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RESEARCH ARTICLE

Ethylenediamine tetra acetic acid (EDTA) enhances the antitumor efficacy of cisplatin against human breast cancer cells *in vitro*

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ABSTRACT

Background: Ethylenediamine tetra acetic acid (EDTA) is used in several biomedical applications. **Aim**: The aim of this study was to investigate the effect of EDTA treatment on anticancer efficacy of cisplatin (Cis) against human breast cancer (MCF-7) cells *in vitro*. **Materials and Methods:** MCF-7 cells were treated either with Cis, EDTA, or their combination for 24h *in vitro*. The percentages (%) of the inhibitory, and the median inhibitory concentration (IC_{50}) of EDTA were determined by MTT assay. The % of Cis and EDTA on early and late apoptosis, necrosis, and cell cycle of MCF-7 were assessed by flow cytometry. **Results:** Our data showed slight antitumor effects for EDTA *in vitro*. However, Cis/EDTA treatment increased the antitumor efficacy **of Cis** as evidenced by increasing IC_{50}, and the percentage of MCF-7 mortality. Cis/EDTA co-treatment also increased the % of apoptotic and necrotic MCF-7 cells post 24 h of treatment (26.57 and 16.28%, respectively). Furthermore, this co-treatment arrested MCF-7 cell cycle at G_0 phase (32.8%) and G_2/M phase (30.25%). **Conclusion:** Co-treatment of EDTA with Cis increased the anticancer efficacy of Cis.

Keywords: EDTA, Cisplatin, MCF-7 cells, In vitro, anti-tumor

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INTRODUCTION

Cancer remains one of the most challenging diseases worldwide. Unlimited efforts are to find new strategies for cancer treatment. Currently, the conventional chemotherapy considers the backbone of cancer treatment (Olga et al., 2021). Even though, chemotherapy is the best choice for treatment but drug resistance and side effects are still major problem post-treatment (El-Naggar et al., 2015). Repeated treatments with the same chemotherapeutic agent led to resistance of tumour cells, therefore, it become less responded to drug action, which in turn led to high mortality among cancer patients. To overcome this problem, treatment protocols have been modified to apply with different molecular targets to reduce tumour cells resistant and to minimize side-effects of chemotherapeutic agents.

The side-effects of chemotherapy remain a major concern for clinicians despite the improved efficacy and enhanced survival offered by modern treatments (Nurgali et al., 2018). Cisplatin (Cis) is one of the most potent chemotherapeutic agents that used in several cancers' treatment protocols (Cepeda et al., 2007). Treatment with Cis is accompanied with severe side effects on different vital organs including liver, heart, and kidneys (Chvetzoff et al., 1998; El-Sawalhi and Ahmed, 2014; El-Naggar et al., 2020). Finding new different approaches to enhance the antitumor effects of Cis and reduce its toxicity is necessary. Therefore, combinatorial therapies of Cis with other agents have been highly considered to provide synergistic effects, reduce toxicities, and decrease drug-resistance (Dasari and Tchounwou, 2014). For instance, Ibuprofen has been reported to accelerate the apoptotic effects of Cis (Endo et al., 2014).

Ethylenediaminetetraacetic acid (EDTA) is a chelating agent used in different biomedical and nutritional purposes. It could alter cell

membranes permeability enhancing absorption of some drugs (Tomita et al., 1996). It is used in heavy metal intoxication treatment (Myint et al., 2009). Furthermore, EDTA was used with other agents to improve therapeutic efficacies (Juzeniene et al., 2007). EDTA improve doxorubicin efficacy via preventing the damages promoted by reactive oxygen species (Hasinoff, 2006). EDTA showed promising in vitro anticancer activities against human cancer cell lines and reported to improve the antitumor efficacy of Cis on colonic cancer in rats (Feril et al., 2017). It has been reported that the treatment with EDTA alone did not show any antitumor activities against Ehrlich ascetic carcinoma (EAC) bearing mice (Song et al., 2014). Consistent with previous findings, treatment with liposomes loaded with EDTA alone did not show any antitumor effects, however, liposomes loaded with EDTA, and doxorubicin could significantly reduce drug toxicity without altering the antitumor activity (Song et al., 2014). EDTA co-treatment with the therapeutic dose of Cis did not alter its antitumor efficacy in vivo (El-Naggar and El-Said, 2020). Treatment with a combination of low dose of Cis and EDTA enhanced Cis antitumor efficacy in vivo and decreased its side effects on liver and kidney tissues (El-Naggar et al., 2019). EDTA administration enhances the antioxidant enzymes activity and decreases the hepatic inflammation and lipid peroxidation in carbon tetrachloride (CCl_4)-induced liver fibrosis (González-Cuevas et al., 2011).

Based on the above information, this study aimed to evaluate the impact of the co-treatment with EDTA on the antitumor efficacy of Cis in vitro on human breast cancer cell lines (MCF-7).

MATERIAL AND METHODS

Chemicals

Cisplatin (cis-diamminedichloroplatinum II) was purchased from the local pharmacy. It is manufactured by EIMC united pharmaceuticals, Egypt. At the time of treatment, Cis was diluted by 0.9% normal saline and adjusted to 2 mg/Kg b.wt in 200 µL. EDTA was purchased from Sigma-Aldrich company. Different concentrations of EDTA were prepared in 0.9% sterilized saline.

Cancer cell lines (MCF-7)

Human breast cancer cell line (MCF-7) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, BioWest, Nuaille, France), 100 U/mL penicillin, 100 mg/mL streptomycin, and 100 mg/mL glutamine at 37 ˚C in a humidified atmosphere containing 5% CO_2. Cells were sub-cultured after every two days.

In vitro cytotoxic effect by MTT assay

To determine the anticancer activity of EDTA alone or with Cis *in vitro*, MCF-7 cells were used. The inhibitory concentration that kills 50% of cells (IC_{50}) was determined by using MTT assay. Briefly, different concentrations (from 600 to 1.17 µM) of EDTA were applied in triplicate to the MCF-7 cells (at 70–80% confluent), and the wells were incubated, then, 10 µL of a 12 mM MTT stock solution [5 mg/mL MTT in sterile PBS saline] was added to each well. This was followed by incubation for 4h at 37˚C. The MTT solution was removed, and the purple formazan crystal formed at the bottom of the wells was dissolved with 100 µL of dimethyl sulfoxide (DMSO) for 20 min. Cis was used as a positive standard. The absorbance at 570 nm was read on an ELISA reader (StatFax-2100, Awareness Technology, Inc.). The concentration of inhibiting 50% of cells (IC_{50}) was calculated with the sigmoidal curve.

Determination of apoptosis and necrotic percentages

Briefly, to determine the apoptotic percentage 24 h post-treatment, MCF-7 cells were re-suspended in 1X binding buffer at a concentration of 3×10^6 cells/ml. Five µl of Annexin-V and 5 µl Propidium Iodide (PI) were added to 100 µl of cell suspension. At that time, the cells were gently shacked and incubated for 15 min at room temperature (25 ˚C) in the dark. Place 400 µl of 1X binding buffer were added and then analyzed by BD FACSCanto™ II flow cytometer.

Cell cycle analysis

Cell cycle analysis was determined as described according to Weir et al. (2007). MCF-7 cells (2

x10^4) were seeded and treated with IC_{50} of Cis, EDTA (300 uM) or with the combination of Cis/EDTA (300µM) for 24 h. Cells were then harvested and fixed overnight in 70 % cold ethanol at 4 °C. After washing with ice-cold PBS, the fixed-cell pellets were collected by centrifugation and re-suspended in PI/RNase staining Buffer, then analyzed on a flow cytometer. Cell-cycle was calculated using CELLQUEST software (Becton Dickinson Immuno-cytometry Systems, San Jose, CA).

Statistical analysis

One–way analysis of variance (ANOVA) was used to assess the significant differences among treatment groups. Dunnet test was used to compare all groups against the control group to show the significant effect of treatment. The criterion for statistical significance was set at p <0.05 or p <0.01. All data are presented as mean ± SD.

RESULTS

***In vitro* treatment with Cis or EDTA increased mortality % of MCF-7 cells in a concentration dependent manner**

Different concentrations of EDTA and Cis were prepared to test the viability and inhibitory percentages of MCF-7 cells post 24h of treatment *in vitro*. The results showed that the inhibitory % of MCF-7 was increased gradually with the increasing Cis or EDTA concentrations. MCF-7 cells were treated with different concentrations (0-600 µM). The results showed that the inhibitory concentration (IC_{50}) of Cis was 37.5 µM, while the IC_{50} of EDTA was 298 µM post 24h of treatment (Table 1 and Figure 1).

Treatment with a combination of EDTA/Cis increases the mortality % of MCF-7 cells *in vitro*

After the assessment of the IC_{50} of Cis and EDTA against MCF-7 cells *in vitro*, the mortality percentages of MCF-7 were determined post treatment with a combination of Cis (IC_{50}) and different concentrations of EDTA (150, 300, and 600 µM) for 24h *in vitro.* The results showed that the combination between Cis (IC_{50})/EDTA (150 µM) increased the percentage of MCF-7 mortality to 60%. Treatment with a combination of Cis (IC_{50})/EDTA (300 µM) increased the inhibitory % of cells to 73.39%, while the treatment with Cis (IC_{50})/EDTA (600 µM) increased the inhibitory % to 94.5% (Table 2).

Combinatorial treatment with EDTA/Cis increased the percentages of apoptotic and necrotic tumor cells

The percentage of apoptotic (early and late stages), and necrotic cells of MCF-7 were assessed post treatment with Cis (IC_{50}), EDTA (300 µM), and their combination for 24h. The results showed that the treatment with IC_{50} of Cis increased the percentages of apoptotic and necrotic MCF-7 cells post 24h of exposure to 16.46 and 9.72%, respectively. Interestingly, the treatment with a combination of Cis/EDTA increased the percentages to 26.57 and 16.28%, respectively, compared to their values post treatment with Cis or EDTA alone (Figure 2 and Table 3). Treatment with EDTA increased the percentage of apoptotic and necrotic tumor cells slightly when compared to untreated MCF-7 cells (Figure 2).

Treatment with EDTA/Cis arrested MCF-7 cell cycle at G_0 and G_2/M phases

The effect of the treatment with either Cis (IC_{50}), EDTA (300 µM) or their combination on cell cycle of MCF-7 were determined. The results showed that the treatment either with EDTA or Cis arrested MCF-7 cell cycle at G_0 and G_2/M phases. Treatment with EDTA or Cis increased G_0 phases from 1.64% in untreated cells into 11.72 and 26.1%, respectively. Treatments with EDTA alone or with Cis alone increased G_2/M from 6.44% in untreated cells into 15.14 and 25%, respectively. Treatment with a combination of Cis/EDTA, interestingly, increased G_0 phase into 32.8% and increased G_2/M phase to 30.25% (Figure 3).

DISCUSSION

Cisplatin (Cis) during treatment bind with DNA to form Cis–DNA adducts (Dasari and Tchounwou, 2014). EDTA is a metal chelating agent that able to remove heavy metals from the body (Myint et al., 2009). This study aimed to address the impact of EDTA treatment on Cis antitumor efficacy *in vitro*.

Table 1. The inhibitory percentages for MCF-7 cells post treatment with cisplatin or EDTA for 24 h *in vitro*.

Conc. (µM)	Inhibitory %	
	Cisplatin	EDTA
0	0	0
1.17	1.15±0.03	0
2.3	3.47±0.07	0.6±0.01
4.7	8.62±0.09	1.35±0.02
9.38	18.41±0.12	3.1±0.035
18.75	31.18±0.35	6.4±0.046
37.5	50.62±0.64	8.9±0.08
75	62.96±0.69	11.5±0.12
150	81.22±1.29	23.8±0.35
300	93.07±1.45	65.01±0.56
600	96.66±1.74	88.5±0.87

Table 2. The inhibitory percentages of MCF-7 cells post treatment with cisplatin (IC_{50})/$EDTA_1$ (150 µM), cisplatin (IC_{50})/$EDTA_2$ (300 µM), and *cisplatin* (IC_{50})/$EDTA_3$ (600 µM) for 24h *in vitro.*

Groups	Inhibitory %
Cisplatin/$EDTA_1$	60 ± 0.67
Cisplatin/$EDTA_2$	73.39 ± 0.75
Cisplatin/$EDTA_3$	94.57 ± 0.98

Table 3. Percentages of apoptosis (early and late), and necrosis of MCF-7 cells post treatment with cisplatin (IC_{50}), EDTA (300 µM), or their combination for 24h *in vitro.*

Condition	Percentage (%)		
	Early apoptosis	Late apoptosis	Necrosis
Untreated MCF-7 cells	0.49±0.03	0.07±0.005	1.08±0.078
Cisplatin (IC_{50})	2.54±0.15	13.92±1.18	9.72±0.91
EDTA (300 µM)	4.22±0.33	3.89±0.35	3.61±0.36
Cisplatin/EDTA (300 µM)	4.61±41	21.96±2.06	16.28±0.87

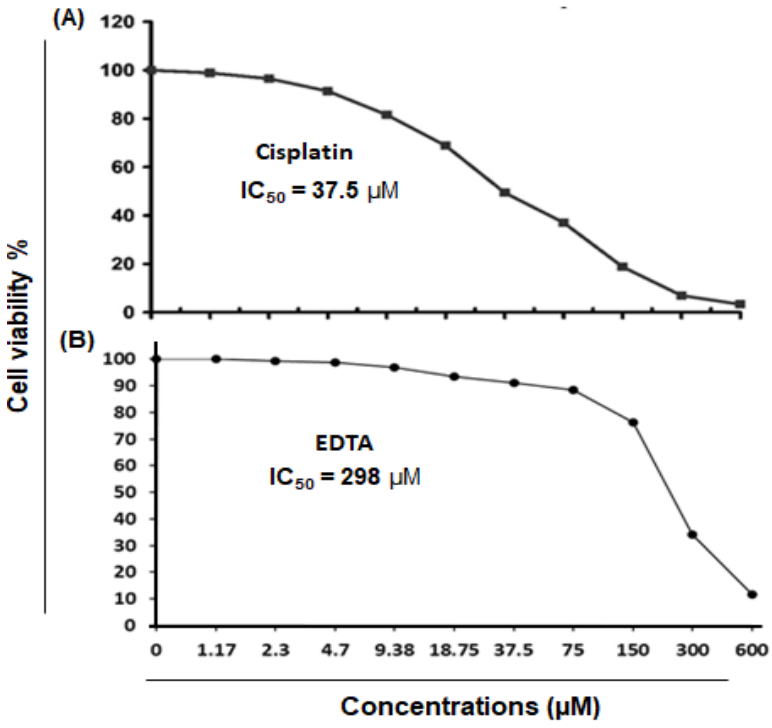

Figure 1. The IC_{50} of cisplatin **(A)** and EDTA **(B)** on MCF-7 cells post 24h of treatment *in vitro*.

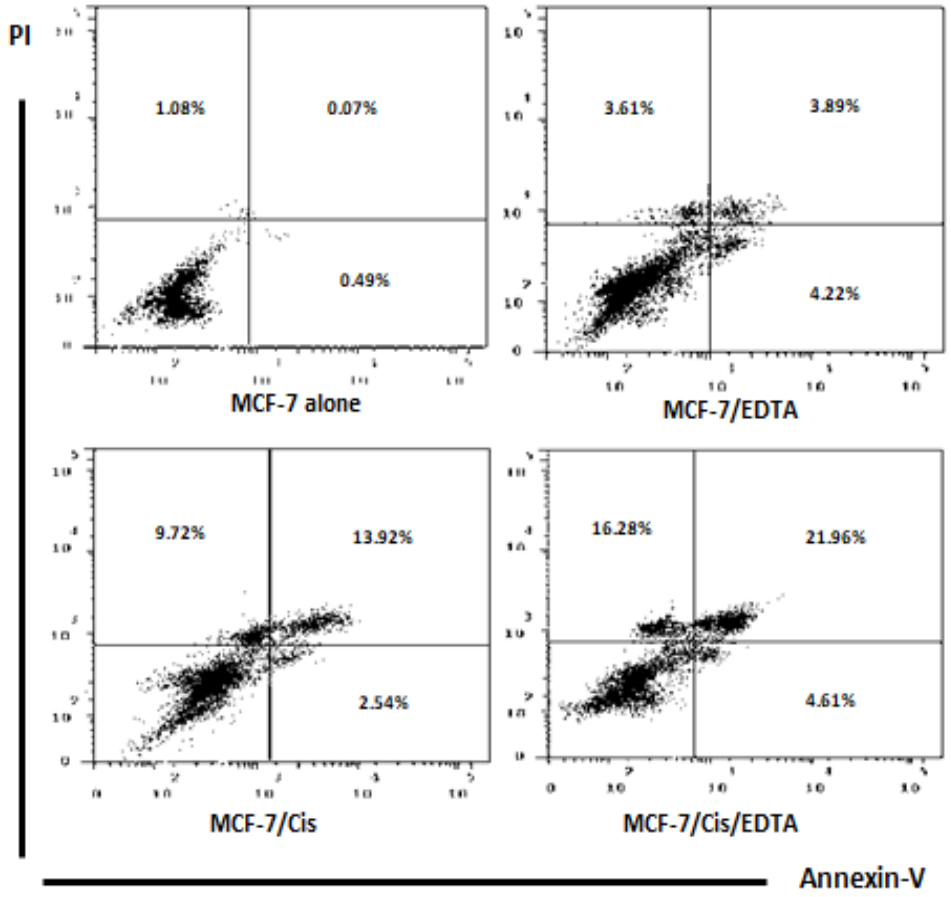

Figure 2. Treatment with Cis/EDTA increased the percentages of apoptotic and necrotic MCF-7 cells post 24h of treatment *in vitro*.

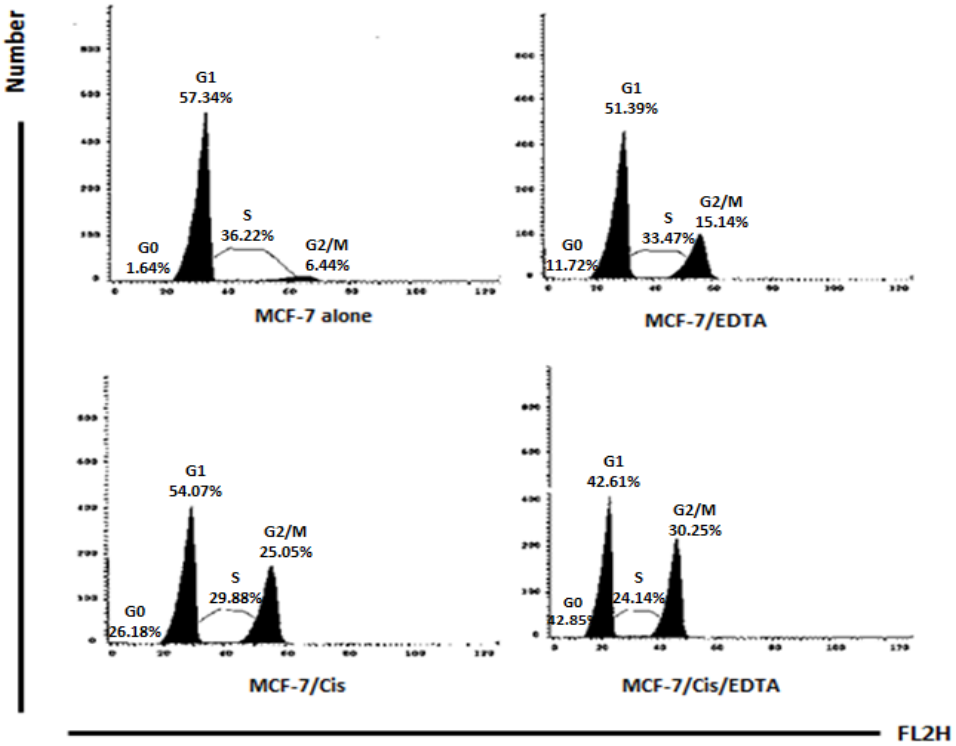

Figure 3. Cis/EDTA arrested G0 and G2/M phases in MCF-7 cell cycle post 24 h of treatment *in vitro*.

Treating MCF-7 cells with EDTA alone increased the percentage of the apoptotic and necrotic tumor cells but slightly than the untreated MCF-7 cells. The results showed that the treatment with EDTA alone showed slight antitumor activity against MCF-7-cells evidenced by slight increase in the percentages of their apoptosis and necrosis. This finding agreed with a previous study (Feril et al., 2017), which showed that EDTA revealed antitumor activity against several tumor cell lines *in vitro*. In contrast, previous *in vivo* study reported that EDTA alone is not a potential anticancer agent in Ehrlich ascitic carcinoma mice model (El-Naggar et al., 2019; El-Naggar and El-Said, 2020).

Treatment of MCF-7 cells with Cis, however, showed an increase in the percentage of apoptotic and necrotic cells post 24 h of exposure, which is in the same line with a previous report (Niknafs, 2011). The data showed that Cis or EDTA treatment increased the inhibitory percentage in a concentration dependent manner. The results demonstrated that the combinatorial treatment with EDTA/Cis significantly increased the percentages of apoptotic and necrotic cells more than their percentages when treated either with EDTA or Cis alone. The enhancement of the antitumor efficacy of the low dose of Cis upon co-treatment with EDTA could be due to an increase in the cellular permeability of EAC cells, which in turn could increase the effect of Cis. These results were in line with previous studies, which reported that EDTA could enhance the antitumor efficacy of low dose of Cis in Ehrlich ascetic carcinoma bearing mice by increasing the percentages of apoptotic tumor cells (Velma et al., 2016; El-Naggar et al., 2019; Liu et al., 2019).

Consistent with the effects on the apoptotic and necrotic percentages, EDTA or Cis induced MCF-7 cell cycle arrest on G_0 and G_2/M phases. EDTA treatment led to a slight arrest on the S-phase; however, Cis induced a significant arrest on G_0 and G_2/M phases. These findings are in agreement with previous reports (Plaimee et al., 2015; Velma et al., 2016; Liu et al., 2019). Interestingly, the combinatorial treatment led to an increase in the percentage of G_0 and G_2/M cell cycle phases when compared to their percentages upon treating MCF-7 either by EDTA or Cis alone. This finding clearly showed that EDTA could enhance the anticancer efficacy of Cis by increasing the mortality percentages of cancer cells by increasing the induction of apoptotic pathway and increasing cell cycle arrest at G_0 and G_2/M during cell division. In summary, these findings collectively indicated that the EDTA treatment may have a potential effect to enhance the chemotherapeutic effect of Cis in vitro against human breast cancer cell line (MCF-7).

CONFLICT OF INTEREST

All authors declare that they have no conflict of interests.

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