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Original Research Article

Diethyldithiocarbamate complexes with metals used as food supplements show different effects in cancer cells

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ABSTRACT

Diethyldithiocarbamate (ditiocarb), a metabolite of the old anti-alcoholic drug disulfiram (Antabuse), forms proteasome-inhibiting metal complexes with copper or zinc that suppress cancer cells both *in vitro* and *in vivo*. The drug has been used in a clinical trial (NCT00742911) along with copper gluconate as a dietary supplement in patients with cancer spreading to the liver. In this study, we demonstrate the effect of synthetic complexes of disulfiram with four various metals (Mn, Fe, Cr and Cu) used as food supplements. These complexes may be spontaneously formed in the blood during the use of disulfiram with divalent metals and thus may suppress the growth of cancer *in vivo*. The cytotoxic effect of the compounds and the compounds' ability to inhibit the cellular proteasome were tested in the osteosarcoma cell line U2OS. After 48 h, copper and manganese complexes exhibited cytotoxic effect on the cell line, in sharp contrast to both iron and chromium complexes.

Introduction

Dithiocarbamate compounds have been effective against cancer in animal models and in the clinical studies (Cvek and Dvorak, 2007; Cvek, 2011). Chemically, dithiocarbamates are the reduced form of thiuram disulfides whit strong complex-forming properties and rich coordination chemistry (Thorn and Ludwig, 1962), which facilitates a large array of uses. For instance, thiuram disulfides and dithiocarbamates are used in the rubber vulcanization process (Nieuwenhuizen et al., 1997) or as pesticides, with potential side effects, in agriculture (Environmental Health Criteria, 1988; Chou et al., 2008). Disulfiram, a thiuram disulfide clinically used under the brand name Antabuse, is converted to diethyldithiocarbamate in the body and shows intriguing biological activity and a safe pharmacological profile (Børup et al., 1992; Cvek and Dvorak, 2007). Most importantly, patients on disulfiram exhibit

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inhibited aldehyde dehydrogenase (ALDH) activity (Suh et al., 2006). ALDH is an essential enzyme in ethanol metabolism, converting acetaldehyde to acetate. Consequently, disulfiram's nonspecific interaction with enzyme sulfhydryl groups (Johansson, 1992) has been utilized in the treatment of chronic alcoholism (Bell et al., 2012). Other cellular effects and properties of these compounds are described in several recent publications (Xu et al., 2011; O'Brian et al., 2012). Among these effects and properties, the most discussed is antitumor activity (Cvek, 2011), which was described, for the first time, in the late 1970s by Lewison (1977). Moreover, it was experimentally demonstrated that the administration of disulfiram prevents induced tumorigenesis in laboratory animals (Wattenberg, 1975; Sunderman et al., 1984). These findings gradually led to the establishment of a double-blind, placebo-controlled phase II clinical trial for disulfiram's main metabolite, diethyldithiocarbamate (ditiocarb), in patients with nonmetastatic, high-risk breast cancer. At 5 years, overall survival was 81% in the ditiocarb group compared with 55% in the placebo group (Dufour et al., 1993). The results of this clinical trial confirmed the potent anticancer activity of diethyldithiocarbamate in humans. The antitumor effect of disulfiram has been potentiated by synchronous administration with zinc gluconate, leading to clinical remission in a melanoma patient with a liver metastasis (Brar et al., 2004). It was consistently found that a complex of ditiocarb and metal (e.g., copper that is formed in the body after disulfiram digestion; Johansson, 1992) is responsible for the antitumor activity, rather than disulfiram or ditiocarb alone (Viola-Rhenals et al., 2006). The diethyldithiocarbamate–copper complex most likely targets the cellular proteasome and can inhibit the proteasome (Chen et al., 2006; Cvek et al., 2008). The proteasome is essential for cancer cells (Drexler et al., 2000) and is involved in many important cell signaling pathways and processes, such as nuclear factor-κB signaling, cell cycle progress, proliferation, the immune response and maintenance of cellular protein homeostasis (Finley, 2009; Bedford et al., 2010). Thus, inhibition of the chymotrypsin (CT)-like activity of the proteasome is selectively lethal to cancer cells in an apoptotic manner (Adams, 2004; Cvek and Dvorak, 2011). Moreover, many organic complexes with diverse metals are likely able to inhibit the proteasome and suppress cancer cells in this way (Chen et al., 2005; Verani, 2012). Given the strong metal-binding properties of diethyldithiocarbamate (Hogarth, 2012), we hypothesize that this compound can chelate other biogenic metals in the human body and create various complexes with diverse biological activities *in vivo*. To examine the biological activity of such complexes, we synthesized diethyldithiocarbamate complexes with metals used as dietary supplements, i.e., Cu, Mn, Fe and Cr (Wong, 2012). Although the exact mechanism of action of disulfiram and its metabolites in cancer cells remains unclear, several clinical trials have been performed in the United States. For instance, disulfiram was used in combination with copper for the treatment of refractory solid tumors involving the liver (ClinicalTrials.gov Identifier NCT00742911) or in combination with arsenic trioxide for the treatment of metastatic melanoma (ClinicalTrials.gov Identifier NCT00571116). These trials suggest the great potential of diethyldithiocarbamate–metal complexes to become a cheap and effective cancer cure.

Materials and methods

Materials

Sodium diethyldithiocarbamate trihydrate, manganese(III) acetate ($Mn(MeCOO)_3$), iron(III) chloride hexahydrate ($FeCl_3{\bullet}6H_2O$), chromium(III) nitrate nonahydrate ($Cr(NO_3)_3{\bullet}9H_2O$), copper(II) chloride dihydrate ($CuCl_2{\bullet}2H_2O$), fetal bovine serum, dimethylsulfoxide (DMSO) and DMEM were purchased from Sigma–Aldrich. Penicillin/streptomycin (100×) was purchased from PAA. A substrate (Suc-LLVT-AMC) for the determination of CT-like 20S proteasome activity was purchased from Sigma–Aldrich. Complete protease inhibitor cocktail tablets and the phosphatase inhibitor PhosSTOP were purchased from Roche Diagnostics GmbH. A mouse monoclonal antibody against Poly (ADP-ribose) polymerase (PARP-1) was purchased from Cell Signaling Technology. Mouse monoclonal antibodies against ubiquitin (P4D1), a rabbit polyclonal antibody against IκBα (C-15), a goat polyclonal antibody against actin (I-19), secondary antibodies and luminol for Western blotting were purchased from Santa Cruz Biotechnology.

Synthesis of the complexes

Tris-(*N*,*N*-diethyldithiocarbamato)-manganese(III), -iron(III) and -chromium(III) complexes were prepared from aqueous solutions of $Mn(MeCOO)_3$, $FeCl_3{\bullet}6H2O$ and $Cr(NO_3)3{\bullet}9H_2O$, respectively, in the presence of a three-fold molar excess of sodium diethyldithiocarbamate trihydrate, with constant stirring and in air. All complexes precipitated as solid powders, which were collected by filtration; washed, first with water and then with EtOH; and dried in vacuo for 24 h (yields: 80%, 74% and 78%, respectively, for the Mn(III), Fe(III) and Cr(III) complexes). The bis-(*N*,*N*-diethyldithiocarbamato)-copper(II) complex was synthesized and described according to a reported method (Hogarth, 2005; Cvek et al., 2008).

Structural characterization of the complexes

The obtained complexes were characterized by IR spectroscopy, ESI mass-spectrometry and elemental analysis. Infrared spectra (4000–400 cm^{-1}) were recorded on a BIO-RAD FTS 3000 MX instrument in KBr pellets. Far infrared spectra (400–200 cm^{-1}) were recorded on a Vertex 70 spectrophotometer, in CsI pellets. Wavenumbers are in cm^{-1}; abbreviations: *s*, strong; *m*, medium; *w*, weak. ESI^+/ESI^- mass spectra were obtained on a VARIAN 500-MS LC ion trap mass spectrometer (solvent: dimethylsulfoxide; flow: 20 μL/min; needle spray voltage: ±5 kV, capillarity voltage: ±100 V; nebulizer gas (N_2): 35 psi; drying gas (N_2): 10 psi; drying gas temperature (N_2): 350 °C). For the MS spectra description, M denotes the metallic complexes. C, H and N elemental analyses were carried out by the Microanalytical Service of the Instituto Superior Técnico, Lisbon. The obtained spectroscopic features are in agreement (Healy and White, 1972a,b; Almeida et al., 1996; Hogarth, 2012) with the proposed η^2-coordination of *N*,*N*-diethyldithiocarbamate to the metal centers.

Tris-(*N*,*N*-diethyldithiocarbamato)manganese(III), [Mn(η2-S2CNEt2)3]: IR (KBr pellet, cm^{-1}): 1470 s, ν(C–N); 1250 m,

$\nu_{ass}(CS_2)$; 973 s, $\nu_s(CS_2)$; 388 m, ν(Mn–S). ESI$^+$: *m*/z 501 ($[M+H]^+$), 457 ($[M\text{-}NEt_2]^{+\bullet}$), 352 ($[M\text{-}S_2CNEt_2]^+$). Anal. Calc. for $MnC_{15}H_{30}N_3S_6$ (499.75 g mol^{-1}): C, 36.1; H, 6.1; N, 8.4; S, 38.5%. Found: C, 35.9; H, 6.1; N, 8.7; S, 38.3%.

Tris-(*N*,*N*-diethyldithiocarbamato)iron(III),[Fe(η2-S2CNEt2)3]: IR (KBr pellet, cm^{-1}): 1490 s, ν(C–N); 1198 m, $\nu_{ass}(CS_2)$; 988 s, $\nu_s(CS_2)$; 371 m, ν(Fe–S). ESI$^+$: *m*/z 502 ($[M+H]^+$), 425 ($[M\text{-}CS_2]^{+\bullet}$). Anal. Calc. for $FeC_{15}H_{30}N_3S_6$ (500.65 g mol^{-1}): C, 36.0; H, 6.0; N, 8.4; S, 38.4%. Found: C, 36.2; H, 5.8; N, 8.4; S, 38.6%.

Tris-(*N*,*N*-diethyldithiocarbamato)chromium(III),[Cr(η2-S2CNEt2)3]: IR (KBr pellet, cm^{-1}): 1505 s, ν(C–N); 1246 m, $\nu_{ass}(CS_2)$; 954 s, $\nu_s(CS_2)$; 391 m, ν(Cr–S). ESI$^+$: *m*/z 498 ($[M+H]^+$), 421 ($[M\text{-}CS_2]^{+\bullet}$), 349 ($[M\text{-}S_2CNEt_2]^+$). Anal. Calc. for $CrC_{15}H_{30}N_3S_6$ (496.80 g mol^{-1}): C, 36.3; H, 6.1; N, 8.5; S, 38.7%. Found: C, 36.1; H, 6.4; N, 8.2; S, 38.9%.

Cell culture

Cancer cell lines were obtained from the Health Protection Agency culture collection (UK).

Cell lines were cultured in complete DMEM or RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 5 mL of penicillin/streptomycin solution at 37 °C in a 5% CO_2 atmosphere. The cells were passaged according to the obtained instructions.

Growth inhibition assay

All cell lines were cultivated for 24 h, and the U2OS line was cultivated for 24 or 48 h with synthetic diethyldithiocarbamate–metal complexes. In parallel, cells were treated with DMSO (0.1% as a solvent control) and Triton X-100 (5%) to assess the minimal and maximal cell damage, respectively. An MTT test was used following the standard protocol. Absorbance was measured spectrophotometrically at 540 nm (TECAN, Infinite M200). The IC50 values were calculated using data from three independent cell passages.

Proteasome activity in whole-cell extracts

Cells were seeded on 100 nm Petri dishes at a density of 3×10^6 cells/dish. After 24-h, the cells were washed twice with 2 mL of ice-cold PBS and scraped into 1000 μL of ice-cold PBS.

The cells were then isolated and suspended in a buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 0.1 μM PMSF) and then centrifuged at 15,000 rpm for 15 min at 4 °C. Subsequently, the cell extract (10 μg) was incubated with 10 μM substrate to measure the CT-like activity (*Suc-LLVY-AMC*) in 90 μL of assay buffer (30 mM Tris–HCl and 0.035% sodium dodecyl sulfate (pH 7.4)) in the presence of an inorganic metal complex and bortezomib or the equivalent volume of solvent (DMSO) as a control. After 2-h of incubation at 37 °C, the inhibition of proteasome activity was measured based on the release of hydrolyzed free AMC groups, fluorometrically at 380/460 nm (TECAN, Infinite M200PRO).

Proteasome activity in U2OS cells

Cells were seeded on six-well plate in a density of 0.5×10^6 cells/well. U2OS cells were treated with diethyldithiocarbamate and bortezomib complexes at three concentrations for 8 h. After incubation, cells were twice washed with 1 mL of ice-cold PBS and scraped in 200 μL ice-cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100 and PMSF 0.1 μM]. U2OS cells were harvested and used for whole cell extract preparation. The cell extracts (10 μg) was incubated in 90 μL of assay buffer (30 mM Tris–HCl and 0.035% sodium dodecyl sulfate (pH 7.4)) with 10 μM of substrate for measuring the CT-like activity. After 2-h of incubation at 37 °C, inhibition of each proteasomal activity was measured by the release of hydrolyzed free AMC as described above.

Western blotting

U2OS cells were seeded on six-well dishes at a density of 1×10^6 cells/well. After 24 h, the culture medium was replaced with fresh medium. Thereafter, the cells were treated for 8 h with complexes and DMSO as a solvent control. Total cell protein extracts were prepared as follows: cells were washed twice with 50 μL of ice-cold PBS and scraped into 200 μL ice-cold RIPA buffer (25 mM Tris–HCl (pH 7.6), 450 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) with protease and phosphatase inhibitors. The mixture was incubated for 15 min on ice, vortexed and centrifuged (13,000 × *g* for 10 min at 4 °C).

Protein concentrations were determined by the Bradford method. SDS-PAGE gels (10%) were run on a Bio-Rad electrophoresis instrument according to a general protocol. Protein transfer onto a 0.45 μM PVDF membrane was performed. The membrane was saturated with 7% nonfat dried milk with primary antibody for 4-h, followed by incubation with a secondary antibody for 1-h at room temperature. Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibody, luminol for western blotting and chemiluminescence film.

IC50 calculations and statistical analysis

The data are reported as the mean ± standard error of mean (SEM) of three independent experiments. For determination of cytotoxicity, the compounds were added to U2OS cells at four concentrations, and the IC50 values were calculated by fitting the data to a four-point dose–response curve using the ED50 Plus v 1.0 (online free software). A Student's *t*-test was used at the significance level $2\alpha = 0.05$ for statistical analysis.

Results

We first investigated the effect of diethyldithiocarbamate–metal complexes on U2OS cells. A dose–response curve of the cytotoxic effect (IC50) was produced using an MTT cell viability assay (Fig. 1). These data demonstrate that the copper complex had a cytotoxic effect on U2OS cells, with IC50 = 13.3 ± 1.32 μM after 24 h (Fig. 1A). After the 48-h period, we recorded a strong cytotoxic effect for the copper complex (IC50 = 2.37 ± 0.12 μM) and a cytotoxic effect for the manganese complex (IC50 = 13.3 ± 1.43 μM) (Fig. 1A and B). The iron and chromium complexes did not have any cytotoxic effect on the U2OS osteosarcoma cell line (Fig. 1A and C). We found that the copper and

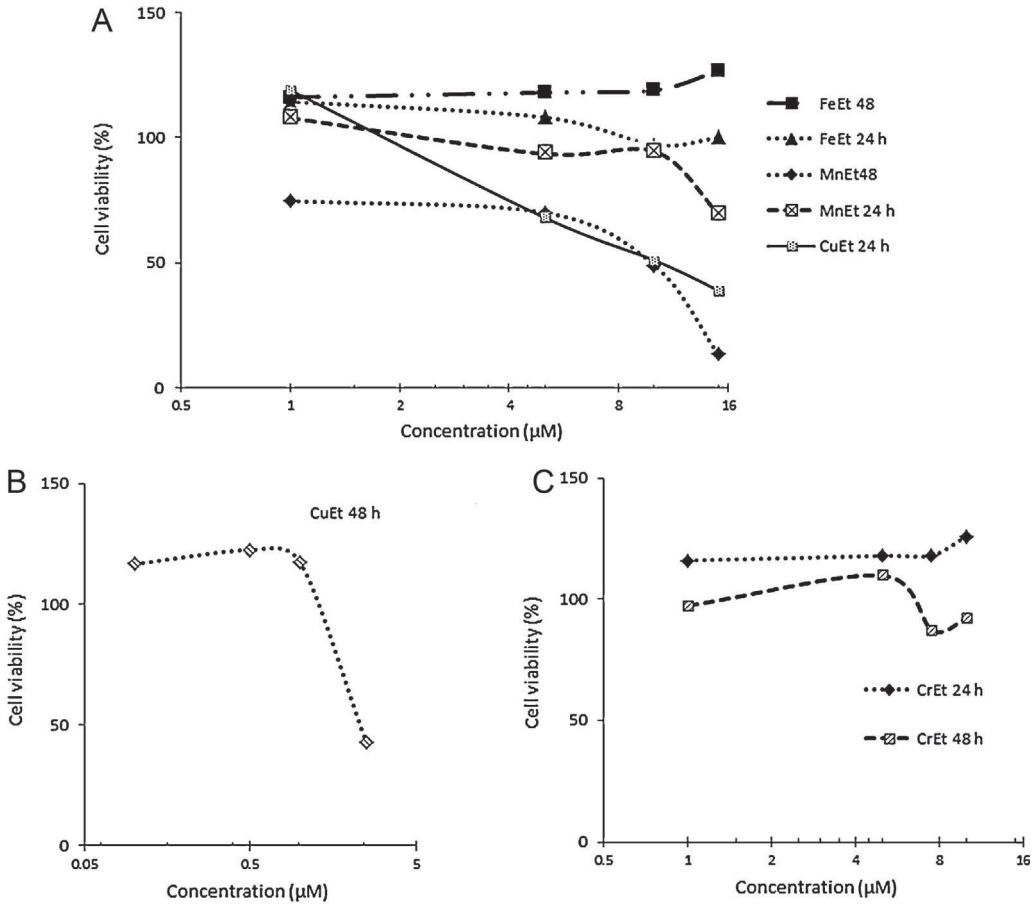

Fig. 1 – The viability (%) of U2OS cells treated of dithiocarbamate metal complexes. U2OS cells were treated with complexes according to solubility in culture medium and cytotoxic effects. (A) Cells were treated with iron, manganese complex after 24 and 48 h and copper complex after 24 h, (B) The strong grow-inhibition effect of copper complex after 48 h treatment and (C) the effect of less soluble chromium complex.

manganese complexes induced inhibition of the CT-like activity of the U2OS cellular proteasome after 8 h, whereas the other tested complexes were inactive against tumor cell proteasomes (Fig. 2A). We used a maximal concentration of 10 μM for the chromium complex because of the lower solubility of this compound and 15 μM for the other complexes. Consistent with the measured toxicity, the copper complex showed higher potency in inhibiting the CT-like activity of the cellular proteasome than the manganese analog. We demonstrated that the addition of any diethyldithiocarbamate–metal complex to the cell lysate in a short period of time did not inhibit the CT-like activity of the proteasome (Fig. 2B). Further experiments were performed to determine whether diethyldithiocarbamate–metal complexes inhibit the cellular proteasome in cancer cells. U2OS cells were treated with diethyldithiocarbamate–metal complexes, bortezomib (a standard proteasome inhibitor) or DMSO (vehicle control) for 8 h; harvested; and used for cell extract preparation followed by Western blot analysis. The accumulation of ubiquitinated proteins could be a sign of the inhibited proteasome. We detected the accumulation of ubiquitinated proteins, which achieved significantly higher levels in the cells treated with bortezomib and copper- and manganese-diethyldithiocarbamate complexes (Fig. 3A). The hypothesis that these compounds inhibit the cellular proteasome was verified by the accumulation of the proteasome substrate IκBα (Fig. 3B), which was recorded together with the accumulation of ubiquitinated proteins. In the cells treated with bortezomib and copper- and manganese diethyldithiocarbamate complexes, we recorded PARP-1 cleavage to an 85 kDa fragment (Fig. 3C), which is associated with apoptotic cell death (Vaux and Strasser, 1996). Thus, our result shows that active diethyldithiocarbamate–metal complexes including copper or manganese are able to suppress cancer cells derived from the human osteosarcoma through the inhibition of the 26S proteasome and the activation of caspases 3 and 7 (responsible for PARP-1 cleavage), thereby inducing apoptosis (Duriez and Snah, 1997).

Discussion

Today, cancer treatment is based on conventional chemotherapy, which yields an improved prognosis, particularly in young patients. However, the general toxicity caused by nonspecific interactions of chemotherapeutic drugs and the development of drug resistance limit chemotherapy's use and

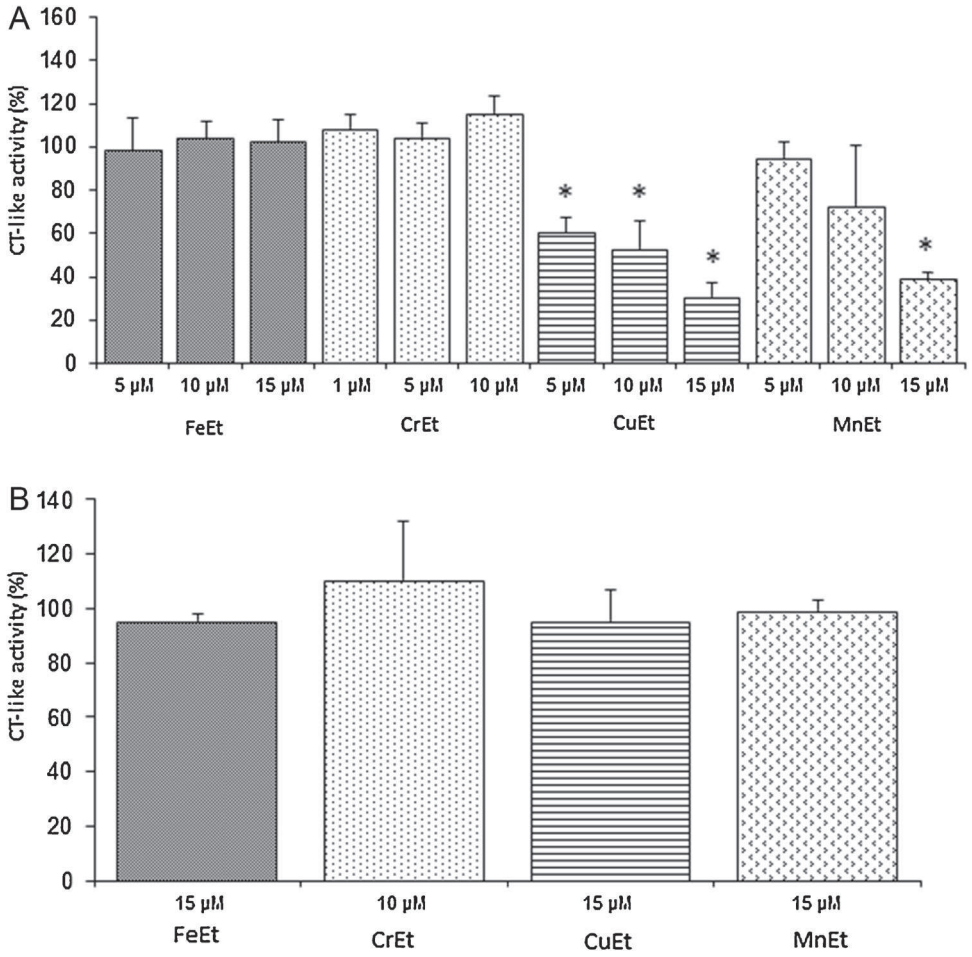

Fig. 2 – The effects of dithiocarbamate metal complexes on proteasome. CT-like activity of proteasome was evaluated in the human osteosarcoma derived cell line U2OS (A) and U2OS cell lysates (B). Cells were pre-treated with complexes for 8 h and the lysates for 15 min. *Statistically significant at $2\alpha = 0.05$.

could seriously damage a patient's health (Chabner and Roberts, 2005; Ta et al., 2009; Gill et al., 2013). Moreover, this insufficiently effective and harmful chemotherapy is significantly expensive. Society needs new effective drugs with a safe pharmacological profile and minimal side effects at a reasonable cost. However, at present, this goal is likely impossible because current drug research consumes particularly high amounts of money and cannot afford to produce lowcost products (Fojo and Grady, 2009). Moreover, new drugs used in oncology are mostly very toxic (Vera-Badillo et al., 2013). An effort to develop a truly effective and safe anticancer drug might be accomplished through other approaches. One approach is drug repurposing or off-label use (Chong and Sullivan, 2007; Boguski et al., 2009). In fact, there are certain old drugs that demonstrate therapeutic activity in diverse diseases, such as thalidomide. This drug was originally designed for the suppression of nausea, but the drug was soon prohibited because of teratogenic activity. Later, thalidomide was utilized as a leprosy treatment (Martínez-Fríaz, 2012). This repurposing could also apply to disulfiram, which shows anticancer activity (Cvek and Dvorak, 2008). The most reported and studied compound is a complex of disulfiram's main metabolite, diethyldithiocarbamate, with copper (Guo et al., 2010; Liu et al., 2012; Skrott and Cvek, 2012). Certain papers have even reported that disulfiram's anticancer effect is copper dependent (Yu et al., 2007; Rae et al., 2013). Here, we show that not only copper but also certain other endogenous metals chelated by diethyldithiocarbamate can suppress cancer cells via proteasome inhibition *in vitro*.

After 8-h treatment with the diethyldithiocarbamate–manganese and –copper complexes, higher levels of ubiquitinated proteins and the accumulation of IκBα, markers of proteasome inhibition, were detected. Nevertheless, the manganese- and copper–diethyldithiocarbamate complexes did not inhibit the 20S proteasome in a short period of time, implying that these complexes most likely inhibit only the 26S proteasome. These results are consistent with the findings of previous studies (Hogarth, 2012; Skrott and Cvek, 2012). Several papers attribute the anticancer effect of the diethyldithiocarbamate–copper complex to Cu-induced oxidative stress (Cen et al., 2002; Morrison et al., 2010; Kwolek-Mirek et al., 2012). Our results suggest that Cu-induced oxidative stress may not be the only mechanism of action of this compound. Moreover, the mechanism of action of diethyldithiocarbamate–metal complexes is not as copper dependent as the process initially appeared but still depends on the

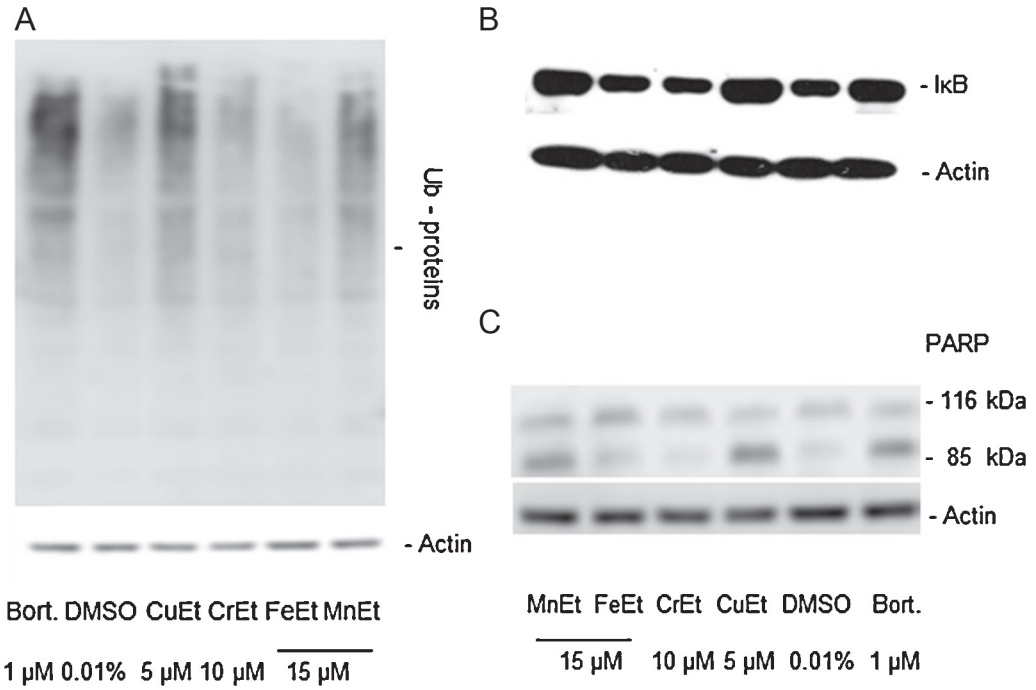

Fig. 3 – Differential effects of dithiocarbamate complexes on U2OS cells after 8 h treatment. (A) Western blot analysis of accumulation of ubiquitinated proteins. (B) Inhibition of the degradation of the proteasome substrate protein IκBα. (C) PARP-1 was cleaved during copper and manganese complex or bortezomib induced programmed cell death.

type of chelated metal ion. This finding is particularly consistent with (Rae et al., 2013). Compared with the manganese complex, the copper complex was more potent against the U2OS cells. The diethyldithiocarbamate–iron and –chromium complexes were unable to inhibit both the 20S and the 26S proteasome. The reason why the chromium and iron–diethyldithiocarbamate complexes were not able to inhibit the proteasome is worthy of further investigation. Manganese–diethyldithiocarbamate is structurally very similar to iron and chromium complexes, so we suggest a degree of metabolic activation of the complex within the cells. In the case of the copper complex, there is already a suggested mechanism of inhibition of the 26 proteasome by the complex, in which dithiocarbamate sulfur interacts with zinc in the Poh1 protein within the proteasome (Cvek et al., 2008). However, whether the manganese complex inhibits the proteasome via the same mechanism as the copper complex remains an open question. The cellular proteasome is a promising target in cancer therapy. The proteasome inhibitors bortezomib and carfilzomib have already been approved by the FDA for cancer treatment, and other compounds are being developed (Cvek, 2012a; Kortuem and Stewart, 2013). Dithiocarbamate complexes with certain endogenous metals, serving as potent proteasome inhibitors could be very effective anticancer drugs with minimal side effects (Børup et al., 1992). Furthermore, disulfiram is a very inexpensive drug whose approval for cancer treatment in combination with certain food-supplement metals would remove the financial burden of cancer therapies from health care systems (Cvek, 2012b). This publication shows the potential of disulfiram to become very effective and safe anticancer drug, especially when combined with metals commonly used as food supplements, such as copper or manganese.

Conflict of interest

None.

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