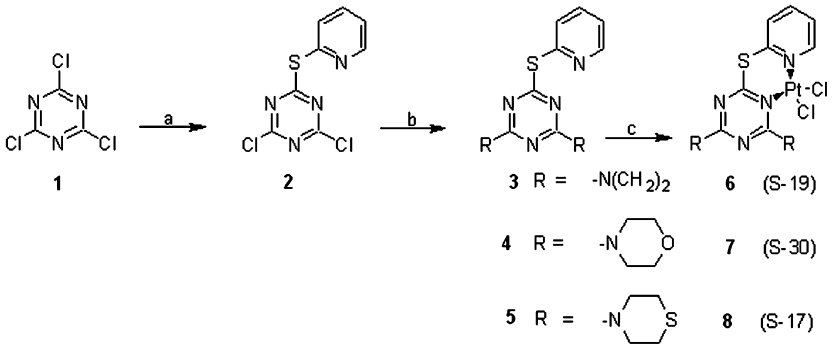

Scheme 1. Synthetic strategy for compound **7** (S-30) and other derivatives.[36] Reagents and conditions: (a) **1** in acetone, cooled to −15 °C; and added 2-thiopyridine [2 equivalents (equiv) in acetone], stirred 30 min at −15 °C, 80%; (b) **2** (1 equiv) in methanol and added morpholine (6 equiv), 25 °C, 8 h, 75%; (c) **4** (1 equiv) in CH_2Cl_2 and added K_2PtCl_4 (1 equiv) in methanol–water mixture (CH_2Cl_2/CH_3OH/H_2O, 10:1:1), N_2, reflux for 8 h, 60%.

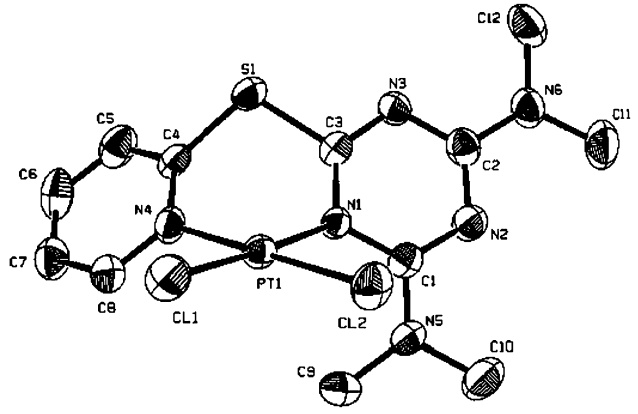

Figure 1. X-ray structural representation of S-19 [Pt ($C_{12}H_{16}N_6SCl_2$)], with the atomic numbering and hydrogen atoms are omitted for clarity.[37]

negative phenotype. Moreover, these breast cancer cells possess a p53 mutant species that can undergo changes to the pseudo-normal wild-type species under certain conditions leading to growth inhibition or apoptosis. Hence, changes to the p53 status could be studied in an aggressive breast cancer phenotype background. S-30 (compound **7**), a dichloro platinum(II) derivative of 2-[(2′-pyridinesulfide)-4,6-bismorpholinyl]-1,3,5-triazine, proved to be the most effective in this class of compounds.

In MDA-MB-468 cells, the platinum(II) complexes were more potent than their respective ligands (IC_{50} $\geqslant$ 100 μM) as determined by the MTT assay (Table 1). In a separate experiment, cells were incubated with S-17, S-19, S-30 or cisplatin up to 10 μM for 24 h. The IC_{50} value of S-30 in MDA-MB-468 cells was 0.39 μM, which is about 5 and 14 times lower than cisplatin (IC_{50} 1.81 μM), S-17 (IC_{50} 3.11 μM), and S-19 (IC_{50} 5.65 μM), respectively.

Table 1.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
3	>100	**6**	5.65
4	>100	**7**	0.39
5	>100	**8**	3.11
		Cisplatin	1.81

In most cycling cells, cytotoxicity is preceded or accompanied by cell cycle arrest.[17] To examine the effect of S-30 on cell cycle progression, we treated cells with varying concentrations of S-30 and analyzed the DNA content of propidium-iodide stained cells by flow cytometry. The G1 cell population increased with time and concentration. At 24 h, 5 μM S-30 caused over a 77.0% accumulation of cells in G1 phase attenuating cells in the S and G2 phases (Fig. 2B). In contrast, cisplatin blocked cell cycle progression in the S phase (G0/G1 = 22.27%, S = 56.01%, and G2/M = 21.72%); S-phase block was also observed in other breast cancer cell lines.[18] This difference in cell cycle arrest by cisplatin and S-30 is interesting and potentially useful in cancer therapy.

Anticancer drugs[19] (including cisplatin) exert their toxicity through induction of apoptosis. To investigate if S-30 induced apoptosis, we examined S-30-treated cells for apoptosis-associated changes. Flow cytometry analysis of S-30-treated cells showed a sub-G1 (A_0) peak (Fig. 2B); this is suggestive of apoptosis. Apoptosis was confirmed by morphology and DNA fragmentation. Representative electron micrographs (Fig. 2D) show that S-30 caused characteristic morphological changes such as membrane blebbing, chromatin condensation, and vacuolization at higher concentrations at 24 h; nevertheless, some chromatin condensation and vacuolisation were also seen at lower concentrations. Apoptotic cells have fragmented nuclear chromatin[20] produced as a result of DNA strand breaks. In situ TUNEL assay showed a dark blue nuclear staining in S-30-treated cells in comparison to control cells, which stained pale red with Nuclear Fast Red (Fig. 2F). Thus, S-30 possesses apoptosis-inducing properties.

Growth arrest and apoptosis occur in response to DNA damage produced by chemotherapeutic drugs.[19] The phosphoprotein p53 exerts its tumor-suppressive effects by inducing arrest and apoptosis, thereby controlling propagation of damaged DNA.[21] p53 mediates G1 arrest through its direct transcriptional target,

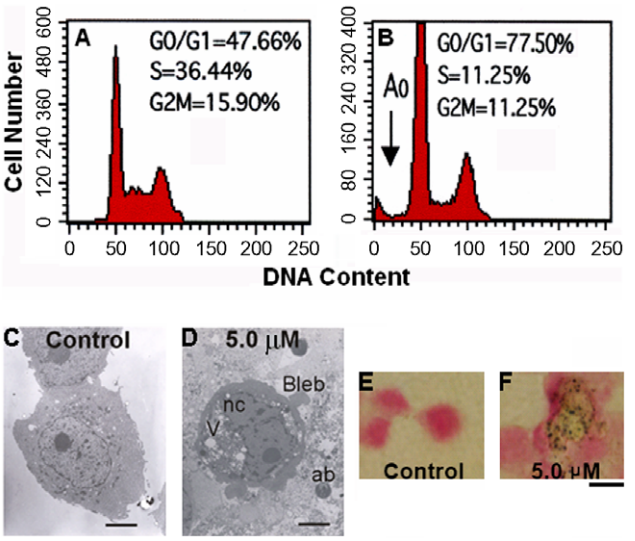

Figure 2. Effect of S-30 on cell cycle distribution and apoptosis; (A) DNA histograms for control; (B) cells treated with 5 μM S-30 for 24 h; histograms are shown in a different scale to magnify the sub-G1 peak, A_0, which is present in cells treated with 5 μM S-30; (C) electron micrographs of control; (D) cells treated with 5.0 μM S-30 for 24 h. At 5.0 μM, as labeled, morphological changes such as blebbing, vacuolization (denoted as **v**), nuclear condensation (denoted as **nc**), and apoptotic bodies (denoted as **ab**) were seen (scale bar-3 μm); (E) control; (F) images of TUNEL stained 5.0 μM S-30-treated cells at 24 h. At 5.0 μM S-30, DNA fragmentation was seen (scale bar-10 μm).

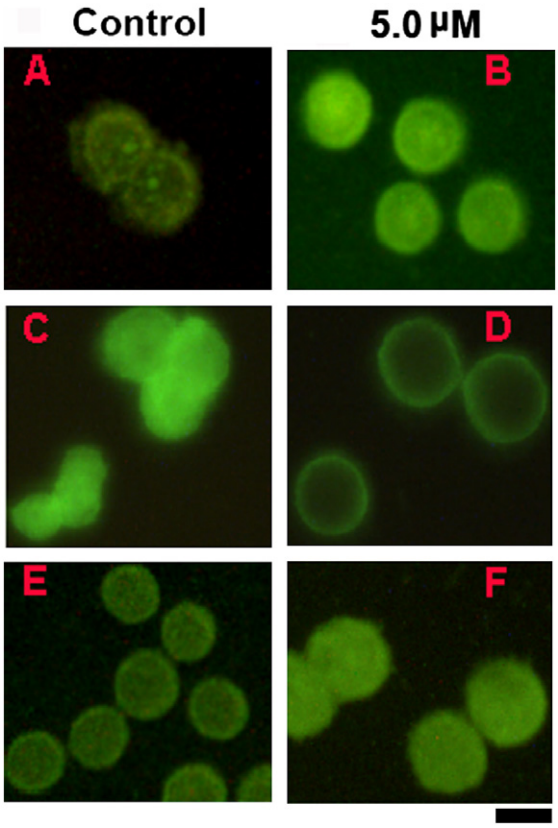

Figure 3. Effect of S-30 on the subcellular localization of p53 and $p21^{WAF1/CIP1}$ in MDA-MB-468 cells. Cells were seeded in chambered glass slides, treated with 5.0 μM S-30 for 3.5 h. (A and B) Cells were stained with wild-type p53-specific PAb 1620. (C and D) Cells were stained with mutant p53-specific PAb 240. (E and F) cells were stained with anti-$p21^{WAF1/CIP1}$. (A and C), and (E) represent corresponding control cells. Cells showed positive staining with PAb 1620 (B) or (D) an absence of reactivity with PAb 240 in the nucleus. S-30 caused an increase in the nuclear staining of $p21^{WAF1/CIP1}$ (F). Scale bar-10 μm.

$p21^{WAF1/CIP1}$.[22] Both p53 and $p21^{WAF1/CIP1}$ are induced by DNA damage.[23–25] MDA-MB-468 cells possess the p53 mutant, $p53^{273His}$. $p53^{273His}$ has properties like its wild-type counterpart ($p53^{273Arg}$)[26] and becomes growth-suppressive under certain conditions.[27,28] Many drugs can cause G1 arrest and G1-restricted apoptosis by restoring the wild-type function of some p53 mutants, including $p53^{273His}$.[29] The nuclear localization of p53 is essential for its growth-suppressive as well as its oncogenic actions.[30] Nuclear accumulation of mutant p53 is associated with poor clinical outcome.[31] MDA-MB-468 cells show positive nuclear localization of mutant p53 under certain conditions.[32] To study the involvement of p53 and $p21^{WAF1/CIP1}$, we performed immunohistochemical analysis of subcellular localization of these proteins. S-30-treated cells displayed a varying pattern of subcellular localization of p53. Fluorescence microscopy showed that at 3.5 h, prior to cell cycle arrest and apoptosis, there was a localization of wild-type p53 species (PAb 1620 reactive) (Fig. 3B) and an absence of mutant p53 species (PAb 240 reactive) in the nucleus (Fig. 3D). S-30 also increased the intensity of nuclear staining of $p21^{WAF1/CIP1}$ protein (Fig. 3F). The prior nuclear localization of wild-type p53 species appears to be part of the lethal signal;[31] such localization and absence of mutant p53 species in the nucleus appear early enough to be causative for p53 involvement in arrest and apoptosis. Thus, S-30 induces the wild-type properties of $p53^{273His}$, which in turn induces $p21^{WAF1/CIP1}$ localization in MDA-MB-468 cells. This suggests that S-30 causes G1 arrest and apoptosis in a p53/$p21^{WAF1/CIP1}$-consistent manner.

Cisplatin generally forms intrastrand and interstrand cross-links with DNA at d(GpG) and d(ApG),[12] and such binding modes have been characterized using short sequences of DNA duplex. The protein data bank[33] (PDB) holds about 29 such structures. The primary mode of binding was first characterized using X-ray crystallography by Dickerson co-workers.[34] In 2006, Calderone et al.[11] have characterized a new target for cisplatin, a protein (Cu/Zn superoxide dismutase, SOD) from bovine origin. According to them, cisplatin selectively binds to His-19 and two other residues (Thr-17 and Gly-31) are within the 3 Å of cisplatin for chain A. These residues are located in the surface of the SOD. Therefore, platinum complexes with bulky ligands can also accommodate without bumping (too close together) other atoms and still be able to alter the function of SOD. Using modeling and docking tools,[35] we have compared by docking S-30 to the three known targets, d(GpG), d(ApG), and SOD, bound to cisplatin. In our modeling experiments, we have used three X-ray structures from PDB, 1LU5[7] for d(GpG), 1A2E[8] for d(ApG), and 2AEO[11] for SOD. S-30 produces more bumping (atoms are too close together) labels when docked to the binding sites of either d(GpG) or

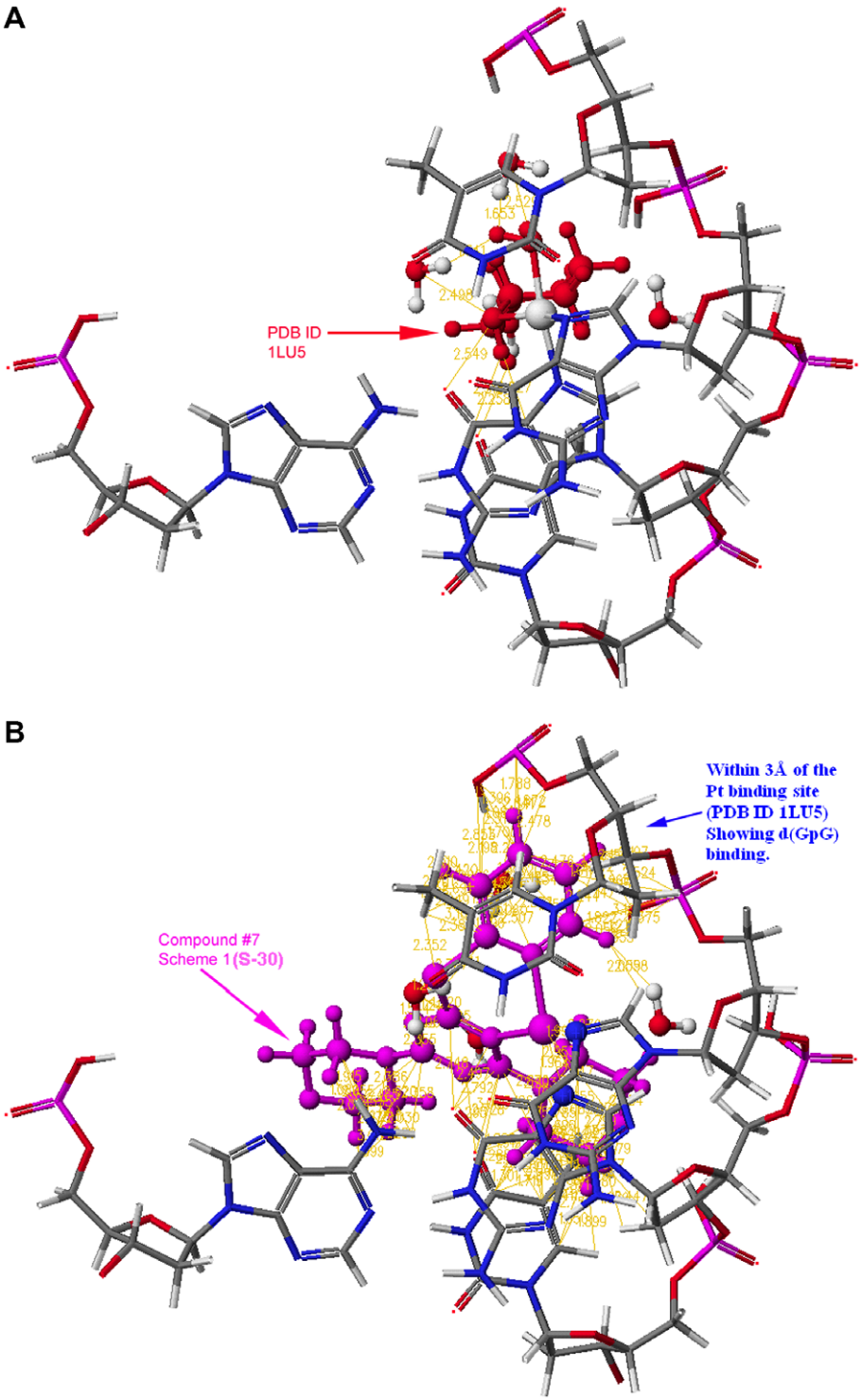

Figure 4. Modeling for the target d(GpG) Pt-binding site using structure of 1LU5; (A) showing the bumping (yellow label) when cisplatin is bound to 1LU5. (B) yellow labels (bumping) are more intense when S-30 is docked to the Pt-binding site.

d(ApG). This indicates that atoms are too close together due to the larger size of S-30. Therefore, S-30 may not be suitable for these sites.

In Figure 4A and B, and in Figure 5A and B docking and bumping labels of cisplatin and S-30 are compared for d(GpG) and d(ApG) targets, respectively. A suitable target that one could speculate for S-30 could be SOD for four reasons (i) bumping labels are relatively less in number so atoms are not too close to each other (Fig. 6A and B), (ii) Pt-binding site is on the surface of the SOD, so S-30 can accommodate for binding and be able to modify protein function upon binding, (iii) analysis of global gene expression (microarray) data shows that SOD is differentially expressed in a number of cancers including breast cancer and in other diseases,

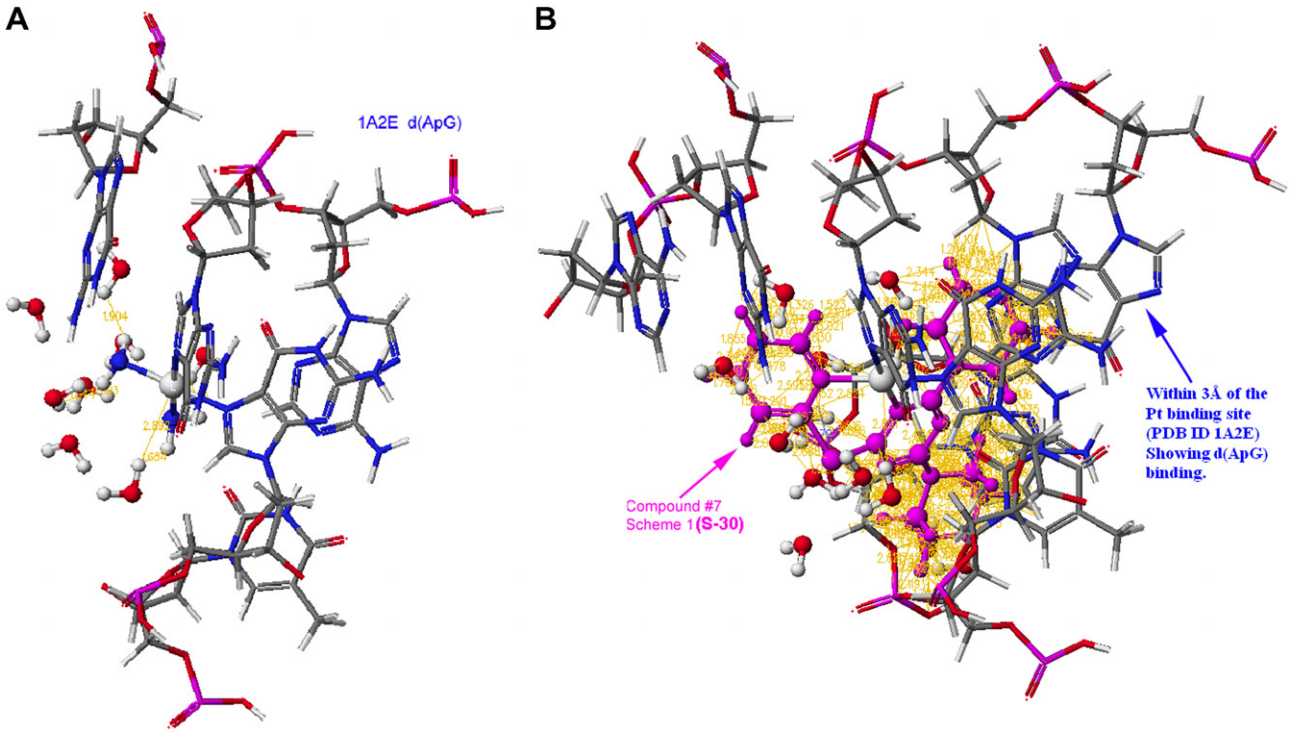

Figure 5. Modeling for the target d(ApG) Pt-binding site using structure of 1A2E; (A) showing the bumping (yellow label) when cisplatin is bound to 1A2E. (B) Yellow labels (bumping, atoms are too close to each other) are much more intense when S-30 is docked to the Pt-binding site.

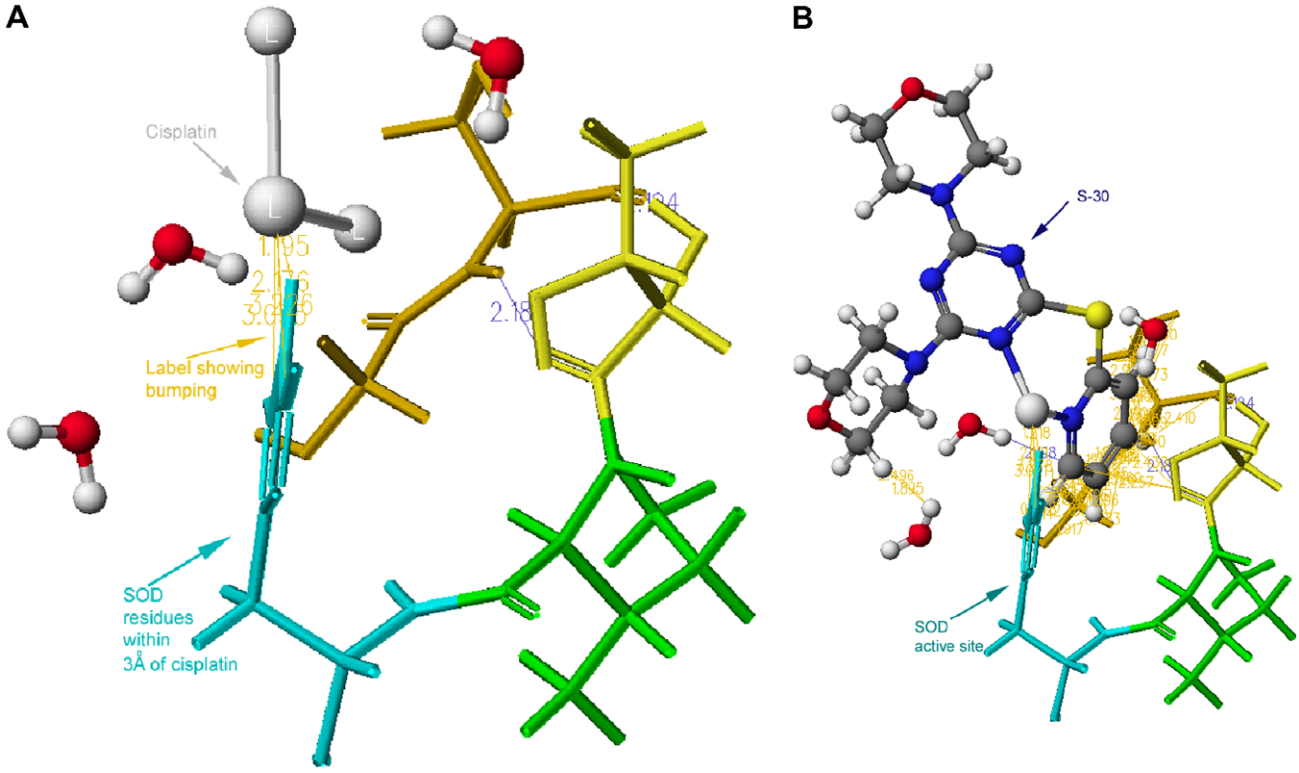

Figure 6. Modeling for the target SOD Pt-binding site using structure of 2AEO; (A) showing the bumping (yellow label) when cisplatin is bound to 2AEO. (B) Yellow labels (bumping) are less compared to d(GpG) or d(ApG) when S-30 is docked to the Pt-binding site.

(iv) SOD1 is involved in a number of pathways including apoptosis. It exerts its apoptotic effect via Tp53/BAD/BAX/BCL2 in the amyotrophic lateral sclerosis (ALS) pathway (Fig. 7).

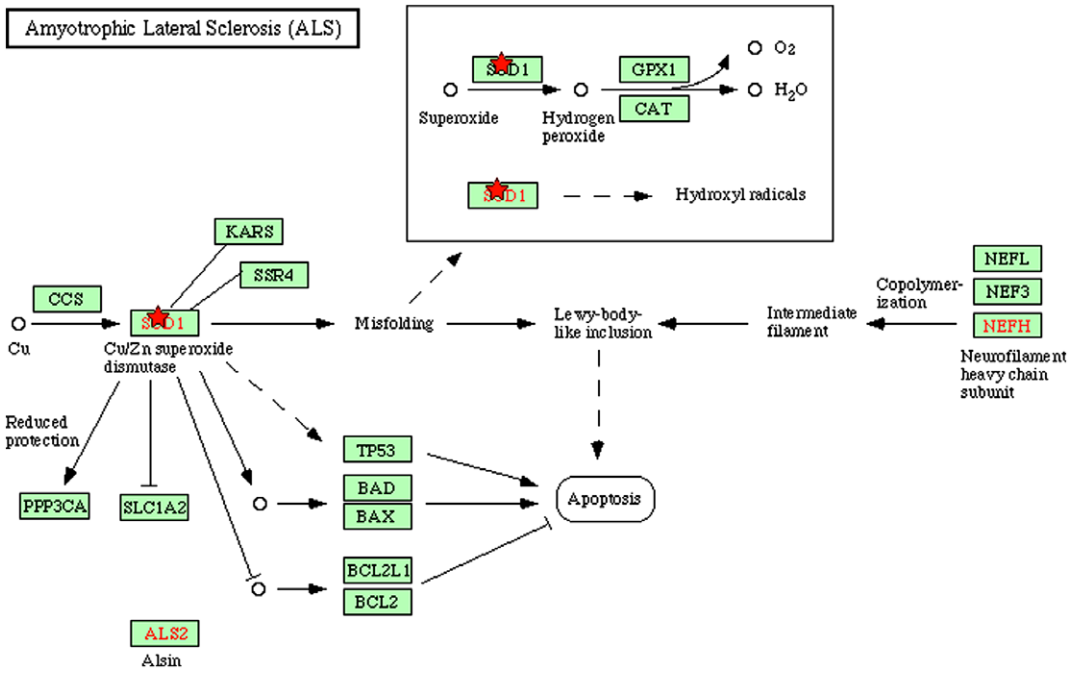

Figure 7. KEGG's Pathway for amyotrophic lateral sclerosis (ALS), showing the role of SOD for apoptosis.

The protein encoded by this gene binds copper and zinc ions, and is one of two isozymes responsible for destroying free superoxide radicals in the body. Mutations in this gene have been implicated as causes of familial amyotrophic lateral sclerosis, Alzheimer's disease, and Type II Diabetes. As we have mentioned earlier, S-30-treated cells displayed a positive subcellular localization of wild-type p53 which may lead to apoptosis as supported by TUNEL assay and other experimental results.

The cisplatin-bound SOD is from bovine origin. So, Blast search of bovine SOD was performed to identify analogous human protein targets which resulted in about 22 human proteins; a multi-sequence alignment of those targets showed >80% sequence identity and >88% similarity. SOD1 is one of them.

Interestingly, SOD1 is differentially expressed in a number of cancers including breast cancer (data not shown). According to Gene Ontology terms, process, function, and components, SOD1 is involved in a number of important biological processes such as superoxide metabolism, response to stress, system development, physiological process, etc. The molecular function of SOD1 is also numerous, some examples of which include catalytic activity, superoxide dismutase activity, and oxidoreductase activity. The components are cytoplasm, intracellular, and cell in which SOD1 is involved. Therefore, SOD1 is an important target for therapy of cancer and other diseases.

S-30 is a small molecule that can be prepared easily with only three high yielding steps, and is highly potent. Results suggest that S-30 is a potential promising agent for cancer therapy.

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36. ^{1}H NMR ($CDCl_3$) δ: 9.26 (d, 1H, 6-pyridyl); 7.92 (t, 1H, 5-pyridyl); 7.73 (d, 1H, 3-pyridyl); 7.42 (t, 1H, 4-pyridyl); 3.86–3.74 (br s, 16H, morpholine and water); Anal. Calcd for [$(C_{16}H_{20}N_6O_2S)PtCl_2 \cdot H_2O$]: C, 29.82; H, 3.44; N, 13.05. Found: C, 30.11; H, 3.23; N, 13.18.
37. Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 634556. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 (0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].