

Synthesis and Structure-Activity Correlation Studies of Metal Complexes of α -N-heterocyclic Carboxaldehyde Thiosemicarbazones in *Shewanella oneidensis*

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Abstract: This investigation involved the synthesis of metal complexes to test the hypothesis that structural changes and metal coordination in pyridine thiosemicarbazones affect cell growth and cell proliferation *in vitro*. Thiosemicarbazones are well known to possess antitumor, antiviral, antibacterial, antimalarial, and other activities. Extensive research has been carried out on aliphatic, aromatic, heterocyclic and other types of thiosemicarbazones and their metal complexes. Due to the pronounced reactivity exhibited by metal complexes of heterocyclic thiosemicarbazones, synthesis and structural characterization of di-2-pyridylketone ⁴N-phenyl thiosemicarbazone and diphenyl tin (Sn) and platinum (Pt) complexes were undertaken. *Shewanella oneidensis MR-1*, a metal ion-reducing bacterium, was used as a model organism to explore the biological activity under aerobic conditions. A comparison of the cytotoxic potential of selected ligand and metal-complex thiosemicarbazones on cell growth in wild type *MR-1* and mutant *DSP-010 Shewanella oneidensis* strains at various concentrations (0, 5, 10, 15, 20 or 25 ppm) was performed. The wild type (*MR-1*) grown in the presence of increasing concentrations of Sn- thiosemicarbazone complexes was comparatively more sensitive (mean cell number = $4.8 \times 10^8 \pm 4.3 \times 10^7$ SD) than the *DSP-010*, a spontaneous rifampicillin derivative of the parent strain (mean cell number = $5.6 \times 10^8 \pm 6.4 \times 10^7$ SD) under comparable aerobic conditions ($p=0.0004$). No differences were observed in the sensitivity of the wild and mutant types when exposed to various concentrations of diphenyl Pt- thiosemicarbazone complex ($p=0.425$) or the thiosemicarbazone ligand ($p=0.313$). Growth of *MR-1* in the presence of diphenyl Sn- thiosemicarbazone was significantly different among treatment groups ($p=0.012$). *MR-1* cell numbers were significantly higher at 5ppm than at 10 to 20ppm ($p=0.05$). The mean number of *DSP-010* variant strain cells also differed among diphenyl Sn- thiosemicarbazone complex treated groups ($p=0.051$). In general, there was an increasing trend in the number of cells from about 5.0×10^8 cells (methanol control group) to about 6.0×10^8 cells (25ppm). The number of cells in methanol control group was significantly lower than cell numbers at 20ppm and 25ppm ($p=0.05$), and numbers at 5ppm treatment were lower than at 20 and 25ppm ($p=0.05$). Furthermore, a marginally significant difference in the number of *MR-1* cells was observed among diphenyl Pt- thiosemicarbazone complex treatment groups ($p=0.077$), and an increasing trend in the number of cells was noted from $\sim 5.0 \times 10^8$ cells (methanol control group) to $\sim 5.8 \times 10^8$ cells (20ppm). In contrast, the *DSP-010* variant strain showed no significant differences in cell numbers when treated with various concentrations of diphenyl Pt- thiosemicarbazone complex ($p=0.251$). Differences in response to Sn- metal complex between *MR-1* and *DSP-010* growing cultures indicate that biological activity to thiosemicarbazone metal complexes may be strain specific.

Keywords: α -N-heterocyclic carboxaldehyde thiosemicarbazones, biological activity, metal complexes and *Shewanella oneidensis*.

Introduction

This investigation involves the synthesis of metal complexes to test the hypothesis that structural changes

and metal coordination in pyridine thiosemicarbazones affects growth and cell proliferation *in vitro*. *Shewanella oneidensis MR-1* [1], a metal ion-reducing bacterium, and *DSP-010*, a spontaneous rifampicillin derivative of

the parent strain, were used as model organisms to explore the biological activity of newly synthesized metal complexes of α -N-heterocyclic carboxaldehyde thiosemicarbazones (HCT) *in vitro*. Studies of metal complexes of thiosemicarbazones have shown that they can be more active in cell destruction, as well as in the inhibition of DNA synthesis, than the uncomplexed ones. Thiosemicarbazones are a class of compounds obtained by condensing thiosemicarbazide with suitable aldehydes or ketones and are well known to possess antitumor [2], antiviral [3], antibacterial [4], antimalarial [5], and other activities. They belong to a large group of thiourea derivatives, the biological activity of which are a function of parent aldehyde or ketone.

Extensive research has been carried out on aliphatic, aromatic, heterocyclic and other types of thiosemicarbazones and their metal complexes [6-13]. Efforts to evaluate structural features essential for biological activity have included:

- 1) Exchange of the sulfur atom of thiocarbonyl group by oxygen or selenium.
- 2) Changing the point of attachment of the thiosemicarbazone moiety in the parent aldehyde or ketone.
- 3) Substitution on the terminal ^4N position.
- 4) Variation of the parent aldehyde or ketone.

The thiosemicarbazone moiety (Figure 1) acts as a chelating agent for metal ions by binding through the sulfur atom and the hydrazine nitrogen atom. Thiosemicarbazone molecule can exist in tautomeric form in solution as thione or thiol (Figure 2). The thione form can act as a neutral bidentate ligand, while the thiol form can be a singly charged bidentate ligand due to loss of its proton. In metal complexes, thiosemicarbazones can act as a tridentate ligand with a donor atom apart from thione/thiol sulfur atom and azomethine nitrogen. The possible ligation in metal complexes can be either as a neutral molecule or as a monobasic anion due to loss of hydrogen from azomethine nitrogen atom.

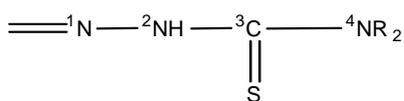


Figure 1: Thiosemicarbazone uncomplexed ligand moiety.

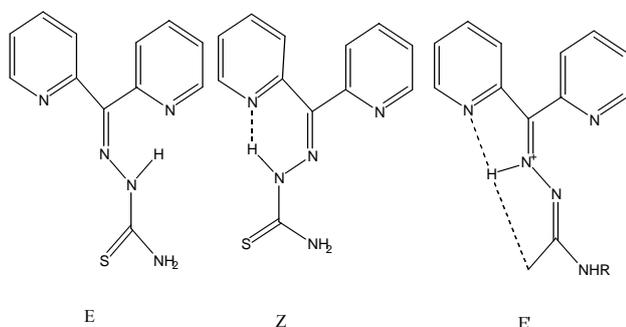


Figure 2: Thiosemicarbazone molecule as thione or tautomeric forms in solution.

The molecular features essential for such activities can be ascertained by designing synthetic routes to

modify, replace, or substitute the derived thiosemicarbazone ligand. In this direction, the phenyl and ethyl derivatives of cyclopentanone thiosemicarbazones were synthesized and characterized [14]. Recently, attention was focused on the derivatives of di-2-pyridylketone thiosemicarbazones as a ligand for tin [15] and other metals. As only a few tin (IV) complexes of these ligands have been reported, the synthesis and chelating behavior of dibutyl and diphenyl tin (IV) along with platinum (IV) complexes of di-2-pyridylketone phenyl thiosemicarbazones and its reactivity in *Shewanella oneidensis*. It is anticipated that data obtained from this study will provide information on the interactions of metal complexes *in vivo* and the development of better therapeutic agents for translational research.

Materials and Methods

Preparation and Characterization of Thiosemicarbazones

Preparation of Di-2-pyridyl Ketone ^4N -phenyl Thiosemicarbazone, $(\text{C}_5\text{H}_4\text{N})_2\text{C}=\text{NNCSNC}_6\text{H}_5$

A solution of di-2-pyridyl ketone (1.84g, 10 mmol) in 50mL ethanol was slowly added with stirring to a solution containing 4-phenyl-3-thiosemicarbazide (1.67g, 10mmol) in ethanol (50 ml) and five drops of concentrated hydrochloric acid. The reaction mixture was refluxed for 2 hours. A yellow solid resulted on cooling. The crude sample was filtered and re-crystallized in ethanol (yield ca.70%) with a melting point 138-140°C. Melting points were determined using mel-temp II apparatus. IR spectra were obtained in 4000-400 cm^{-1} range in KBr pellets on a Nicole 670 FT-IR spectrophotometer [$\nu_{\text{N-H}}$ 3150 cm^{-1} (br); $\nu_{\text{C=N}}$ 1588 cm^{-1} (sh), 1531 cm^{-1} (sh), 1480 cm^{-1} (sh),1455 cm^{-1} (sh); $\nu_{\text{C=S}}$ 999 cm^{-1} (sh), 917 cm^{-1} (sh)]. The absorption spectra recorded on Cary 3E UV-Vis spectrophotometer using 1 cm quart cuvettes in DMSO as solvent. The visible spectra of the ligand showed strong absorbance at 345 nm, and 280 nm indicating π - π^* transition of pyridyl ring and imine function of thiosemicarbazone moiety, and their n- π^* transitions.

Preparation of Metal Complexes

A typical method of preparation involved either refluxing or stirring the metal salts dissolved in anhydrous ethanol in 1:1mole ratio.

Diphenyltin Dichloride Complex

The tin complex of di-2-pyridylketone ^4N -phenyl thiosemicarbazone was prepared by the following procedure: to a hot ethanolic solution (50 mL) of the ligand (5 mmol, 1.67g) was added a solution in ethanol (50 mL) of diphenyltin dichloride (5 mmol, 1.72g). The mixture was refluxed for about 30 minutes and the resulting solution cooled to obtain a yellow solid (yield ca. 65%) with a melting point 138°C-140°C. A comparison of IR spectrum with the ligand showed a shift of the band from $\sim 3300 \text{ cm}^{-1}$ to 3450 cm^{-1} (br) is

attributable to ν_{N-H} . This indicates that the ligand is coordinated in its deprotonated form. The shift of imine $\nu_{C=N}$ band from $\sim 1530\text{ cm}^{-1}$ to $\sim 1550\text{ cm}^{-1}$ indicates the coordination of azomethine nitrogen N(2) to the metal ion. The band $\nu_{C=S}$ at about $\sim 850\text{ cm}^{-1}$ of the ligand is shifted to lower frequencies by $\sim 40\text{--}50\text{ cm}^{-1}$ suggests the coordination of metal through sulfur atom. The bands at about $\sim 510\text{ cm}^{-1}$ can be assigned to the metal nitrogen binding. The absorption spectrum of the tin complex was similar to that of the ligand. Conductivity measurements of the metal complexes were determined in DMSO using YSI model 35-conductance meter. The low molar conductance values ($< 10\text{ Smol}^{-1}\text{cm}^2$) of the tin and Pt-complexes indicate the non-electrolytic nature.

Dichloroplatinum (II) Complex

To a suspension of platinum (II) dichloride (g) in methanol (50 mL) was added 5.0 mmol methanolic solution (50 mL) of the ligand (1.67g), and the mixture was refluxed for about 1 hour. The resulting mixture was left to air at room temperature to obtain a dark red colored solid (yield 80%) with a melting point 102°C - 103°C . The IR spectrum has shown a shift of $10\text{--}30\text{ cm}^{-1}$ for $\nu(C=N)$ absorption band indicating the positive involvement of the azomethine nitrogen in bonding to the metal ion. Similarly the $\nu(C=S)$ band shifted by $5\text{--}10\text{ cm}^{-1}$ indicating the coordination of thiocarbonyl sulfur to the metal ion. The molar conductivity of the complex in DMSO indicated that the platinum complex ($\sim 50\text{ Smol}^{-1}\text{cm}^2$) is nonpolar in nature.

Bacterial Strains

Wild-type *Shewanella oneidensis MR-1* and *DSP-010*, a spontaneous rifampicillin derivative of the parent strain (a kind gift from the Environmental Science Group at Oak Ridge National Laboratory) were grown under aerobic condition in Luria-Bertani (LB) medium at 37°C . This species of *Shewanella* is gram-negative, facultative anaerobes. Bacterial cell concentrations used in the experiments were equivalent to an optical density of 0.4 at 600 nm (approximately 8×10^8 cells/mL = 0.18 g/L). The optical densities of the cell suspensions were measured spectrophotometrically at 600 nm using a Bio-RAD Smart Spec instrument. To an exponentially growing culture of *MR-1* and *DSP-010* cells, uncomplexed ligand, Pt- and Sn- complexes were added at 0, 5, 10, 15, and 25 ppm ($\mu\text{g/ml}$). Negative controls containing media alone or solvent controls (methanol) were included in each growth curve experiments. Cells were incubated for 48 h at 37°C and the bacterial growth was measured at 600nm. Mean cells per milliliter and optical densities at 600nm [$\text{OD}_{600\text{nm}}$] were plotted against Ligand, Pt- and Sn-metal concentrations for *Shewanella oneidensis MR-1* and *DSP-010* cells.

Statistical Analysis

A one-way analysis of variance (ANOVA) test was used to determine whether there are significant differences in mean numbers of *S. oneidensis* cells exposed to the test chemicals. The Fisher's PLSD test was used for pair-wise comparisons among treatment

groups. The Student's *t*-test was applied for comparing paired data sets (significance at a *p*-value ≤ 0.05).

Results

In this study, wild-type *Shewanella oneidensis* demonstrated biological activity when exposed to newly synthesized Pt-, and Sn- complexes of α -N-heterocyclic carboxaldehyde thiosemicarbazones. Wild type (*MR-1*) grown in the presence of increasing concentrations of Sn- thiosemicarbazone complexes was comparatively more sensitive (mean cell number = $4.8 \times 10^8 \pm 4.3 \times 10^7$ SD) than the *DSP-010*, a spontaneous rifampicillin derivative of the parent strain (mean cell number = $5.6 \times 10^8 \pm 6.4 \times 10^7$ SD) under comparable aerobic conditions (Unpaired *t*-test, *t*-value = -4.003, *df* = 28; *p* = 0.0004; *n* = 15). No differences were observed in the sensitivity of the wild and mutant types when exposed to various concentrations of diphenyl Pt-thiosemicarbazone complex (Unpaired *t*-test, *t*-value = -0.81; *p* = 0.425) or the thiosemicarbazone ligand (Unpaired *t*-test, *t*-value = -1.03, *p* = 0.313).

Growth of *MR-1* in the presence of diphenyl Sn-thiosemicarbazone was significantly different (ANOVA, *df* = 6,14; *F*-value = 4.259; *p* = 0.012) among treatment groups (Figure 3). *MR-1* cell numbers were significantly higher at 5ppm than at 10 to 20ppm (Fisher's PLSD test, *p* = 0.05). The mean number of *DSP-010* variant strain cells also differed among diphenyl Sn- thiosemicarbazone complex treated groups (ANOVA, *df* = 6,13; *F*-value = 2.897; *p* = 0.051) (Figure 3). No differences were observed in the number of cells in the negative control and methanol control groups (Fisher's PLSD test, *p* > 0.05). In general, there was an increasing trend in the number of cells from about 5.0×10^8 cells (methanol control group) to about 6.0×10^8 cells (25ppm). The number of cells in methanol control group was significantly lower than cell numbers at 20ppm and 25ppm (Fisher's PLSD test, *p* = 0.05), and numbers at 5ppm treatment were lower than at 20 and 25ppm (Fisher's PLSD test, *p* = 0.05).

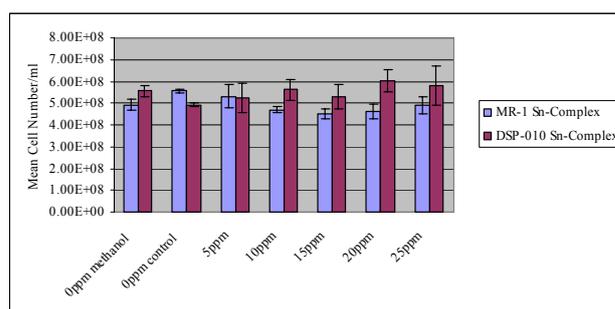


Figure 3: Effects of various concentrations of diphenyl Sn- thiosemicarbazone metal complex on *Shewanella oneidensis MR-1* and *DSP-010* grown aerobically in LB broth. Mean cell/ml was determined at 37°C after a 48 hour time period for three independent experiments. Error bars are for standard deviation.

Furthermore, a marginally significant difference in the number of *MR-1* cells was observed among diphenyl Pt- thiosemicarbazone complex treatment groups (ANOVA, *df* = 6,14; *F*-value = 2.464; *p* = 0.077), and an increasing trend in the number of cells was noted from

~5.0 X 10⁸ cells (methanol control group) to ~5.8 X 10⁸ cells (20ppm) (Figure 4). In contrast, the *DSP-010* variant strain showed no significant differences in cell numbers (Figure 4) when treated with various concentrations of diphenyl Pt- thiosemicarbazone complex (ANOVA, df=6,14; F-value=1.492; p = 0.251).

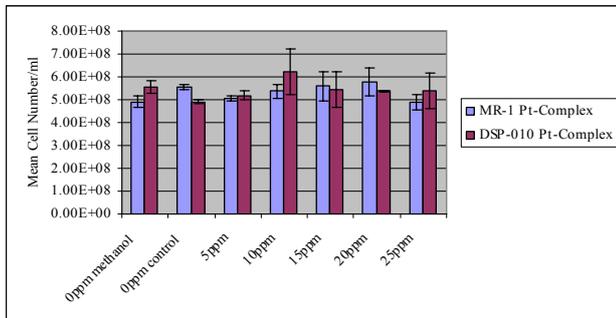


Figure 4: Effects of various concentrations of diphenyl Pt- thiosemicarbazone metal complex on *Shewanella oneidensis MR-1* and *DSP-010* grown aerobically in LB broth. Mean cell/ml was determined at 37°C after a 48 hour time period for three independent experiments. Error bars are for standard deviation.

MR-1 cells showed no significant differences in numbers (Figure 5) when treated with various concentrations of the uncomplexed thiosemicarbazone ligand alone (ANOVA, df=6,14; F-value=0.235; p = 0.235). A similar observation was noted when *DSP-010* variant strain was treated with various concentrations of the ligand (ANOVA, df=6,14; F-value=2.004; p-value = 0.133) (Figure 5).

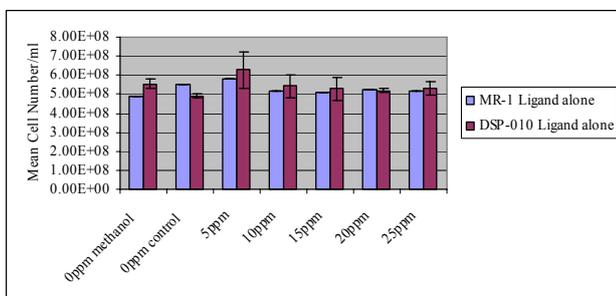


Figure 5: Effects of various concentrations of uncomplexed thiosemicarbazone ligand on *Shewanella oneidensis MR-1* and *DSP-010* grown aerobically in LB broth. Mean cell/ml was determined at 37°C after a 48 hour time period for three independent experiments. Error bars are for standard deviation.

Discussion

Micro-organisms require the presence of a number of metals that play essential biochemical roles such as catalysts, enzyme co-factors, activity in redox processes and stabilizing protein structures [16]. Metals may accumulate above normal physiological concentrations by the action of unspecific, constitutively expressed transport systems, whereby they become toxic. Intracellular metals can exert a toxic effect by forming coordinate bonds with anions blocking functional groups of enzymes, inhibiting transport systems, displacing

essential metals from their native binding sites and disrupting cellular membrane integrity [17]. There are five basic mechanisms that convey an increased level of cellular resistance to metals: (1) efflux of the toxic metal out of the cell; (2) enzymic conversion; (3) intra- or extracellular sequestration; (4) exclusion by a permeability barrier; and (5) reduction in sensitivity of cellular targets. Our study indicates that *S. oneidensis MR-1* is susceptible to growth inhibition by tin complex. In contrast, *DSP-010*, a spontaneous rifampicillin derivative of the *MR-1* parent strain, demonstrates marked growth in the presences of di-2-pyridylketone ⁴N phenyl thiosemicarbazone as a ligand and tin or platinum complexes. For diphenyl Sn- thiosemicarbazone, untreated and methanol solvent controls groups were not significantly different (Fisher's PLSD test; p=0.114) therefore, the observed differences of *DSP-010* cell growth among treatments as compared to *MR-1* are most likely due to the effect of Sn on *Shewanella* mutant bacteria.

Growth comparison of *Shewanella oneidensis MR-1* and *DSP-010* strains in the presence of thiosemicarbazone ligand, diphenyl Pt- and Sn-thiosemicarbazone complexes demonstrated strain specific differences under aerobic conditions. No significant change occurred with *MR-1* or *DSP-010* strains grown in the presence of the uncomplexed ligand. Similarly, wild type and mutant strains of *S. oneidensis* did not demonstrate significant growth differences in the presences of diphenyl Pt- thiosemicarbazone. However, an unpaired t-test indicated significant difference between *MR-1* and *DSP-010* after 48-hour growth in the presence of increasing concentrations of diphenyl Sn-thiosemicarbazone. Differences in response to Sn- metal complex, between *MR-1* and *DSP-010* growing cultures indicate that biological activity to thiosemicarbazone metal complexes may be strain specific.

The possible reduction of inorganic Sn(IV) to Sn(II) may explain the growth advantage of *DSP-010* mutant strain over wild-type *MR-1* with di-2-pyridylketone thiosemicarbazone derivatives. In literature, microbial resistance mechanisms have been widely studied [18,19]. However, very little is known about the reduction of inorganic Sn(IV) to Sn(II) in microbes. It may be hypothesized that heavy metal complexes such as diphenyl tin (IV) thiosemicarbazone are unable to escape sequestration, metal efflux or metal reduction systems thereby causing toxicity in *Shewanella oneidensis MR-1*. In contrast, *DSP-010* may be able to effectively reduce inorganic Sn(IV) to Sn(II) thus allowing growth in increasing concentrations of Sn- metal complexes. Alternately, *DSP-010*, may be able to mobilize and remove di-2-pyridylketone ⁴N-phenyl thiosemicarbazone, diphenyl tin and platinum complexes more efficiently through membrane transport systems. Future binding studies should address the uptake and binding affinities of diphenyl thiosemicarbazones in *Shewanella oneidensis MR-1* and *DSP-010*.

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