

## Biological Activity of Metal-edds (ethylenediaminedisuccinate) Complexes in K562 and PBMC Cells

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O efeito do ácido *S,S*-etilenodiaminodi-succínico (edds) na supressão da oxidação do ácido ascórbico catalisada por metais (Mn, Fe, Co, Ni, Cu, Zn) foi testado *in vitro* através da oxidação da sonda fluorescente cloreto de 1,2,3-diídrorodamina. A atividade pró-oxidante do ferro não foi totalmente suprimida, mesmo sob excesso molar de quatro vezes do ligante. O efeito do meio de cultura na toxicidade dos complexos para células mononucleares do sangue periférico (PBMC) e da linhagem K562 foi estudado. A citotoxicidade de Fe-edds foi abolida na presença de Trolox ou de proteínas do soro. Os prováveis mecanismos de toxicidade celular foram investigados através do bloqueio de transportadores de monocarboxilatos (MCT) e estudos de ciclo celular por citometria de fluxo. Células tratadas com os complexos metálicos e com o ácido  $\alpha$ -ciano-4-hidroxicinâmico, um conhecido bloqueador de MCT, mostraram recuperação de viabilidade, sugerindo que esses transportadores possam estar envolvidos na internalização dos complexos metal-edds. O ácido livre promoveu a parada do ciclo celular na fase G0/G1 (PBMC) ou S (K562), sugerindo dano direto ao DNA ou interferência na sua duplicação.

The effect of *S,S*-ethylenediaminedisuccinic acid (edds) on the quenching of metal-catalyzed (metal = Mn, Fe, Co, Ni, Cu, Zn) oxidation of ascorbic acid was tested *in vitro* via oxidation of the fluorescent probe 1,2,3-dihydrohodamine dihydrochloride. The pro-oxidant activity of iron was not fully suppressed, even at a four-fold molar excess of the ligand. The effect of serum on the toxicity to peripheral blood mononuclear cells (PBMC) and K562 cells was investigated. The cytotoxic effect of Fe-edds was abrogated in the presence of Trolox or serum proteins. The probable pathways of cell toxicity were investigated through blocking of the monocarboxylate transporters (MCT) in association with cell cycle studies by flow cytometry. Cells treated with metal complexes and  $\alpha$ -cyano-4-hydroxycinnamic acid, a known MCT inhibitor, showed recovery of viability, suggesting that MCT proteins may be involved in the internalization of metal-edds complexes. The free acid induced cell cycle arrest in G0/G1 (PBMC) and S (K562) phases, suggesting direct DNA damage or interference in DNA replication.

**Keywords:** K562, edds, antitumor, pro-oxidant, cell cycle, PBMC

### Introduction

Ethylenediaminedisuccinic acid (edds) was the first natural aminopolycarboxylic acid discovered, isolated from a culture of *Amycolatopsis orientalis*. It was first detected due to its ability to inhibit Zn<sup>2+</sup>-dependent phospholipase C activity. Edds has two chiral carbon atoms, resulting in three possible stereoisomers [*S,S*], [*R,S*] and [*R,R*].<sup>1,2</sup>

Technical applications of edds usually involve remediation of soils contaminated with heavy metals, either in

extraction columns,<sup>3</sup> induced *in situ* phytoextraction<sup>4,5</sup> or soil washing in permeable barriers.<sup>5,6</sup> Comparative tests involving [*S,S*]-edds and edta (ethylenediaminetetraacetic acid) were performed to determine their relative efficiencies in inducing phytoextraction of heavy metals; [*S,S*]-edds was more efficient for Cu<sup>2+</sup> removal, while edta removed Pb<sup>2+</sup> preferentially. However, since edds is more biodegradable than edta, the former has been recommended as a potential replacement for the latter in environmental applications.<sup>7,8</sup> The *S,S* stereoisomer was found to give rise to slightly more stable complexes (around 0.3 – 0.5 log units) than the *R,S* isomer, suggesting a small stereospecificity in metal

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coordination.<sup>9</sup> [S,S]-edds is also efficient for radiochemical decontamination.<sup>10</sup>

There are relatively few studies addressing the biological activity of this chelator. It is toxic to algae<sup>11</sup> and inhibits the formation of biofilms by *Xylella fastidiosa*.<sup>12</sup> Its antiviral activity *in vitro* and *in vivo*, which has been demonstrated against cytomegaloviruses, is probably due to inhibition of ribonucleotide reductase activity.<sup>13</sup>

Metal complexes of [S,S]-edds are also highly biodegradable,<sup>14</sup> but there are no reports of their behavior when in biological medium. Their stability is comparable to that of edta-containing analogues; therefore, they might be involved in altered metal mobilization and availability when present in the environment. An understanding of the biological activity of metal-edds complexes is important both for decontamination applications and for the development of metallopharmaceuticals.

In this paper, we report the biological and pro-oxidant activities of several M-[S,S]-edds complexes (M = Fe<sup>3+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Mn<sup>2+</sup>) in K562 and PBMC cells. Also, in this study the protective effect of serum proteins against toxicity was verified. We found that the oxidant activity of iron was not fully inhibited by edds, but Fe-edds cell toxicity was halted by the presence of serum and Trolox, which acted as antioxidants. In the absence of medium, blocking of membrane monocarboxylate transporters decreased K562 mortality caused by Fe- and Co-edds complexes, which implies that this transport route may be used for the internalization of these complexes. Free edds induced cell cycle arrest in G0/G1 (PBMC) and S (K562) phases, indicating DNA damage or interference with DNA replication.

## Experimental

**Abbreviations:** edds, S,S-ethylenediaminedisuccinic acid; PBMC, Peripheral Blood Mononuclear Cells; 4-HCA,  $\alpha$ -cyano-4-hydroxycinnamic acid.

### Reagents

The following reagents were used without further purification: MnSO<sub>4</sub>·H<sub>2</sub>O, FeSO<sub>4</sub>·7H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O, NiSO<sub>4</sub>·6H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, CoSO<sub>4</sub>·7H<sub>2</sub>O, edta disodium salt, nitrilotriacetic acid (nta), L-ascorbic acid, NaCl (Cromoline, Diadema, Brazil); Octaquest® E30 ([S,S]-edds trisodium salt; The Associated Octel Co., UK); Trolox, Chelex®,  $\alpha$ -cyano-4-hydroxycinnamic acid (4-HCA; Sigma-Aldrich, St. Louis, MO); N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Vetec, Rio de Janeiro, Brazil); 1,2,3-dihydrorhodamine dihydrochloride (DHR;

Biotium, Hayward, CA). Ascorbic acid and DHR were prepared as concentrated stocks of 8 mmol L<sup>-1</sup> in water or 50 mmol L<sup>-1</sup> in dimethyl sulfoxide, respectively, kept frozen as aliquots and thawed immediately before use. HEPES-Buffered Saline (HBS) was prepared with 20 mmol L<sup>-1</sup> HEPES, 150 mmol L<sup>-1</sup> NaCl, pH 7.4. Phosphate Buffer Saline (PBS) was prepared with 0.14 mol L<sup>-1</sup> NaCl; 2.6 mmol L<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O and 7.4 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, pH 7.4. When required, metal-free HBS was prepared by treating the solution with Chelex® resin (10 mg mL<sup>-1</sup>).

### Instruments

Spectra were recorded using a Shimadzu UV-1650PC (UV-Visible) or a Bomem MB-100 spectrophotometer in KBr pellets (infrared). Fluorescence measurements were performed in a Tecan GENios microplate reader (Tecan, Austria;  $\lambda_{exc}$  = 485 nm;  $\lambda_{em}$  = 535 nm) in 96-well microplates (TPP, Switzerland). Flow cytometry measurements were carried out using a FACS Calibur (Becton Dickinson, CA) with laser excitation at 488 nm.

### Complexes

M-edds (M = Mn<sup>2+</sup>, Fe<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup> or Zn<sup>2+</sup>) complexes were prepared once a week through the dissolution of the appropriate mass of metal salt in a 0.5 mol L<sup>-1</sup> edds aqueous solution to give a 1:1 metal:edds final stoichiometry. Stock solutions were not kept for longer than one week due to the development of mold.

### Pro-oxidant activity

The ability of edds to prevent the metal-catalyzed oxidation of ascorbic acid was assessed by a slight modification of a previously reported method.<sup>15</sup> Metal-nta stock solutions were prepared by mixing 100 mmol L<sup>-1</sup> nta (titrated to pH 7.0 with NaOH) with the appropriate mass of metal salt to produce a metal:nta molar ratio of 1:10. The objective of this procedure was to prevent metal hydrolysis by protecting the metal ion in a well-defined chelate. The nitrilotriacetic acid was chosen as the protective ligand because the formation constants of its metal complexes are lower than those of metal-edds analogues,<sup>16</sup> and thus the metal can be transferred to edds in the reaction medium.

The metal-nta solutions were further diluted in water to give a final metal concentration of 5  $\mu$ mol L<sup>-1</sup>. 20  $\mu$ L aliquots of these solutions were then transferred, in duplicate, to a microplate and mixed with 20  $\mu$ L aliquots of different edds solutions ([edds]<sub>final</sub> = 0, 2.5, 5, 10 or 20  $\mu$ mol L<sup>-1</sup>). The mixtures were then immediately mixed with to 180  $\mu$ L of

a fluorogenic solution consisting of 40  $\mu\text{mol L}^{-1}$  ascorbic acid and 50  $\mu\text{mol L}^{-1}$  DHR in metal-free HBS. Fluorescence reading was performed at 37°C for 40 minutes.

### Cell studies

*In vitro* studies were performed using K562 human leukemia cells cultured in complete RPMI-1640 medium (RPMI, 2% glutamax, 40 mg  $\text{L}^{-1}$  gentamicine, 10% heat-inactivated fetal calf serum) at 37 °C and 5%  $\text{CO}_2$ . Cell viability was determined through Trypan Blue counting. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors by centrifugation in a Ficoll-Paque<sup>TM</sup>PLUS gradient (GE Biosciences). Briefly, blood samples were diluted 1:1 with PBS and layered over Ficoll-Paque<sup>TM</sup>PLUS. After centrifugation at 900 $\times g$  for 30 minutes at 20 °C, PBMC were harvested at the interface, washed in PBS and immediately processed.

To assess the toxicity of the metal-edds complexes and the effect of serum proteins, triplicates of 1.0 mL of a cell suspension in either complete RPMI medium or PBS (*ca.* 10<sup>6</sup> cells  $\text{mL}^{-1}$ ) were transferred into a 24-well plate and treated with M-edds solutions at final concentrations of 0, 10, 100 and 1000  $\mu\text{mol L}^{-1}$  of M-edds. After 24 hours of incubation, cells were centrifuged at 500 $\times g$  for 3 minutes and resuspended in 50% Trypan Blue in PBS. Live and dead cells were counted.

The inhibition of cell monocarboxylate transporters (MCT) was assessed in a similar setup, except that cells were kept in PBS and treated with 10 mmol  $\text{L}^{-1}$  4-HCA for 24 h before the addition of 100  $\mu\text{mol L}^{-1}$  M-edds complexes (M = Fe, Ni, Co). Controls with no 4-HCA were kept under the same conditions. To assess the role of pro-oxidant activity in cell viability, cells were kept in PBS and treated with 100  $\mu\text{mol L}^{-1}$  of the antioxidant Trolox immediately before the addition of Fe-edds (final concentrations: 100 and 1000  $\mu\text{mol L}^{-1}$ ). Controls had no Trolox added. Analysis of variance (ANOVA,  $p < 0.05$ ) followed by group comparison using Fisher's LSD test (Protected

t-Test) were performed with GB-STAT<sup>®</sup> software (version 9.0; Dynamic Microsystems).

For the flow cytometry experiments, the cells (1.0 mL aliquots; 1.0 $\times 10^6$   $\text{mL}^{-1}$ ) were incubated in complete RPMI for 1 h at 37 °C and 5%  $\text{CO}_2$  in the presence of 10  $\mu\text{mol L}^{-1}$  M-edds or edds alone. Cells were then centrifuged and stained with propidium iodide (PI) in hypotonic fluorochrome solution (50  $\mu\text{g mL}^{-1}$  PI in 0.1% sodium citrate, 0.1% Triton X-100) and PI fluorescence was measured by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA). Data were acquired using CellQuest (BD, CA) and analyzed with FlowJo (TreeStar, CA) software. The percentage distribution of the cell population among the different phases of the cell cycle was calculated using the Watson Pragmatic method (G2 defined as 2 $\times$ G1).

## Results and Discussion

### Complexes

The spectroscopic data indicated that edds forms stable, hexadentate complexes with most transition metals. The infrared analysis of the crystals obtained by recrystallization (Table 1) showed the characteristic displacement of the asymmetric carboxylate stretching to lower frequencies for the complexes, as previously established,<sup>17,18</sup> implying that this group is involved in the coordination to the metal. UV-visible measurements of aqueous solutions of  $[\text{M}(\text{H}_2\text{O})_6]^{2+}$  and M-edds (M = Co, Ni, Cu;  $[\text{M}] = [\text{edds}] = 5 \mu\text{mol L}^{-1}$ ) were also consistent with the formation of complexes, with the displacement of the absorption maxima ( $\lambda_{\text{abs}}$ ) to 492 nm (Co-edds<sup>19</sup>), 369 and 583 nm (Ni-edds<sup>20</sup>) and 670 nm (Cu-edds<sup>21</sup>). Upon oxidation to Fe<sup>III</sup> during complex formation, Fe-edds solutions turn yellowish-brown and display a small shoulder at *ca.* 490 nm.

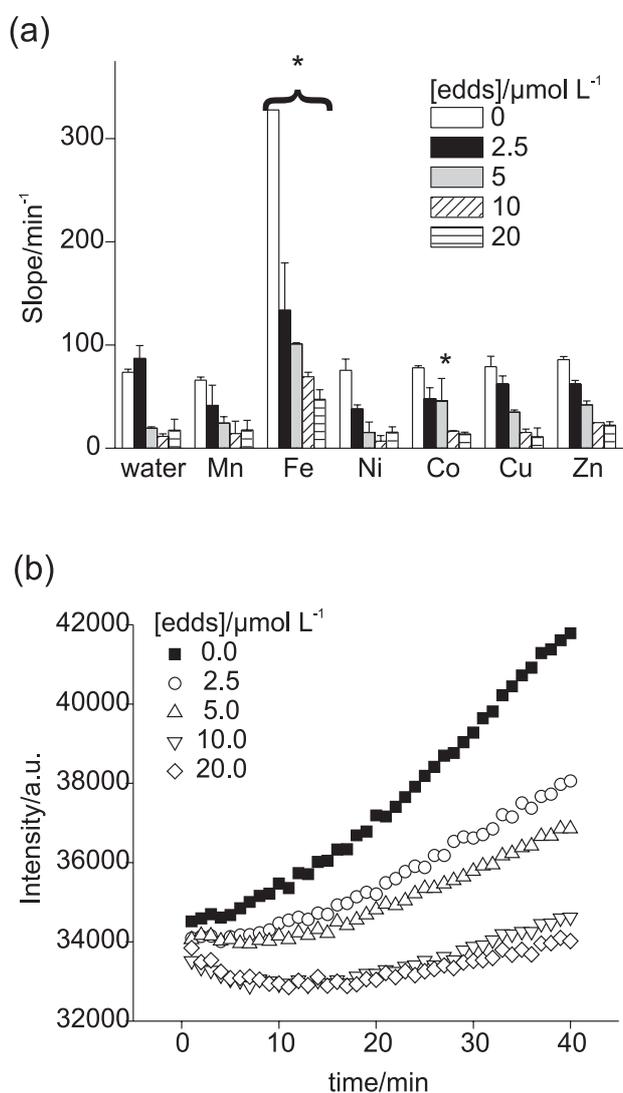
### Pro-oxidant activity

The DHR probe (non-fluorescent in its reduced form) undergoes unspecific oxidation and develops fluorescence

**Table 1.** Main infrared absorptions ( $\text{cm}^{-1}$ ) for metal-edds complexes

Complex	Literature <sup>18</sup>			Present study		
	$\nu(\text{C-O})_{\text{as}}$	$\nu(\text{C-O})_{\text{s}}$	$\nu(\text{C-N})$	$\nu(\text{C-O})_{\text{as}}$	$\nu(\text{C-O})_{\text{s}}$	$\nu(\text{C-N})$
Mn <sup>II</sup> -edds	1630, 1590	1400	1060, 1030	1630	1394	1032
Fe <sup>III</sup> -edds	1635	1400	1080, 1060	1635	1391	1060, 1106
Co <sup>II</sup> -edds	-	-	-	1609	1395	1061, 1107
Ni <sup>II</sup> -edds	1680, 1570	1410	1115, 1085	1603	1403	1056, 1112
Cu <sup>II</sup> -edds	1610	1400	1115, 1090	1608	1396	1063, 1108
Zn <sup>II</sup> -edds	1630, 1595	1400	1060, 1035	1605	1400	-

in an oxidant concentration-dependent manner. In our experimental setting, the slope of a kinetic fluorescence curve is directly dependent on the amount of free radicals produced during the metal-catalyzed oxidation of ascorbic acid in the reaction media. Metal ions, such as  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$ , are known to catalyze this reaction<sup>22,23</sup> when in their free form, but a plethora of chelators might decrease their activity provided that they can bind to the metal through available coordination sites.<sup>24</sup> Thus, we aimed to verify whether edds might behave as an antioxidant in this metal-promoted oxidation by removing the metal through the formation of a stable chelate.



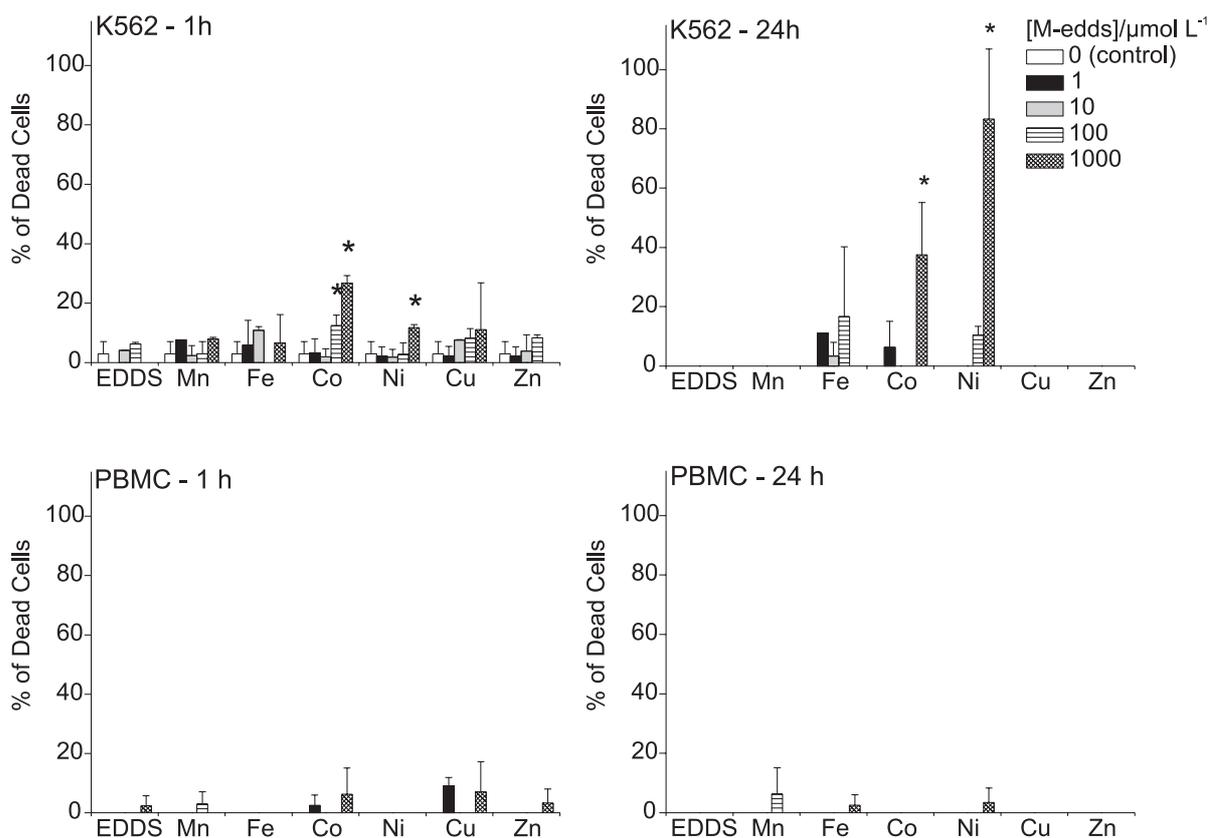
**Figure 1.**  $\mu\text{mol L}^{-1}$ . Asterisks indicate significant differences from the control (water) according to analysis of variance (ANOVA,  $p < 0.05$ ) and comparison of the groups using Fisher's LSD test (Protected t-Test). (b) Example of a typical fluorescence kinetic curve. The figure depicts fluorescence intensity (in arbitrary units) versus time for Fe (5  $\mu\text{mol L}^{-1}$ ) in the presence of different concentrations of edds. The slopes were calculated from  $t = 15$  min onwards.

We observed that, except for Fe, all tested metals behaved similarly to the control with respect to the decrease in the rate of ascorbate oxidation versus [edds] (Figure 1). Traces of iron present in the buffer may be responsible for the apparent "redox activity" of water alone, which subsided with increasing [edds]. However, this should not exclude the occurrence of important redox reactions promoted by other cations, since the experimental setting was optimized for clinical evaluation of redox-active iron.<sup>15</sup> As expected, the Zn-edds complex did not show any redox activity.

"Free" iron displays high redox activity (Figure 1). Interestingly, iron-catalyzed ascorbate oxidation was not totally suppressed, even at a four-fold molar excess of edds. Two important conclusions might be drawn from this observation. First, edds is not an antioxidant *per se*. Second, iron chelates such as Fe-edta, which present at least one free coordination site, are known to promote the formation of reactive oxygen species.<sup>24</sup> Indeed, Fe-edta is an excellent catalyst of ascorbate oxidation.<sup>25</sup> Our observations indicate that this is also the case for Fe-edds, since  $\text{Fe}^{3+}$  tends to form less stable bonds with relatively soft bases like the N atoms of edds, which implies that these groups are readily replaced by solvent molecules, the metal remaining redox-active. On the other hand, Kovaleva *et al.*<sup>18</sup> identified a peak at  $1725 \text{ cm}^{-1}$  attributed to the  $\nu(\text{C}=\text{O})_{\text{as}}$  of a free carboxylic group in Fe-edds. They suggested that the nitrogen atoms, three oxygen atoms from the carboxyl groups and one water molecule form the coordination sphere of  $\text{Fe}^{3+}$  in Fe-edds. We did not identify this particular stretching frequency in our experiments (Table 1). Kovaleva's model also explains the persistence of available positions (the water molecule) through which ascorbate could interact with Fe and initiate a cascade of oxidation. It should be noted that complete blocking of all available coordination sites, in order to prevent unwanted redox side-reactions *in vivo*, is one of the requisites for molecules employed in iron chelation therapy.<sup>15</sup>

#### Cell studies

Initially, we compared the effect of the incubation time of M-edds complexes on K562 and PBMC viability (Figure 2). The tumor line was less resistant to the toxic effects of some of the complexes (Fe-edds, Co-edds and Ni-edds) after 24 h when compared to PBMC. In fact, virtually no toxic effects were observed for non-tumoral ex-vivo cells even at high M-edds concentrations and prolonged exposure. Edds is known to capture  $\text{Zn}^{2+}$  in biological media,<sup>2</sup> therefore it can compete with enzymes for this metal and induce loss of enzymatic functions. Curiously, free edds did



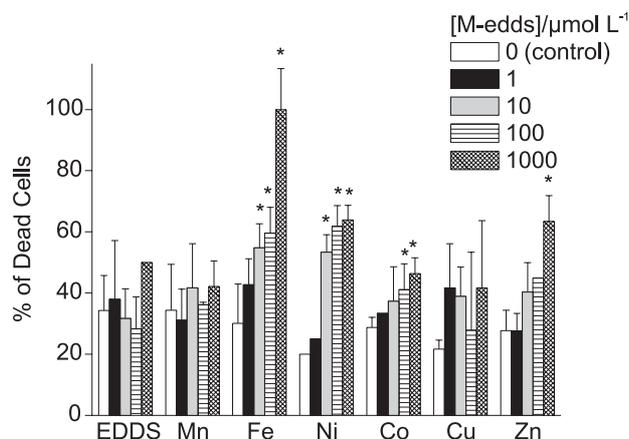
**Figure 2.** Cytotoxicity of M-edds complexes to K562 and PBMC cells after 1 and 24 h incubation (mean  $\pm$  S.D.) in complete RPMI medium. Asterisks indicate significant differences from the control according to analysis of variance (ANOVA,  $p < 0.05$ ) and comparison of the groups using Fisher's LSD test (Protected t-Test). The Trypan Blue exclusion test was used to evaluate cell viability.

not show high toxicity to the cells (Figure 2), indicating that the toxicity of M-edds complexes, when present, depends mainly on the nature of the metal being transported into the cell. Iron, cobalt and nickel complexes were the most toxic to K562 cells, albeit at the relatively high concentrations of 100 and 1000  $\mu\text{mol L}^{-1}$ . This observation supports previous claims that edds is a useful chelator to reduce metal contamination risks,<sup>1</sup> but at the same time indicates that some of the M-edds complexes could be effective metallodrugs for tumor chemotherapy.

Since the toxicity of metallodrugs *in vivo* is critically determined by the outcome of their interactions with plasma proteins,<sup>26</sup> and serum deprivation may stress cells leading to higher susceptibility to toxicants and apoptosis, we conducted a set of experiments to assess the toxicity of M-edds complexes to K562 in the presence and absence of serum (Figure 3).

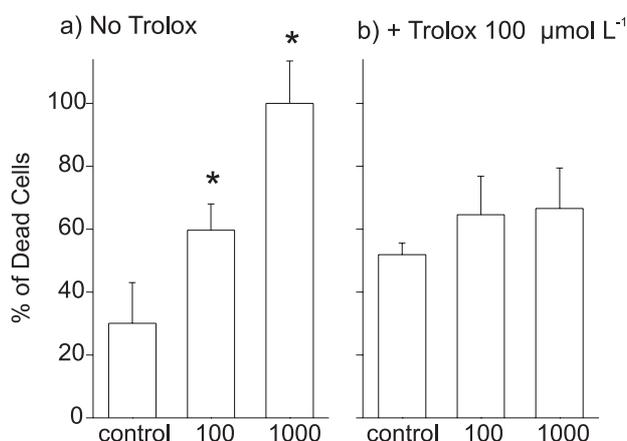
As anticipated, all control groups displayed higher mortality when treated with PBS only (compare right upper panel of Figure 2 with Figure 3). Also, Zn-edds was more toxic under this condition than in the presence of the culture medium. Interestingly, Fe-edds displayed a greatly

increased rate of mortality in the absence of serum. Proteins such as albumin have extensive antioxidative effects *in vivo*, and we previously noted (Figure 1) that Fe-edds is



**Figure 3.** Cytotoxicity of M-edds complexes to K562 cells after 24 h incubation in PBS (mean  $\pm$  S.D.). Asterisks indicate significant differences from the control according to analysis of variance (ANOVA,  $p < 0.05$ ) and comparison of the groups using Fisher's LSD test (Protected t-Test). The Trypan Blue exclusion test was used to evaluate cell viability.

an effective pro-oxidant of ascorbate (and, presumably, of other biological substrates). Therefore, we hypothesized that the observed decreased viability of cells treated with Fe-edds in PBS might be the result of an oxidative aggression towards the cells, as observed by other researchers for other iron complexes.<sup>27,28</sup> To test this point, we conducted a comparative study on the viability of cells challenged with Fe-edds, using cells which had been treated or not treated with the antioxidant Trolox in the absence of serum (Figure 4). The presence of the antioxidant suppressed toxicity even at the highest concentrations of Fe-edds.

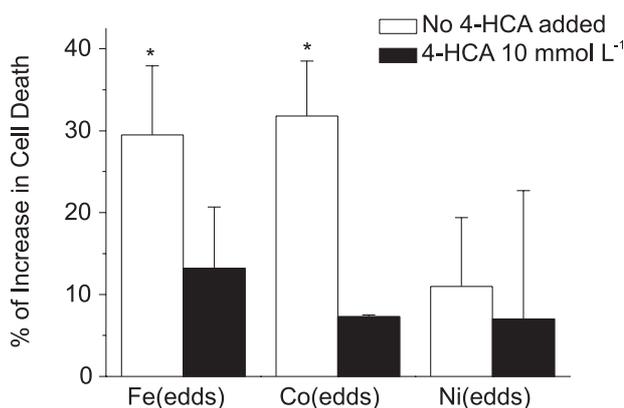


**Figure 4.** Effect of 100 µmol L<sup>-1</sup> Trolox on K562 cell viability after treatment with 100 µmol L<sup>-1</sup> and 1000 µmol L<sup>-1</sup> (mean ± S.D.) of Fe-edds in PBS. Asterisks indicate significant differences from the control according to analysis of variance (ANOVA,  $p < 0.05$ ) and comparison of the groups using Fisher's LSD test (Protected t-Test). The Trypan Blue exclusion test was used to evaluate cell viability.

To gain some insight into the mechanisms of biological action, the cell toxicity studies in the absence of serum were repeated in the presence and absence of 4-HCA, a known inhibitor of membrane monocarboxylate transporter (MCT) proteins.<sup>29-31</sup> This family of proteins is involved in the transport of short chain carboxylates (pyruvate, lactate), ketone bodies and hormones through several biological interfaces.<sup>32</sup> This test was not performed with cells grown in complete RPMI due to the fact that, as previously observed, cell toxicity in this case was difficult to observe.

We observed (Figure 5) that 4-HCA-treated cells resisted Fe- and Co-edds toxicity. Ni-edds did not show a similar recovery, probably because the concentration was too high to verify the role of MCT blocking. This indicates that membrane monocarboxylate transporters may be required for the internalization of these metal complexes. Our results do not rule out other internalization routes such as passive diffusion. However, for the anionic M-edds complexes, passive diffusion through cell membranes is unlikely, since none of these complexes showed a detectable

octanol-water partition (data not shown). A previous report accounted for the facilitated transport of aluminum citrate by MCT1, suggesting that the transporter would recognize a free citrate carboxylate.<sup>33</sup> However, it should be noted that potential metal ligands, such as acetoacetate, which lack this free carboxylic acid motif, are recognized MCT substrates.<sup>32</sup> To our knowledge, the present work is the first report of complexes lacking a free carboxylic function in the chelating agent being transported by MCTs.



**Figure 5.** Increase of K562 mortality in relation to controls promoted by 100 µmol L<sup>-1</sup> M-edds (M= Fe, Co, Ni) in the presence or absence of 10 mmol L<sup>-1</sup> 4-HCA (mean ± S.D.) in PBS. Asterisks indicate significant differences from the control according to analysis of variance (ANOVA,  $p < 0.05$ ) and comparison of the groups using Fisher's LSD test (Protected t-Test). The Trypan Blue exclusion test was used to evaluate cell viability.

The mechanisms by which metal ions cause cell cycle arrest are not fully understood, since cell cycle regulation is a complex process with several steps, which is prone to interference by contaminants. Induction of apoptosis in tumor cells is an important strategy for the development of improved platinum-based metallodrugs. However, the biological response to these drugs seems to be dependent on both the cell line and the metal complex structure.<sup>34</sup> For instance, vanadium compounds have been shown to induce both apoptosis and S phase arrest<sup>35</sup> depending on the experimental model. Exogenous chelators also interfere with the cell cycle, the best known examples coming from studies of siderophores employed in iron chelation therapy, which may exert useful antitumoral effects by deactivating the metalloenzyme ribonucleotide reductase and inducing G1/S arrest.<sup>36</sup>

As with the previous results (Figure 2), we observed that cell mortality (apoptosis) was higher for K562 cells irrespective of the treatment (Figure 6 and Electronic Supplementary Information), which indicates that at least some tumor cell lines are less capable than normal cells of coping with the stress presented by the metal complexes.

In both K562 and PBMC, the free acid edds induced a significant phase-specific arrest (G0/G1 in PBMC and S in K562). K562 cells treated with Mn-edds showed a decreased population in the G2 phase as compared to the control, which may be due to the slightly increased number of dead cells and/or cells in other phases.

Leukemic cell lines such as K562 are susceptible to a number of inducers of apoptosis, and a fraction of terminally differentiated cells undergo apoptosis during maturation as well.<sup>37</sup> Our data corroborate these findings, in the sense that K562 cells were more sensitive to M-edds toxicity than PBMC cells in all cases.

The G1 checkpoint controls the progression through the cell cycle by checking for cell size, presence of growth factors, presence of nutrients and DNA damage. Progression to the S phase occurs after proteins of the Rb family are phosphorylated and unbound from E2F transcription factors in the DNA molecule. Cells with damaged DNA are prevented from progressing to the S phase by the p53

protein which, among other roles, blocks Rb phosphorylation.<sup>38</sup> Our results suggest that edds alone, and possibly Mn-edds, might either induce direct DNA damage or block p53 activity in K562 leukemic cells. Further experiments, *e.g.* an evaluation of nucleotide excision fragments or impaired expression of the p53 protein, and the role of edds in relation to zinc-finger transcription factors, should be performed in order to uncover the precise mechanism of action of these metal complexes.

## Conclusions

Biodegradable ethylenediamminedisuccinic acid (edds) has been proposed as an environmentally friendly alternative to persistent edta for heavy metal remediation and decontamination. The biological activities studied here suggest that this acid is not toxic *per se* to human cells, and the toxicity of M-edds complexes is dependent upon the nature of M. The toxicity of Fe-edds may, in part, be due to its ability to induce redox reactions, although other models of oxidative damage (lipid peroxidation, DNA damage) should be investigated. Monocarboxylate transporters may be involved in the internalization of M-edds complexes. In our study, M-edds complexes also displayed some anti-proliferative activity, which should be explored further for the development of new metallodrugs for cancer chemotherapy.

## Acknowledgements

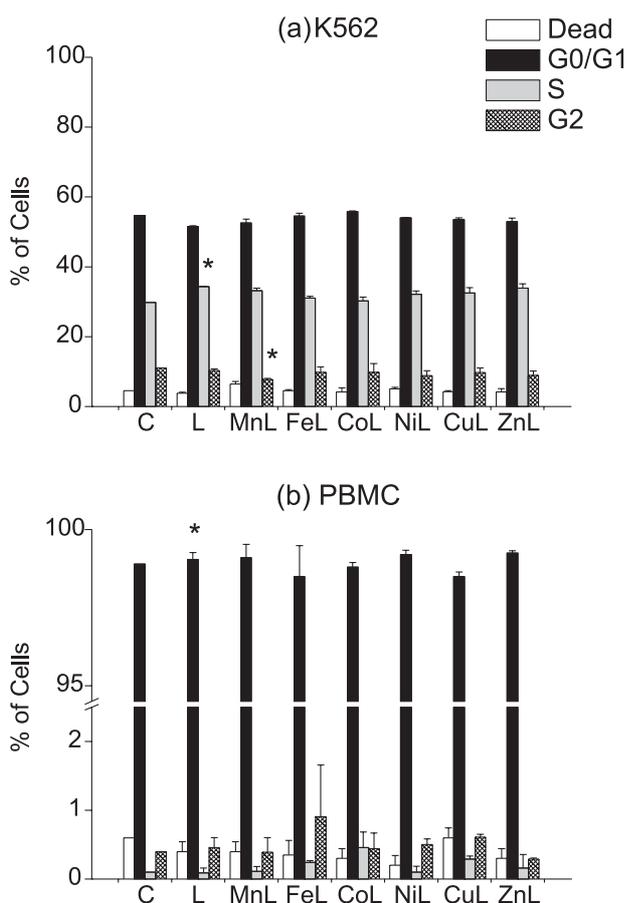
This research was funded in part by CNPq (Brazilian Council for Scientific and Technological Development) and FAPESP (São Paulo State Council for Scientific Research). Octel Performance Chemicals kindly provided us with a sample of edds. Dr. Heraldo Possolo de Souza (School of Medicine, University of São Paulo) kindly permitted the use of the fluorescence microplate reader.

## Supplementary Information

Cell cycle arrest induced by different M-edds complexes of K562 and PBMC cells after 1 h treatments (mean  $\pm$  S.D.) in complete RPMI medium. C = control; L = edds; [edds] = [M-edds] = 1, 100 and 1000  $\mu\text{mol L}^{-1}$ . Supplementary data are available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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**Figure 6.** Cell cycle arrest induced by different M-edds complexes of K562 and PBMC cells after 1 h treatments (mean  $\pm$  S.D.) in complete RPMI medium. C = control; L = edds; [edds] = [M-edds] = 10  $\mu\text{mol L}^{-1}$ . Asterisks indicate significant differences from the control according to analysis of variance (ANOVA,  $p < 0.05$ ) and comparison of the groups using Fisher's LSD test (Protected t-Test).

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