BioMetals (2007) 20:61–72
DOI 10.1007/s10534-006-9015-1

Characterization and *in vitro* cytotoxicity testing of ethanolamine-derived cadmium chelating agents

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Received 15 March 2006; accepted 11 April 2006

Key words: cadmium, xanthate, dithiocarbamate, *in vitro* testing, chelation

Abstract

We have synthesized and characterized the new cadmium chelating agent potassium bis(2-hydroxyethyl)aminoethyldithiocarbonate hemihydrate, K[bhexan]·0.5H_2O (**2**), that is structurally related to the known effective *in vivo* cadmium chelating agent potassium bis(2-hydroxyethyl)dithiocarbamate, K[bhedtc] (**1**). The corresponding cadmium complex of **2** differs from di(bis(2-hydroxyethyl)dithiocarbamato)cadmium(II), $Cd(bhedtc)_2$ (**3**), in that the insoluble compound exhibits an elemental composition consistent with a cadmium:ligand ratio of 2:1. The cytotoxicity of the **1**–**3** was investigated using the human osteoblast-like cell line, Saos-2. Compounds **1** or **2** did not affect cell adherence or cell viability in the 100–500 μM concentration range studied, whereas **3** resulted in a concentration-dependent increase in loss of cell adherence and decrease in cell viability. Overall, the results of the loss of cell adherence, trypan blue exclusion and MTT assays showed that administration of **3** (cadmium complex of **1**) resulted in cytotoxicity lower than that of cadmium chloride, but higher than that of the chelator **1** alone. The effect of simultaneous addition of cadmium chloride and **1** or **2** on cell viability was also assessed using the MTT assay. For the 100 μM cadmium chloride experiments, cell viability comparable to control cells was achieved for both **1** and **2** in the 100–500 μM concentration range studied. Cell viability comparable to control cells was achieved for **1** but not **2** in the 100–500 μM concentration range studied for the 200 μM cadmium chloride experiments. Thus **1** appears more effective than **2** in the ability to mediate the cytotoxic effects of cadmium *in vitro* upon concomitant administration.

Introduction

Cadmium toxicity

There is intense research interest on the adverse effects of the heavy metals cadmium, lead, and mercury on human health (Järup & Alfvén 2004; Kazantzis 2004). Cadmium, in particular, has been implicated in renal dysfunction, bone and liver disease, and several types of cancer including prostate and lung cancer (Järup *et al*. 1998b; Järup & Alfvén 2004; Nordberg 2004). Cadmium increasingly persists in the environment as a result of agriculture, smoking, industry, and electronic waste (including associated NiCad batteries; Jones & Cherian 1990; Järup *et al*. 1998a; Van Assche 1998; Hileman 2006). A recent report concerning human exposure to environmental chemicals determined that approximately 5% of the U.S. population aged 20 years and older have concentrations of urinary cadmium that heighten the risk of developing kidney dysfunction and low bone

mineral density (CDC 2005). These findings demonstrate the need for more research that addresses the role of cadmium in human health.

Cadmium cellular response

Cadmium toxicity has been investigated in many cell types (Gennari *et al*. 2003; Ridd *et al*. 2004; Fotakis & Timbrell 2006). The cytotoxic action of cadmium involves a variety of cellular responses including metabolic changes, sequestering by metal binding proteins, alterations in proto-oncogene expression, inhibition of DNA repair processes, induction of programmed cell death, or apoptosis, and oxidative stress (Beyersmann & Hechtenberg 1997; Waisberg *et al*. 2003).

Several reports indicate a possible role for oxidative stress in cadmium-induced toxicity (Ercal *et al*. 2001; Gennari *et al*. 2003; Lemarié *et al*. 2004; Wätjen & Beyersmann 2004). Exposure to cadmium inhibits antioxidative defense reactions, and by this mechanism indirectly evokes increased amounts of reactive oxygen species (ROS; Thévenod *et al*. 2000; Ercal *et al*. 2001; Wätjen & Beyersmann 2004). The binding of cellular antioxidants, such as the tripeptide glutathione (GSH), to non-redox cycling thiophilic heavy metals (cadmium, lead, and mercury) was found to reduce the concentration of active cellular antioxidants to the point where adverse oxidation occurs to cellular components such as lipids, proteins and DNA, leading to what is referred to as oxidative stress (Ercal *et al*. 2001). To subvert oxidative stress induced by the presence of cadmium, specific chelating agents could be administered to bind cadmium, leaving sufficient amounts of antioxidants to neutralize ROS. In the case of the chelating agent pyrrolidine dithiocarbamate, the compound can also scavenge ROS directly. Administration of this compound averted cadmium-induced apoptosis in cultured proximal tubule cells (Thévenod *et al*. 2000).

Cadmium chelation

One approach to reduce the toxic action of cadmium, both in the treatment of acute intoxication, and in the mobilization of intracellular cadmium to prevent or alleviate chronic toxicity, has been to use chelating agents (Andersen 1999; Kelley *et al*. 1999). Administration of monoisoamyl *meso*-2,3-dimercaptosuccinate (M*i*-ADMS) has shown effectiveness in reducing typical histopathologic signs of apoptosis and the associated DNA fragmentation when administered up to 1 h after rats were treated with cadmium chloride (Yan *et al*. 1997). Examples of α-mercapto-β-(5-substituted, 2-furyl) acrylic acids (Tandon *et al*. 2000) and α-mercapto-β-substituted aryl acrylic acids (Chatterjee *et al*. 2004) have shown the ability to sequester intracellularly bound cadmium *in vivo* and *in vitro*, respectively. However, substitution of the furan ring of the α-mercapto-β-(5-substituted, 2-furyl) acrylic acids dropped the cadmium mobilizing efficacy relative to the parent compound, and led to excretion and or concentration of endogenous metals (zinc, copper) in the livers and/or kidneys of rats (Tandon *et al*. 2000).

Other sulfur containing molecules such as dithiocarbamates and xanthates are also known to form stable complexes with cadmium (Jiang *et al*. 2002; Cesur 2003). A comparative study examined the effect of vicinal dithiols [2,3-dimercaptopropan-1-ol (BAL) and *N*-(2,3-dimercaptopropyl) phthalamidic acid (DMPA)] and a dithiocarbamate [sodium *N*-(4-methoxybenzyl)-D-glucamine dithiocarbamate (MeOBGDTC)] on the biliary excretion of cadmium in rats (Jones *et al*. 1991a). This study showed that while all compounds increased the biliary excretion of cadmium, the most effective was MeOBGDTC whose administration resulted in a 580% increase in biliary cadmium content.

Attaining the appropriate balance between lipophilicity and hydrophilicity of cadmium chelating agents and the corresponding complexes is critical to the efficacy of mobility and excretion of cadmium in biological systems. Lipophilicity has been shown to be key in the mobilization of intracellular cadmium (Jones *et al*. 1991b). However, administration of sodium diethyldithiocarbamate (Na[Et_2dtc]), a dithiocarbamate incorporating two lipophilic ethyl side chains, was shown to adversely affect the function and structure of type II pulmonary epithelial cells *in vitro* (Tátrai *et al*. 2001b). Simultaneous administration of Na[Et_2dtc] and cadmium chloride in Sprague–Dawley rats altered the distribution of cadmium by increasing the cadmium content in the lungs, and caused more serious structural changes than cadmium chloride exposure alone (Tátrai *et al*. 2001a). In contrast, the substitution of hydroxy

groups for hydrogens at the end of the ethyl groups of Na[Et_2dtc] to give sodium bis(2-hydroxyethyl)dithiocarbamate, Na[bhedtc] changes both the lipophilicity and hydrophilicity, and subsequent biological profile, of the compound significantly. Prior injection of Na[bhedtc] afforded significant protection against cadmium chloride-induced developmental toxicity and reduced kidney and liver cadmium concentrations in hamsters relative to cadmium chloride treated controls (Hatori *et al*. 1990).

Na[bhedtc], sodium *N*-methyl-*N*-hydroxyethyldithiocarbamate (Na[Mehedtc]), and sodium *N*-ethyl-*N*-hydroxyethyldithiocarbamate (Na[Ethedtc]) were compared for their relative efficacies in promoting organ mobilization and excretion of cadmium (Gale *et al*. 1984). Mobilization of renal, intestinal, and testicular cadmium was observed for all compounds in the following order of efficiency: Na[Ethedtc] > Na[Mehedtc] > Na[bhedtc]; this correlated with octanol/aqueous partition coefficients determined for the cadmium complex of each of the above dithiocarbamates. However, only Na[bhedtc], the least lipophilic and most hydrophilic dithiocarbamate evaluated, promoted cadmium excretion. The more lipophilic dithiocarbamates promoted redistribution of cadmium to the liver.

Existing chelating agents capable of mobilizing aged cadmium deposits in experimental animals, which have been related to kidney dysfunction and low bone mineral density, are too toxic for human application (Andersen 1999). In the present study, we have synthesized a xanthate, potassium bis(2-hydroxyethyl)aminoethyldithiocarbonate hemihydrate, K[bhexan]$\cdot 0.5H_2O$ (**2**), that is structurally related to the known effective *in vivo* cadmium chelating agent potassium bis(2-hydroxyethyl)dithiocarbamate, K[bhedtc] (**1**), and tested both in a human *in vitro* system. The goal of this study was to compare the abilities of two structurally related cadmium chelating agents to affect the *in vitro* cytotoxicity of cadmium chloride. To our knowledge, this is the first time that the effect of xanthate cadmium chelation has been evaluated *in vitro*. The cytotoxicity testing was undertaken using a human osteoblast-like cell line Saos-2. Extensive research has been done to evaluate the effect of cadmium on the skeleton, linking exposure to increased risk of bone fractures and the development of osteoporosis in humans (Staessen *et al*. 1999; Järup & Alfvén 2004). Thus, the use of Saos-2 cells can provide insight into chelation and cytotoxicity of cadmium that is relevant to human health.

Materials and methods

Materials

Deionized Elix 5 (Millipore corp.) filtered water was utilized throughout. All other chemicals and solvents were obtained commercially and used as received. The human osteoblast-like cell line, Saos-2, was purchased from American Type Culture Collection. Cytotoxicity was evaluated using a Vybrant MTT Cell Proliferation Assay Kit (V-13154; Molecular Probes).

Methods

Compound characterization

UV–vis spectra (200–850 nm) were recorded on an Oceanoptics CHEMUSB2-UV–VIS Spectrophotometer. Melting points were obtained with a LAB. Devices MELTEMP II capillary apparatus and are reported without correction. Elemental analyses (C, H, N, Cd and I) were carried out at the Desert Analytics Laboratory. Complimentary Cd analyses were performed using a PerkinElmer AAnalyst 400 atomic absorption spectrometer (AAS). Suspensions of cadmium complexes in 5% hydrochloric acid were decomposed with heat and the resulting solutions analyzed by flame AAS in the 0–2 ppm concentration range. Infrared spectra (4000–400 cm^{-1}) were measured as potassium bromide pellets using a Nicolet Avatar 360 FT-IR spectrometer. ^1H and $^{13}C\{^1H\}$ NMR spectra were recorded on a Varian Mercury 300 NMR spectrometer at the Department of Chemistry, Boise State University, operating at 300 and 75 MHz, respectively, and referenced to internal tetramethylsilane (TMS) or 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP-d_4) for spectra recorded in D_2O. Electrospray mass spectra (ES) were recorded using a HP API/MS or Applied Biosystems Pulsar Qtrap TOF (high resolution spectra) instrument at The Central Analytical Laboratory at the University of Colorado at Boulder.

Cell culture

Saos-2 cells were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in air containing 5% carbon dioxide. For experiments, cells were plated at a density of 6×10^5 cells/well in six-well culture plates or 2×10^5 cells/well in 96 well culture plates, and the medium was changed after 24 h. Treatment was initiated at 48 hrs with various concentrations of the desired compounds as aqueous solutions; controls received medium only. For the testing of $Cd(bhedtc)_2$ (**3**), the compound was dispensed as 10 μl aliquots of a *N*,*N*-dimethylformamide (DMF) stock solution into the media to make the desired concentration; 10 μl DMF was added to the controls. Cells were analyzed after 24 h of treatment. Non-adherent cells in the removed medium were counted using a hemocytometer and light microscopy to assess loss of cell adherence.

Determination of cytotoxicity

Cell viability was determined using the trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays. Adherent cells were washed with phosphate-buffered saline (PBS), removed off the plate with 0.25% trypsin/EDTA, and stained with 0.05% trypan blue. Cells were counted using a hemocytometer and light microscopy. Cells with an intact cell membrane did not uptake the trypan blue dye and were considered alive. As a second marker for cytotoxicity, the conversion of a water-soluble MTT into an insoluble formazan by mitochondrial dehydrogenase was assessed. Adherent cells were washed with PBS and incubated with 10 μl of MTT (5 mg/ml in PBS) for 4 hrs at 37 °C. After solubilizing in dimethyl sulfoxide (DMSO) the absorbance was measured at 540 nm. Values for light absorption were directly proportional to the viability of the cells.

*Synthesis of potassium bis(2-hydroxyethyl)dithiocarbamate, K[bhedtc] (**1**).* This compound was synthesized as described in the literature (Nakashima & Kida 1982) but at 3/8th the prescribed scale to give white crystals (12.78 g, 61%). λ_{max}/nm (H_2O) 258 (ε/dm^3 mol^{-1} cm^{-1} 1.3×10^4), 289 (1.3×10^4) and 360 (73). Mpt. 194 °C. ν_{max}/cm^{-1} (KBr pellet): 3416 s (OH), 2962 m (CH), 1466 s (C=N), 1193 s (CN), 1149 s (CO), 1019 s (CC) and 958 s (CS). ^{1}H NMR (D_2O): 4.67 (2 H, s, *H*OCH$_2$), 4.27 (4 H, t, $^3J_{HH} = 20.3$ Hz, *C*H$_2$N) and 3.93 (4 H, t, $^3J_{HH} = 20.3$ Hz, *C*H$_2$OH). ^{13}C{^{1}H} NMR (D_2O): 213.76 (R_2NCS_2), 61.69 (*C*H$_2$N) and 59.16 (*C*H$_2$OH). *m*/*z* (ES$^-$; MeOH) 218 ((M – H$^+$)$^-$, 1.8%) and 180 ((M – K$^+$)$^-$, 100%). HRMS Calcd for $C_5H_{10}NO_2S_2$: 180.0158. Found: 180.0150.

*Synthesis of potassium bis(2-hydroxyethyl)aminoethyldithiocarbonate hemihydrate, K[bhexan]·0.5H$_2$O (**2**).* Potassium hydroxide (2.311 g, 41.19 mmol) was dissolved in excess triethanolamine (20.0 ml, 150 mmol) through heating and rapid stirring to give a clear yellow solution. On cooling to room temperature, carbon disulfide (2.50 ml, 41.4 mmol) was added drop-wise to the viscous mixture resulting in a yellow emulsion. After 10 min of stirring, diethyl ether (40.0 ml) was added to the emulsion. The resulting mixture was stirred overnight. The product was filtered, and excess triethanolamine removed via thorough washing with ethanol. The solid was air dried to give a butter-yellow crystalline powder of potassium bis(2-hydroxyethyl)aminoethyldithiocarbonate hemihydate (6.185 g, 55%). λ_{max}/nm (H_2O) 224 (ε/dm^3 mol^{-1} cm^{-1} 8.2×10^3), 303 (1.8×10^4) and 382 (56). Mpt. 139–141 °C. Found C, 30.97; H, 5.35; N, 4.92. $C_7H_{14}KNO_3S_2 \cdot 0.5H_2O$ requires C, 30.86; H, 5.55; N, 5.14%. ν_{max}/cm^{-1} (KBr pellet): 3179 s (OH), 2852 m (CH), 1283 m (C=O), 1218 m (CN), 1114 s (CO), 1038 s (CC) and 692 m (CS). ^{1}H NMR (D_2O): 4.69 (2 H, s, *H*OCH$_2$), 4.56 (2 H, t, $^3J_{HH} = 4.50$ Hz, NCH$_2$*C*H$_2$OCS$_2$), 3.71 (4 H, t, $^3J_{HH} = 5.70$ Hz C*H*$_2$OH), 2.98 (2 H, t, $^3J_{HH} = 4.50$ Hz, S$_2$COCH$_2$C*H*$_2$N) and 2.78 (4 H, t, $^3J_{HH} = 5.70$ Hz, C*H*$_2$N). ^{13}C{^{1}H} NMR (D_2O): 234.53 (RO*C*S$_2$), 73.31 (NCH$_2$*C*H$_2$OCS$_2$), 61.36 (*C*H$_2$OH), 58.19 (*C*H$_2$N) and 54.80 (S$_2$COCH$_2$*C*H$_2$N). *m*/*z* (ES$^-$; MeOH) 525 ((2 M – H$^+$)$^-$, 4.8%), 487 ((2 M – K$^+$)$^-$, 43%), 411 (((2 M – K$^+$) – CS$_2$)$^-$, 10%), 262 ((M – H$^+$)$^-$, 100%), 224 ((M – K$^+$)$^-$, 7.1%) and 186 (((M – H$^+$) – CS$_2$)$^-$, 60%). HRMS Calcd for $C_7H_{14}NO_3S_2$: 224.0420. Found: 224.0391.

*Synthesis of di(bis(2-hydroxyethyl)dithiocarbamato)cadmium(II), Cd(bhedtc)$_2$ (**3**).* To a solution of **1** (30.40 g, 138.6 mmol) in deionized water

(295 ml), a solution of cadmium iodide (25.34 g, 69.20 mmol) in ethanol (438 ml) was slowly added with constant stirring. During the reaction, the mixture changed color from yellow to white. After 30 min, the reaction mixture was refrigerated for 5 days. The resultant white crystalline powder was filtered, washed with ethanol, and dried in air. The crude product was recrystallized from 300 ml of 1:1 DMF/water. The product was dried under vacuum at 45 °C for 12 h to give white crystals of di(bis(2-hydroxyethyl)dithiocarbamato)cadmium(II) (16.93 g, 52%). λ_{max}/nm (DMF) 284 (ε/dm^3 mol^{-1} cm^{-1} 1.6×10^4) and 343 (1.5×10^2). Mpt. 150–150.5 °C. Found C, 25.35; H, 4.30; Cd, 24.43; N, 5.90. $C_{10}H_{20}CdN_2O_4S_4$ requires C, 25.40; H, 4.26; Cd, 23.77; N, 5.92%. ν_{max}/cm^{-1} (KBr pellet): 3241 s (OH), 2930 m (CH), 1486 s (C = N), 1071 s (CO), 1050 m (CN), 1025 s (CC) and 985 s (CS). ^{1}H NMR (DMF-d_7): 5.05 (4 H, t, $^3J_{HH}$ = 5.25 Hz, $HOCH_2$), 4.13 (8 H, t, $^3J_{HH}$ = 5.55 Hz, CH_2N) and 3.92 (8 H, td, $^3J_{HH}$ = 5.55 Hz, $^3J_{HH}$ = 5.25 Hz, CH_2OH). $^{13}C\{^1H\}$ NMR (DMF-d_7): 206.22 (R_2NCS_2), 60.50 (CH_2N) and 59.32 (CH_2OH). m/z (ES^-; DMF) 601 ($(M + I^-)^-$, 100%).

*Reaction of potassium bis(2-hydroxyethyl)aminoethyldithiocarbonate hemihydrate, K[bhexan]·0.5H_2O (**2**) and cadmium iodide*. To a solution of **2** (3.434 g, 12.61 mmol) in deionized water (47.9 ml), a solution of cadmium iodide (2.383 g, 6.506 mmol) in deionized water (34.2 ml) was added with constant stirring. The product precipitated instantaneously. After 1 h, the reaction mixture was filtered, and the solid washed with water and ethanol, and dried in air. The resultant bright sticky yellow precipitate was dried under vacuum at room temperature for 24 h. The washing and drying process was repeated until the percent mass loss was constant – four cycles were necessary; an average 6.3% mass loss was attributed to compound dissolution for the last three cycles. This gave butter yellow crystals (0.5692 g). Decomposes at 300 °C. Found C, 14.95; H, 2.60; Cd, 42.87; I, 1.16; N, 2.73. ν_{max}/cm^{-1} (KBr pellet): 3375 m (OH), 2922 m (CH), 1738 s, 1455 m (CO), 1254 s (CN) and 1047 s (CO), 859 m (CC) and 722 w (CS).

Statistics

Data represents the mean ± standard error of the mean (SEM) of at least three separate experiments. Data were analyzed using a one-way analysis of variance followed by Tukey test for multiple comparisons, or by a Student's t-test for comparison between two groups. A p-value < 0.05 was considered significant.

Results

Synthesis

Cadmium chelating agents potassium bis(2-hydroxyethyl)dithiocarbamate, K[bhedtc] (**1**), and potassium bis(2-hydroxyethyl)aminoethyldithiocarbonate hemihydrate, K[bhexan]·0.5H_2O (**2**) were prepared from the corresponding ethanolamine, potassium hydroxide, and carbon disulfide (Figure 1). The reaction of cadmium iodide and each ligand in a 1:2 molar ratio both gave isolable cadmium complexes. The cadmium complex of **1**, di(bis(2-hydroxyethyl)dithiocarbamato)cadmium (II), $Cd(bhedtc)_2$ (**3**), has the prescribed cadmium:ligand ratio. However, elemental and AAS data (Table 1) indicate that the cadmium:ligand ratio of the cadmium complex of **2** is 2:1. Characterization of the cadmium complex of **2** is limited by the insolubility of the compound – it is insoluble in water, methanol, ethanol, methylene chloride, chloroform, ethyl acetate, diethyl ether, acetone, DMF, and DMSO at room temperature. Heating of DMF and DMSO suspensions of the cadmium complex of **2** led to visible decomposition of the yellow compound. Thus, this compound was not used in the cytotoxicity studies.

In vitro cytotoxicity testing

The cytotoxicity of the cadmium chelating agents **1** and **2**, and complex **3** were investigated using the human osteoblast-like cell line Saos-2.

Cadmium chloride treatment induced a significant loss of cell adherence (Figure 2). In contrast, the addition of the cadmium chelating agents **1** or **2** did not affect cell adherence in the concentration range studied. The addition of complex **3** resulted in a concentration-dependent increase in loss of cell adherence. However, 200 μM complex **3** was needed to induce a significant loss of cell adherence comparable to 100 μM cadmium chloride (Figure 2C).

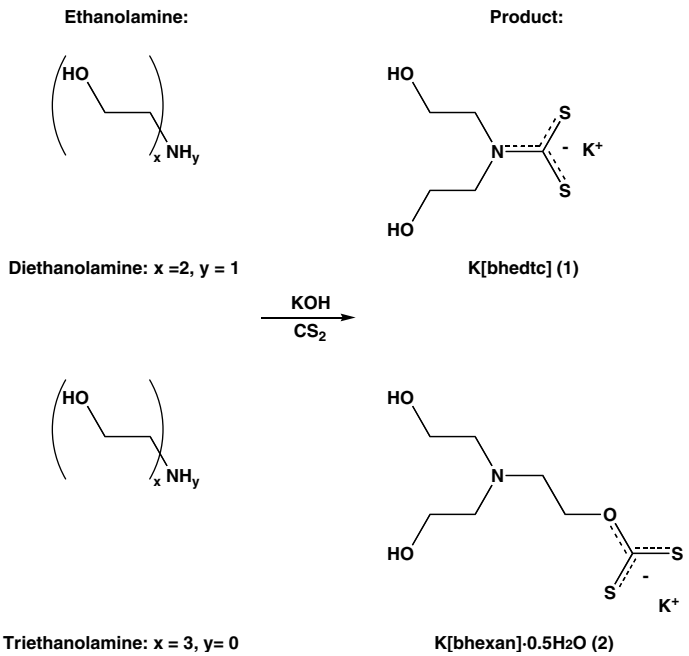

Figure 1. Synthesis of K[bhedtc] (**1**) and K[bhexan]·0.5H_2O (**2**) from di- and tri-ethanolamine, respectively.

Table 1. Calculated and actual %Cd values for the cadmium complex of **2**

Elemental analysis calculation/result	%Cd calculated	%Cd actual ± SEM
Cd:[bhexan]$^-$ ratio of 1:2	20.04	
Cd:[bhexan]$^-$ ratio of 1:1	33.38	
Cd:[bhexan]$^-$ ratio of 2:1	50.06	
AAS		45.98 ± 1.82
Desert analytics		42.87 ± 0.01

To further evaluate the effect of cadmium and the compounds on Saos-2 cells, viability was assessed using trypan blue exclusion, which detects a change in cell membrane integrity. Consistent with loss of cell adherence results, cadmium chloride treatment significantly decreased cell viability compared to untreated control cells (Figure 3), whereas treatment with 100–500 μM cadmium chelating agents **1** or **2** had no detectable effect on cell viability (Figures 3A, B). The addition of complex **3** resulted in a concentration-dependent decrease in cell viability (Figure 3C). Three-hundred μM complex **3** was needed to induce a significant decrease in cell viability comparable to 100 μM cadmium chloride (Figure 3C).

The MTT assay, which detects changes in energy metabolism, was used to further evaluate the cytotoxic effect of cadmium and the compounds on Saos-2 cells. The cytotoxicity profile for cadmium chloride, cadmium chelating agents **1** and **2**, and complex **3** were similar using both trypan blue exclusion and MTT assays (Figures 3 and 4). Although, cells exposed to cadmium chelating agent **2** exhibited reduced viability of approximately 90% compared to untreated control cells (Figure 4B), this difference was not found to be statistically significant.

The effect of simultaneous addition of cadmium chloride and cadmium chelating agents **1** or **2** on Saos-2 cell viability was also assessed using the MTT assay (Figure 5). The cytotoxic effect of 100 μM cadmium chloride was negated by simultaneous administration of 100 μM **1** (Figure 5A). In addition, simultaneous addition of 100 μM cadmium chloride with higher concentrations of **1** maintained Saos-2 cell viability at levels comparable to that of the control. In the 200 μM cadmium chloride experiments, the simultaneous administration of 300 μM or greater **1** resulted in cell viability that was not statistically significantly different from that of the control.

For the cadmium chelating agent **2**, simultaneous administration of 100 μM cadmium chloride and 300 μM or higher **2** resulted in cell viability that was statistically indistinguishable

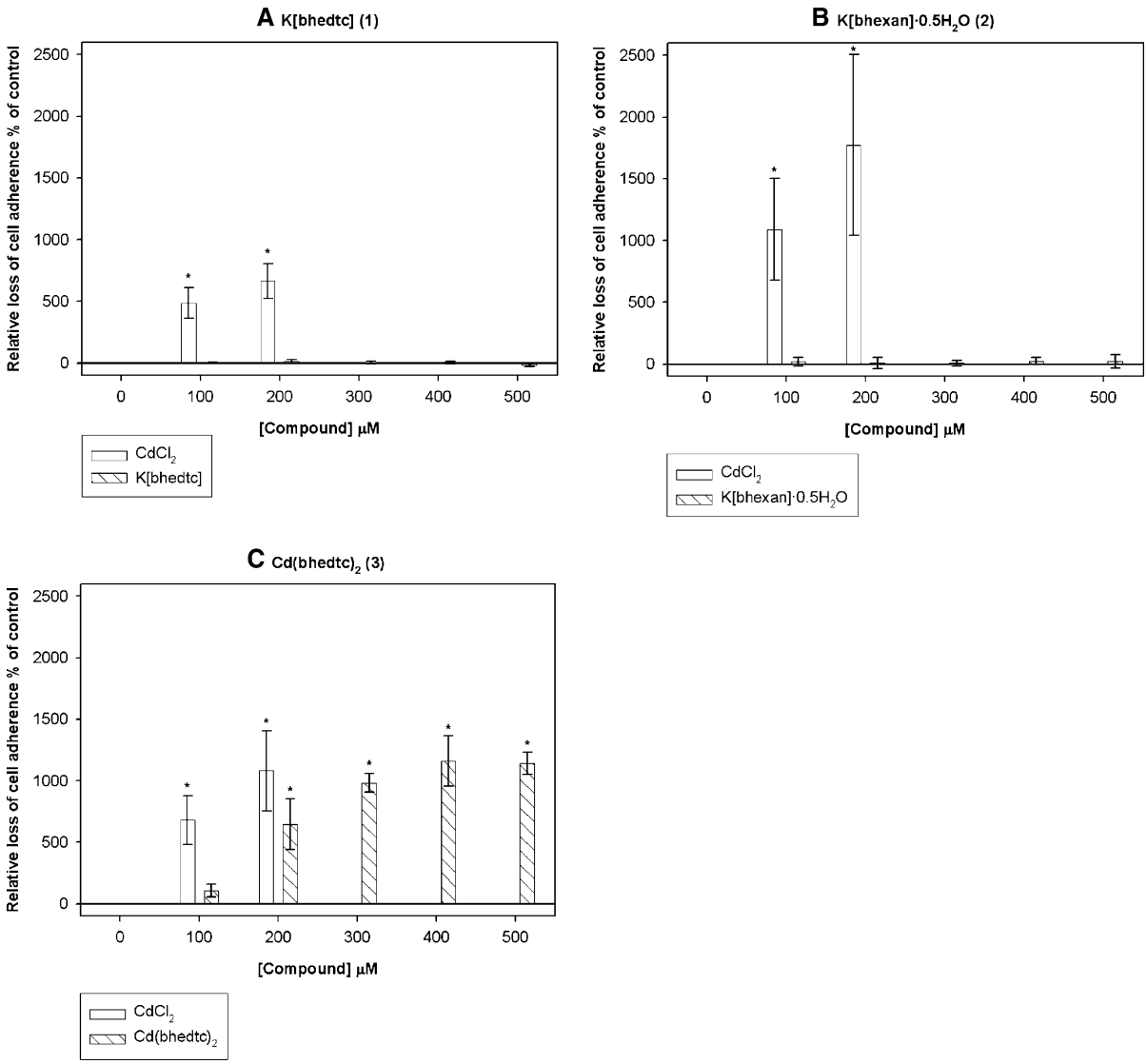

Figure 2. Effect of cadmium chloride and cadmium chelating compounds on cell adherence. Cells were treated with cadmium chloride or (A) K[bhedtc] (**1**), (B) K[bhexan]·0.5H_2O (**2**), or (C) $Cd(bhedtc)_2$ (**3**). The culture medium was collected and non-adherent cells were counted using a hemocytometer. Data are expressed as percent of control. Each bar represents the mean ± SEM for at least three separate experiments. *Indicate values significantly different compared to control.

from that of the control (Figure 5B). However, for the 200 μM cadmium chloride experiments, the highest cell viability attained (27 ± 9%) was with the addition of 400 μM **2**.

Discussion

Synthesis

The group 12 divalent cations Zn^{2+} and Cd^{2+} commonly form four coordinate complexes with dithiocarbamates and xanthates, with a metal:ligand ratio of 1:2. However, the nature of the resulting complex is seldom monomeric. In the case of the reaction of dibutylamine, carbon disulfide, and zinc chloride, the product isolated was the binuclear zinc(II) complex $Zn_2[(n\text{-}Bu)_2NCSS]_4$ ascertained by X-ray diffraction (Zhang *et al.* 2003). For the reaction of sodium *n*-butyl xanthate and cadmium nitrate tetrahydrate, the product was the polymeric compound $[Cd(S_2CO\text{-}n\text{-}C_4H_9)_2]_n$ (Jiang *et al.* 2002). The report of the synthesis of $Cd(bhedtc)_2$ (**3**) concluded that a solid

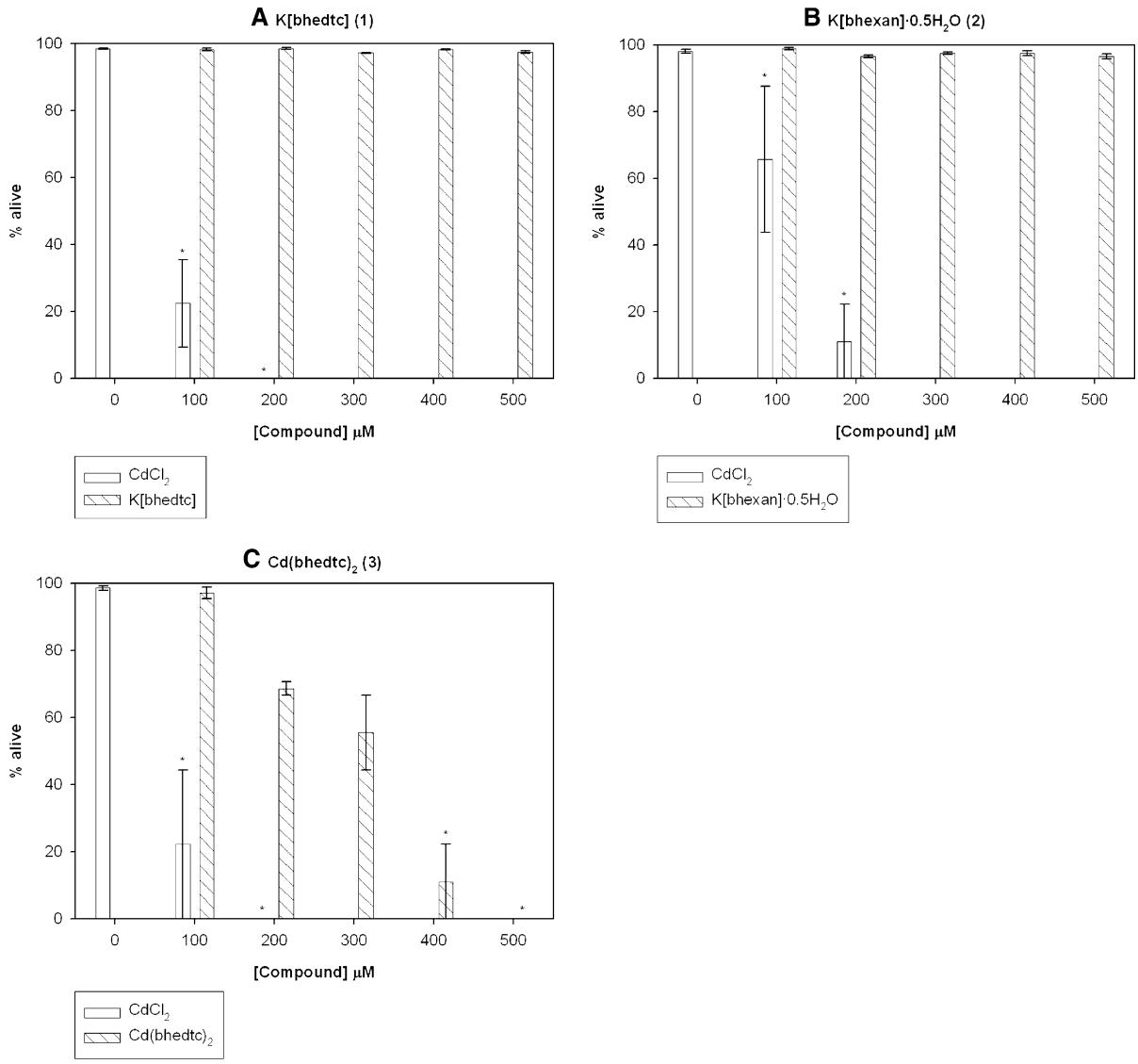

Figure 3. Cell viability measured by a trypan blue exclusion assay. After treatment with cadmium chloride or (A) K[bhedtc] (**1**), (B) K[bhexan]·$0.5H_2O$ (**2**), or (C) $Cd(bhedtc)_2$ (**3**), cells were collected and stained with trypan blue. Data are expressed as percent alive. Each bar represents the mean ± SEM for at least three separate experiments. *Indicate values significantly different compared to control.

state structure incorporating weak intermolecular cadmium–sulfur interactions was possible given the finding that the $[bhedtc]^-$ ligand was anisobidentate (Pages *et al*. 1985). Further, it was suggested that such a structure was responsible for the complex being almost insoluble in water in spite of the presence of hydroxyl groups on the ligand, and might explain why the cadmium complex is excreted in bile rather than in urine when the ligand is used to treat cadmium poisoning (Gale *et al*. 1983).

The isolation of **2** from the reaction of triethanolamine, potassium hydroxide, and carbon disulfide (Figure 1) was notable because mono-, di- and tri-xanthate products are possible. The need to use more than a three-fold excess of triethanolamine to dissolve the potassium hydroxide starting material in the synthesis

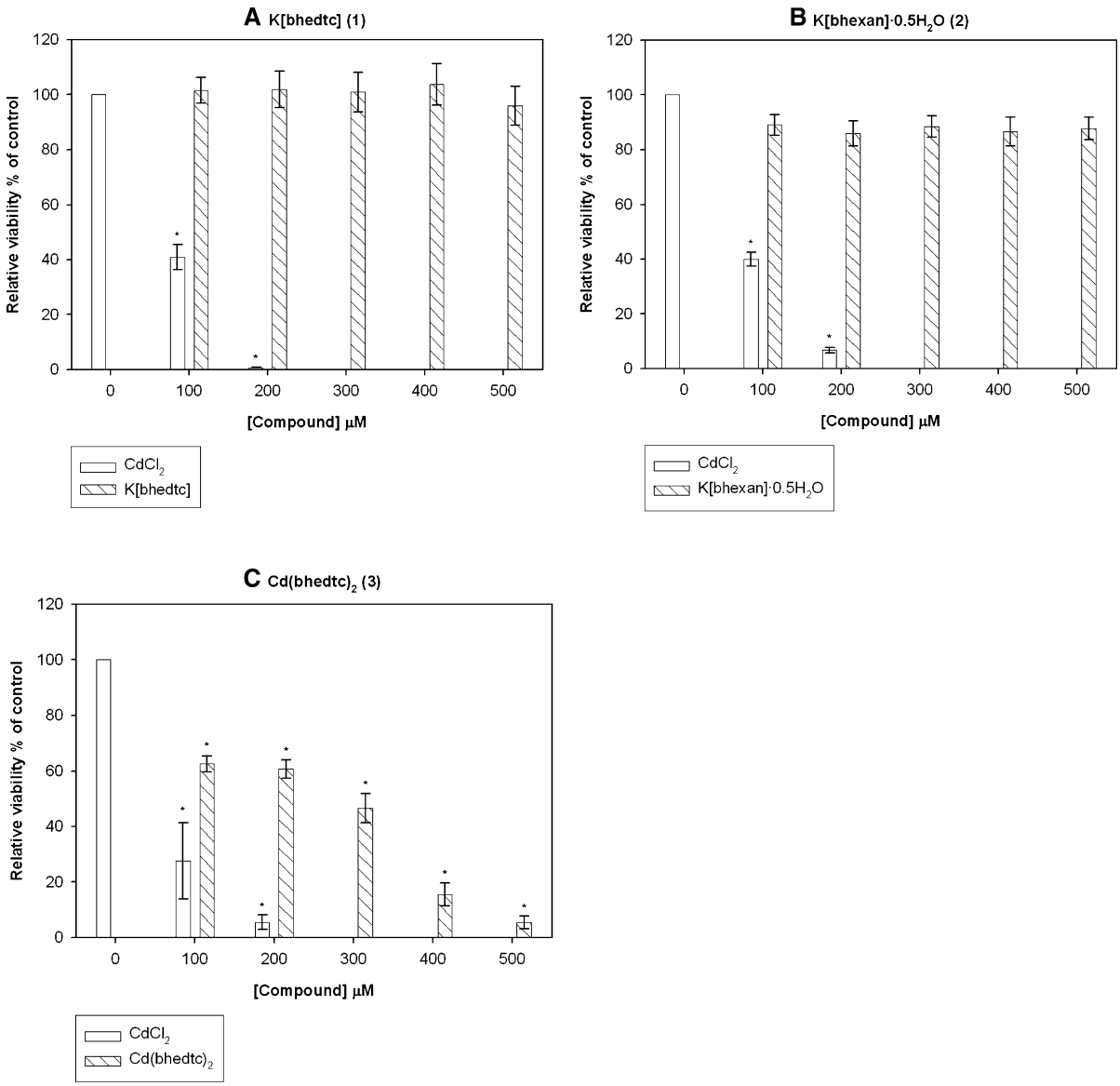

Figure 4. Cell viability measured by a MTT assay. After treatment with cadmium chloride or (A) K[bhedtc] (**1**), (B) K[bhexan]·0.5H_2O (**2**), or (C) $Cd(bhedtc)_2$ (**3**), cells were collected and viability assessed using the MTT assay. Data are expressed as percent of control. Each bar represents the mean ± SEM for at least three separate experiments. *Indicate values significantly different compared to control.

appeared to favor formation of the desired product.

The cadmium chelating agent **2** was synthesized because it contains the same bis(2-hydroxyethyl)amino moiety found in **1**, but a different metal-binding sulfur-based functional group. *In vitro* cytotoxicity comparison therefore between the successful *in vivo* cadmium chelating agent **1** (Gale *et al*. 1984; Hatori *et al*. 1990) and the new structurally related prototype **2** was expected to allow the relative merits of using dithiocarbamate versus xanthate function groups in cadmium chelating agents to be evaluated.

The cadmium complex of **2** was prepared and submitted for elemental analysis in three separate experiments. In all three cases the relative ratio of the average element percentage determined/atomic mass for C:H:N was approximately 7:14:1; this is

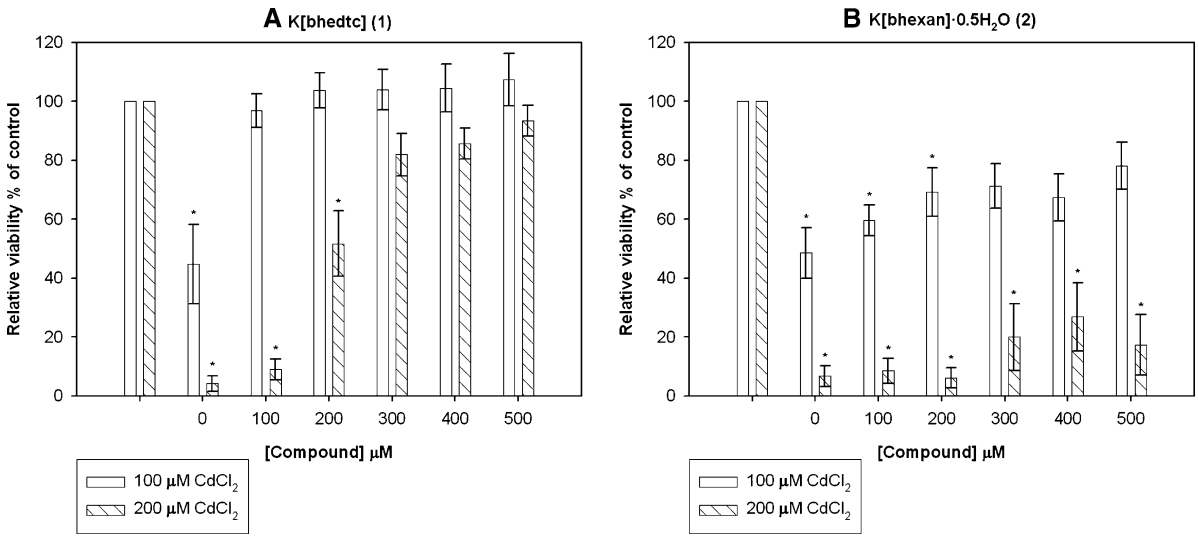

Figure 5. Effect of simultaneous addition of compound (A) K[bhedtc] (**1**) or (B) K[bhexan]·0.5H_2O (**2**) with 100 or 200 μM cadmium chloride on cell viability measured by a MTT assay. Data are expressed as percent of control. Each bar represents the mean ± SEM for at least three separate experiments. *Indicate values significantly different compared to control.

consistent with the formulation of the $[bhexan]^-$ ligand ($C_7H_{14}N_1O_3S_2$). Infrared data also suggested the presence of **2** in the insoluble product. For the latter two elemental analyses, the %Cd and %I were determined in light of the poor agreement of the carbon, hydrogen, and nitrogen data obtained for the compound submitted initially as $Cd(bhexan)_2$. The presence of iodine in the latter two elemental analyses suggested that the product was impure. The likely sources of iodine containing impurities are the starting material cadmium iodide and the byproduct potassium iodide. After consideration of the available data and noting the necessity for charge balance, the best formulation of the product was $[[Cd_2(bhexan)(OH)]\cdot 0.05CdI_2\cdot 2H_2O]_n$. Although the maximum percentage differential between calculated and determined data for this formulation is not optimal (1.4% for cadmium), comparison of the data shown in Table 1 shows that the cadmium:$[bhexan]^-$ ratio of the cadmium complex of **2** is closest to 2:1.

At first glance, the likelihood of cadmium existing as the Cd_2^{2+} dication appears unusual given that such a species is considered metastable (Ortiz & Ballone 1991). However, this species has been structurally characterized by X-ray methods in $Cd_2(AlCl_4)_2$ (Faggiani *et al.* 1986), and even Zn_2^{2+} has been recently identified as sandwiched between two $[C_5Me_5]^-$ rings in decamethyldizincocene (Resa *et al.* 2004). Thus it is feasible that the Cd_2^{2+} dication could be a component to this polymeric product, especially as a potential reducing agent (excess **2**) is present in the reaction mixture.

In vitro cytotoxicity testing

One approach in cadmium intoxication is the chelation of cadmium. The development of effective cadmium chelating agents has a multitude of applications including treatment of heavy metal poisoning and/or bioaccumulation in humans, recycling, and environmental clean up applications. Treatments for acute human oral cadmium intoxication or poisoning exist; oral administration of *meso*-2,3-dimercaptosuccinic acid (DMSA) combined with parenteral administration of the calcium salt of diethylenetriaminepentaacetic acid (DTPA) is considered the optimum chelation treatment (Andersen 1999). However, existing chelating agents capable of mobilizing aged cadmium deposits in experimental animals, which have been related to kidney dysfunction and low bone mineral density, are too toxic for human application (Andersen 1999). In the present study, we have synthesized a xanthate, potassium bis(2-hydroxyethyl)aminoethyldithiocarbonate hemihydrate, K[bhexan]·0.5H_2O (**2**), that is structurally related to the effective *in vivo* cadmium chelating

agent potassium bis(2-hydroxyethyl)dithiocarbamate, K[bhedtc] (**1**), and tested both in a human *in vitro* system. We used a human osteoblast-like cell line, Saos-2, that retains osteoblastic functions since cadmium is known to have deleterious effects on the skeleton and more specifically can alter osteoblast function (Staessen *et al*. 1999; Järup & Alfvén, 2004).

Loss of cell adherence, trypan blue exclusion and MTT assays indicate that cadmium chloride is cytotoxic to Saos-2 cells, whereas the cadmium chelating agents **1** and **2** induce minimal cytotoxicity. The results of the three assays for $Cd(bhedtc)_2$ (**3**) show that complexation reduces the cytotoxicity of cadmium chloride *in vitro* by a factor of two.

We found that simultaneous administration of 100 or 200 μM cadmium chloride and 100–500 μM **1** significantly enhanced the relative viability of cells compared to administration of 100 or 200 μM cadmium chloride alone (Figure 5A). Analogous results for **2** (Figure 5B) showed significant enhancement of the relative viability of cells compared to administration of 100 μM cadmium chloride, but not 200 μM cadmium chloride. Overall, both **1** and **2** reduce cadmium-induced cytotoxicity *in vitro*.

Future work will focus on evaluating the pharmacological efficacy of **1** and **2**, and other prototype cadmium chelating agents. This will include experiments that assess intracellular cadmium mobilization (Nerudová *et al*. 1991; Fisher 1995).

Acknowledgements

This work was supported by NIH Grant Number P20 RR016454 from the INBRE Program of the National Center for Research Resources. The authors acknowledge the help of Kendra G. Coonse in the statistical analysis.

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