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Suppression of survival in human SKBR3 breast carcinoma in response to metal–chelator complexes is preferential for copper–dithiocarbamate

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Abbreviations:
Cu[DEDTC]$_2$, bis-[diethyl dithiocarbamate] copper(II)
Cu[8-OHQ]$_2$, bis-[8-hydroxyquinoline] copper(II)
Cu/ZnSOD, copper/zinc superoxide dismutase
H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate
MnSOD, manganese superoxide dismutase
ROS, reactive oxygen intermediates
Zn[DEDTC]$_2$, bis-[diethyl dithiocarbamate] zinc(II)
Zn[8-OHQ]$_2$, bis-[8-hydroxyquinoline] zinc(II)

ABSTRACT

Since diethyl dithiocarbamate (DEDTC) forms complexes with either zinc or copper, and 8-hydroxyquinoline (8-OHQ) also complexes with copper, we now compared the cytotoxic activity of Cu[DEDTC]$_2$, Zn[DEDTC]$_2$ and Cu[8-OHQ]$_2$. This report shows that at nanomolar levels, only copper–[DEDTC]$_2$, suppresses proliferation and clonogenicity of SKBR3 human breast carcinoma, concurrently with induction of apoptosis-associated PARP fragmentation. Susceptibility to these agents was paralleled by reactive oxygen generation (ROS) and greater expression of anti-oxidant enzymes like MnSOD and catalase, with no comparable effect on Cu/Zn superoxide dismutase. The lethal effects of Cu[DEDTC]$_2$ manifested when adding the two separate aqueous components or the preformed synthetic complexes in DMSO, was prevented by *N*-acetyl cysteine or glutathione, with no comparable protection afforded by non-thiol anti-oxidants like mannitol or DMSO. Exogenously added catalase also protected cells from Cu[DEDTC]$_2$, suggesting that this complex may kill after the levels of superoxide anion [$O_2^{\bullet -}$] dismutated by MnSOD increase hydrogen peroxide-related stress. Cu[DEDTC]$_2$ also induced p21WAF1, a cdk inhibitor usually not inducible in mutant p53 tumors like SKBR3 carcinoma, correlating with dephosphorylation of the Sp1 transcription factor. Concentrations of Cu[DEDTC]$_2$ cytotoxic for SKBR3 carcinoma did not induce comparable damage versus normal diploid human WI-38 fibroblasts. In contrast to the cytotoxic effect of nM levels of Cu[DEDTC]$_2$ against SKBRR3 cells, no response was seen in the same cells exposed to 20 μM *cis*-platin. Since neither DEDTC bound to zinc, nor copper bound to 8-OHQ showed comparable cytotoxicity, our results suggest that the greater activity of copper–DEDTC reflects a specific structure–activity relationship for the active complex. Since Cu[DEDTC]$_2$ shows more effectiveness than other metal–chelator complexes, it may be worth further investigation as an alternative to cancer therapies.

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1. Introduction

Apoptosis is important in normal development, since dysregulation in programmed cell death may cause or contribute to various diseases, including autoimmunity, or cancer [1]. Radicals play an important role in apoptosis, and both induction of apoptosis by reactive oxygen species (ROS) and ROS production by apoptotic cells have been described [2]. The anti-oxidant and radical scavenger pyrrolidine dithiocarbamate (PDTC) has been shown to regulate apoptosis in lymphocytes and tumor cell lines [2–4]. In human colorectal carcinoma, PDTC concentrations of 70 μM produced G1 accumulation and/or the appearance of a subG1 peak, suggesting that PDTC causes cell cycle arrest or apoptosis [4]. Others showed that as little as 25 μM PDTC reduced rat aortic smooth muscle cell viability by 25%, whereas 150 μM PDTC reduced viability by 73% [5]. Some mechanisms to explain the induction of apoptosis by dithiocarbamates is through their ability to chelate metals and raise their intracellular concentration [6], and by inhibiting the activation of the transcription factor NFκB [7] through a zinc requirement [8]. However, copper and zinc also seem to play an important role in dithiocarbamate toxicity since PDTC failed to reduce smooth muscle cell (SMC) viability in medium without Cu(2+) or Zn(2+), but addition of either Cu(2+) or Zn(2+) resulted in a dose-dependent increase in PDTC-induced cell death [8]. Moreover, both Cu(2+) and Zn(2+) showed synergistic effects, suggesting that the induction of apoptosis by PDTC requires Cu(2+) and Zn(2+) and is dependent on cell type [9]. Others recently demonstrated that PDTC induces cell death in vascular smooth muscle (SMC) cells, in serum-supplemented medium but not in serum-deprived conditions. However, addition of copper, iron and zinc restored the cytotoxic effect of PDTC. These data indicated that metals such as copper, iron, and zinc in serum may mediate the cytotoxic effect of PDTC [10]. With reference to other dithiocarbamates, diethyl dithiocarbamate (DEDTC) was used as an investigational agent to ameliorate chemotherapy- or radiotherapy-induced myelosuppression [11]. In mice, treatment with 300 mg/kg of DEDTC ameliorates myelotoxicity induced by several, but not all, chemotherapeutic agents [11]. As a mechanism for this amelioration, 30 μM DEDTC is proposed to hasten bone marrow recovery by augmenting stromal cell production of a factor(s) with hematopoietic colony-stimulating activity [12] and induction of cytokine release [13]. However, controversy exists regarding the toxicity of DEDTC, particularly their effect on the hematopoietic and/or immune system [13]. It has been reported that a 60-min pretreatment with 30 μM diethyl dithiocarbamate (DEDTC) did not inhibit colony formation in human CD341 bone marrow cells. However, 1 μM copper sulfate greatly potentiated the toxicity of DEDTC in myeloid progenitor cells. The increased susceptibility to DEDTC toxicity with the addition of copper was also evident in erythroid colony formation, but not until DEDTC levels reached 0.2 μM [14]. The various results demonstrate that dosing regimen, exposure times and metal concentrations drastically alter dithiocarbamate ability to protect some cells from apoptosis [11,12] or induce toxicity in others [9], highlighting the importance of experimental concentration of the dithiocarbamates and co-factors in the evaluation of the type of response. These findings are consistent with independent studies demonstrating that short-term incubation with DEDTC actually protects certain cell types from apoptosis. In more recent studies, it has been demonstrated that PDTC when bound to copper becomes effective in killing melanoma cells but not normal melanocytes [15]. In these studies, 0.17 μM disulfiram which originates DEDTC or DEDTC at the same concentration, showed highly selective and significant toxicity against A375 and c81–46a melanoma cells dependent on addition of 1 μM of $CuCl_2$ [15]. Moreover, the authors showed that the reaction of disulfiram and $CuCl_2$ in unbuffered aqueous solution directly affords the $Cu[DEDTC]_2$ complex in high yield, suggesting that the anti-melanoma active species is in fact attributable to the $Cu[DEDTC]_2$ complex. However, the same group showed that only melanoma cultures show markedly reduced viability compared with normal melanocytes [16]. More recently, studies against LN Cap prostate carcinoma, used a 1:1 complex of PDTC to copper, to show 50% inhibition of the prostate carcinoma cells at 1 μM and greater than 90% inhibition at 5–10 μM of the complex [17]. Since these various studies suggest a potentially useful effect of copper chelating complexes like dithiocarbamates in tumor suppression at low copper levels, and others have reported that different organic copper-containing compounds, such as 8-hydroxyquinoline [8-OHQ] copper(II), are effective tumor cell apoptosis inducers [18], we now investigated whether DEDTC complexes with copper or zinc are equally cytotoxic [16] or whether copper–DEDTC was comparably effective to copper–[8-OHQ] complexes [15,16,18]. Since earlier studies demonstrated activity of $Cu[DEDTC]_2$ complex against melanoma versus melanocytes [15,16], but most melanomas harbor wt p53 [19], like LNCap prostate carcinoma [17], we now used SKBR3 human breast carcinoma as target cells, since they harbor a dysfunctional tumor suppressor p53 with inactivating point mutations at codon 175 and also overexpress erbB2, parameters contributing to poor prognosis and low therapeutic response to anti-cancer drugs [20,21]. To compare the response of the more active combination versus SKBR3 cells with control normal cultures, we used WI-38 human diploid fibroblasts, known to be non-tumorigenic and to undergo early replicative senescence [22]. Since responses to dithiocarbamates may be linked to expression of anti-oxidant enzymes like MnSOD [23,24], Cu/ZnSOD [25] or catalase [26] or changes in the cyclin-dependent inhibitor p21WAF1 [27], and expression of all these molecules has been linked to the Sp1 transcription factor [28], whose transcriptional activity is up-regulated by dephosphorylation [28–30], we have also investigated whether differential expression of these anti-oxidant enzymes and p21WAF1 is linked to changes in Sp1 phosphorylation and metal–chelator specificity.

2. Materials and methods

2.1. *Preparation and characterization of $Cu[DEDTC]_2$, $Zn[DEDTC]_2$ and $Cu[8\text{-}OHQ]_2$ complexes*

Chelators like DEDTC or 8-OHQ were mixed with copper or zinc at a 2:1 chelator–metal mM ratio [15,18] and the resulting water-insoluble complexes were isolated by filtration, washed

and subsequently re-dissolved in DMSO. UV–vis spectra of the different compounds were obtained with a UV–vis Milton Roy 2000 spectrophotometer with a diode diffraction array. Since DEDTC or 8-OHQ and copper or zinc at μM levels were soluble in H_2O, we used mixtures of chelators like DEDTC or 8-OHQ and either copper or zinc at μM in H_2O for some assays for comparison with identical μM of the preformed DMSO complexes diluted 1:1000, for comparison with the corresponding controls in H_2O, or 0.1% DMSO, unless otherwise indicated.

2.2. Quantitative assesment of cytotoxicity

For this, we used fluorescent assays of relative cell viability/cytotoxicity with Alamar Blue (resazurin) which measures intracellular redox activity by quantitating the cell-catalyzed conversion of non-fluorescent resazurin to resorufin, which is highly fluorescent [31]. When added to a 10% final concentration after the appropriate treatment, the dye is non-toxic, allows fluorescent quantitation, permits re-use for further investigation such as morphological, biochemical and clonogenic analyses. As such, this assay is valuable as an endpoint of cytotoxicity, rather than as a kinetic measure for monitoring cell growth [31]. For this, cells (5–10,000) were allowed to adhere overnight in 96 well TC microtiter dishes. After the treatments indicated in each case, 10% Alamar Blue (BioSource, Camarillo, CA, USA) was added and fluorescence was measured 4 h later in a Fluoroskan Accent microplate reader with an excitation of 568 nm and an emission of 585 nm [31,32].

2.3. Immune blotting

Cell lysates were prepared and SDS-PAGE and bi-directional immune blots were done as previously described using 70 μg protein per lane [32] including prestained MW markers (Life Technologies, Gaithersburg, MD, USA) during SDS-PAGE electrophoresis to help identify specific changes in protein expression. After blocking nitrocellulose membranes with 5% skimmed milk in Tris-buffered saline pH 7.5 for 2 h at room temperature, membranes were reacted overnight with specific antibodies in the same blocking solution. For reprobing immune blots, these were incubated in stripping buffer (2% SDS, 62.5 mM Tris–HCl, pH 6.8, 0.1 M β-mercaptoethanol) for 30 min at 60 °C, followed by washing the membrane, re-blocking it and reaction with a new set of antibodies. Antibodies used for specific immune blotting included: PARP (SC-7150), p21WAF1 (SC-387), cyclin A (SC-239), NFκB p65 (SC-8008) were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibody to MnSOD Cat #06–984 and antibody to Cu/ZnSOD #06–482 were obtained from Upstate Biotechnology, Lake Placid, NY, USA. Antibody to catalase was kindly given by Dr. Larry Oberley, University of Iowa, and antibody to Sp1 was kindly provided by Dr. Stephen Jackson, Wellcome CRC Institute, Cambridge, England. After extensive washing with Tris-buffered saline containing 0.05% Tween 20, membranes were reacted with either anti-mouse IgG-peroxidase or Protein A-peroxidase, depending on whether primary antibodies were mouse or rabbit antibodies. Finally, detection was achieved by Super Signal-mediated chemiluminescence [32].

2.4. Detection of reactive oxygen species

Induction of reactive oxygen species (ROS) was evidenced in adherent cells incubated at different intervals with DEDTC and $CuCl_2$, washed in PBS and then exposed for 30 min to 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) in DMSO, obtained from Molecular Probes (Eugene, OR, USA). Cells were immediately visualized by fluorescence microscopy in a Nikon Eclipse TS 100F inverted microscope. In this assay, green fluorescence (495 nm/527 nm) is detected until the acetates in H_2DCFDA are removed by intracellular esterases and oxidation via ROS occurs within the cells [33].

2.5. Colony formation assay

Exponentially growing cells were seeded at 5000 cells per well in 96-well plates and allowed to attach for 18 h. Subsequently, metal complexes or the indicated reagents were added directly to cells (corresponding to a final DMSO concentration of 0.1%). Control plates received the same volume of DMSO alone. After 48 h, cells were rinsed, and drug-free medium was added. Cultures were transferred to 3.5 cm plates, observed daily for 10–15 days and then were fixed and stained with modified Wright–Giemsa stain (Sigma). Colonies of multiple cells were scored as survivors [34].

2.6. Densitometry

Fluorescent imaging of activity gels with Sypro Ruby and visible image capture of differential protein expression was achieved with a Fluor-S Imager (Bio-Rad) followed by quantitation of bands with the Gel-Pro software (Media Cybernetics, Silver Spring, MD).

3. Results

3.1. Neither Co, Fe or Zn potentiate the cytotoxic effect of DEDTC against SKBR3 cells

Since DEDTC is a metal chelator, our preliminary studies were devoted to find which metals potentiated the effect of DEDTC to a greater extent. We used DEDTC at 5 μM which is much lower than the 70 μM dithiocarbamate concentration found to be not cytotoxic for colorectal carcinoma cells [4], investigating the effect of adding 0.4 μM of $CoCl_2$, $FeCl_2$, $ZnCl_2$ and $CuCl_2$. For this assay, we used triplicates with 5000 cells of SKBR3 cells per 96 well plate and 36 h later quantitation of relative cytotoxicity was assessed by changes in Alamar Blue fluorescence (see Section 2). This revealed that neither DEDTC at 5 μM nor the metals used at 0.4 μM had any cytotoxicity. However, the only combination that potentiated the effect of DEDTC was $CuCl_2$, with no effect whatsoever of the other metals used (Fig. 1).

3.2. $Cu[DEDTC]_2$ is more effective than $Zn[DEDTC]_2$ or $Cu[8\text{-}OHQ]_2$ in suppression of SKBR3 carcinoma proliferation and survival at nM concentrations

Based on the prior experiments suggesting that only copper stimulated the DEDTC toxicity and considering a recent report

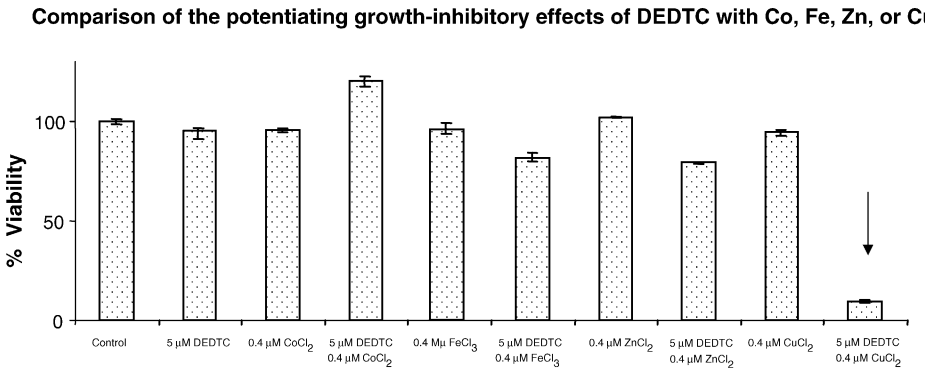

Fig. 1 – Comparison of the potentiating growth-inhibitory effects of DEDTC with Co, Fe, Zn or Cu. SKBR3 cells were seeded in triplicate 96 well plates for 20 h followed by a 48 h exposure to DEDTC, Co, Fe, Zn or Cu, either alone or together at the concentrations indicated in each case. Note that only the DEDTC–Cu combination was effective in significantly diminishing the relative viability estimated by Alamar Blue fluorescence [31]. Similar results were obtained in three different experiments.

showing that other organic copper complexes like Cu[8-OHQ]$_2$ were significant inducers of apoptosis, we compared the efficacy of Cu[DEDTC]$_2$, Zn[DEDTC]$_2$ and Cu[8-OHQ]$_2$, to define whether the chelator or the ligand was important in this toxicity. As expected, these various combinations yielded specific complexes, which changed depending on the metal–ligand combination, as shown by their specific UV spectra (Fig. 2). To monitor relative cytotoxicity, we used again Alamar Blue quantitative fluorescence [31,32]. Since others demonstrated that DEDTC–copper is active against melanoma at a 2:1 ratio [15], this ratio of chelator to metal was now used in our experiments. This revealed that only the aqueous mixture of

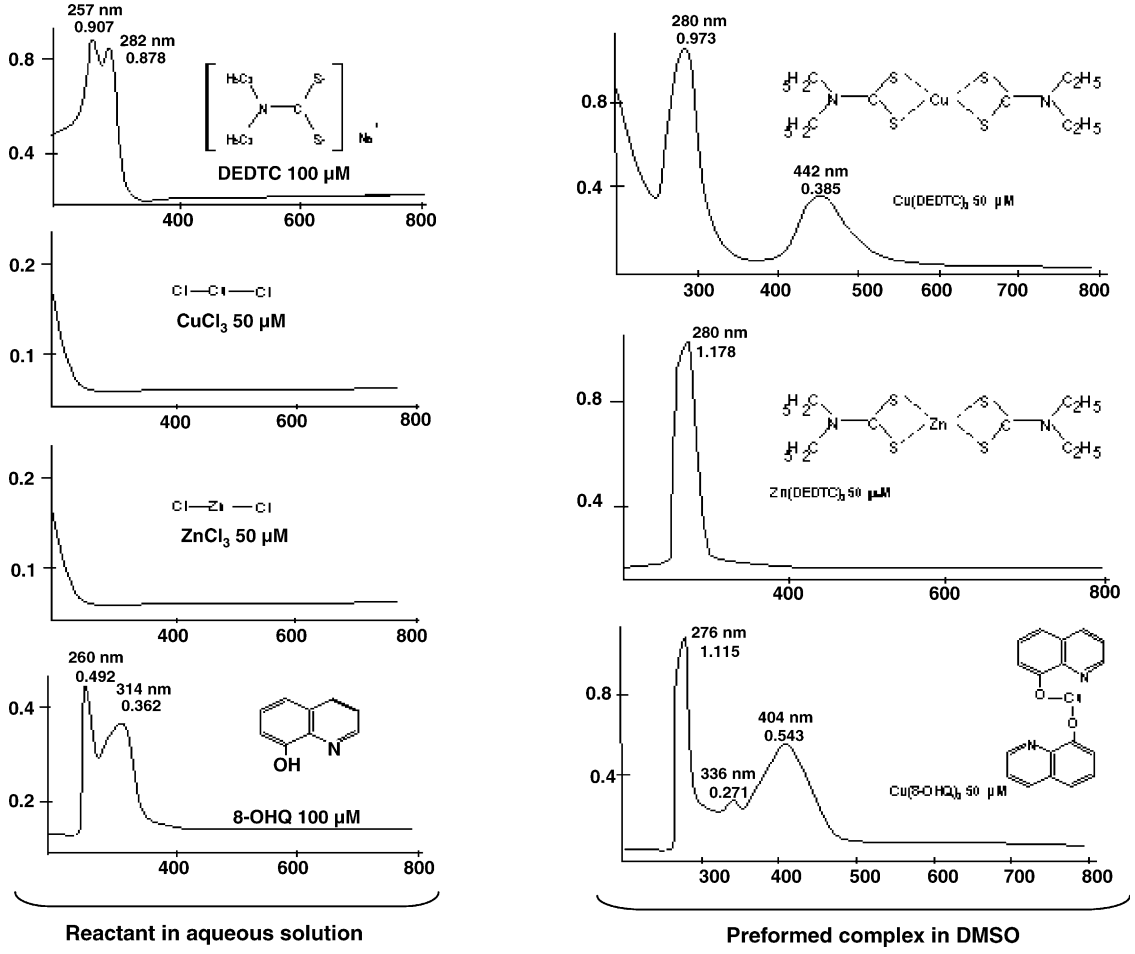

Fig. 2 – DEDTC, 8-OHQ form complexes with copper *Left*: UV–vis spectra of unreacted DEDTC, Cu, Zn and 8-OHQ. *Right*: Interaction between Cu[DEDTC]$_2$, Zn[DEDTC]$_2$ and Cu[8-OHQ]$_2$, showing spectral changes as a result of complex formation.

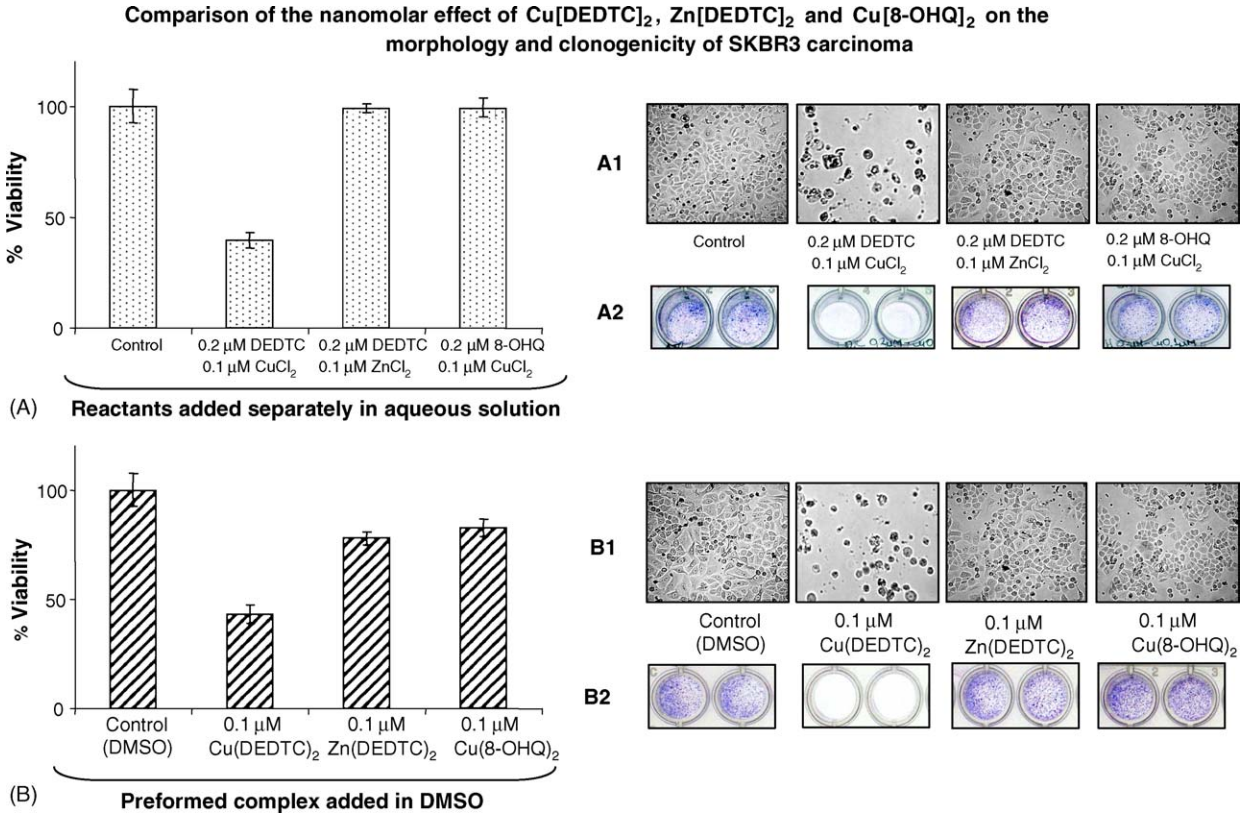

Fig. 3 – Comparison of the nanomolar effect of $Cu[DEDTC]_2$, $Zn[DEDTC]_2$ and $Cu[8\text{-}OHQ]_2$ on the morphology and clonogenicity of SKBR3 carcinoma SKBR3 carcinoma cells were seeded in triplicate 96 well plates for 20 h followed by a 48 h exposure to the indicated concentrations $Cu[DEDTC]_2$, $Zn[DEDTC]_2$ and $Cu[8\text{-}OHQ]_2$. Note in (A) and (B) Alamar Blue fluorescence data; (A1 and B1) morphological changes induced by the complexes, showing maximal cell damage in those treated with $Cu[DEDTC]_2$ in aqueous form or as a preformed complex, in agreement with the Alamar Blue studies; (B1 and B2), clonogenicity studies showing suppression of SKBR3 viability only in cells treated with $Cu[DEDTC]_2$. Similar results were obtained in four different experiments.

0.1 μM Cu plus 0.2 μM DEDTC was effective in producing cytotoxicity of SKBR3 cells (Fig. 3A). Moreover, when the same 2:1 ratio was used to isolate the respective complexes $Zn[DEDTC]_2$ and $Cu[8\text{-}OHQ]_2$, only 0.1 μM of the $Cu[DEDTC]_2$ was effective (Fig. 3B). To confirm that the fluorometric Alamar Blue assay was correctly reflecting cytotoxicity, this was also monitored morphologically (Fig. 3A1 and B1). This confirmed morphological damage only in SKBR3 cells exposed to the 2:1 DEDTC–copper combination (Fig. 3 A1 and B1). Moreover, clonogenicity assays further confirmed that only DEDTC plus copper as aqueous reactants or as preformed complexes were effective in suppressing colony formation by SKBR3 carcinoma (Fig. 3A2 and B2). In separate results, we confirmed that wt p53 C8161 melanoma and mut p53 WM164 melanoma were also affected by the copper–DEDTC combination (Table 1), which was clearly more cytotoxic than the zinc–DEDTC combination (not shown). Another noteworthy information was that when the different complexes shown in Fig. 2 were physically isolated, purified and redissolved in DMSO, they showed an efficacy similar to that seen when the reactants were added separately in the same ratio in an aqueous unbound form (Fig. 3).

3.3. *$Cu[DEDTC]_2$ induces p21WAF1 and catalase together with PARP cleavage*

To help define the mechanism of action of the copper–DEDTC combination, we initially asked whether it induced apoptosis-

Table 1 – Cytotoxic effect of DEDTC–Cu against different cell lines

Cell line	Inhibitory concentration 50 (IC50)	% Viability[a] ± S.D.
SKBR3 carcinoma mutant p53	0.2 μM DEDTC to 0.1 μM $CuCl_2$	40.43 ± 8.41
WM164 melanoma mutant p53	0.6 μM DEDTC to 0.3 μM $CuCl_2$	48.95 ± 4.66
C8161 LPC melanoma wt p53	0.4 μM DEDTC to 0.2 μM $CuCl_2$	44.06 ± 4.58
C8161 bcl-2 melanoma wt p53	0.8 μM DEDTC to 0.4 μM $CuCl_2$	54.7 ± 3.3

[a] Assays were carried out in triplicate measuring relative Alamar Blue fluorescence, as indicated under Section 2.

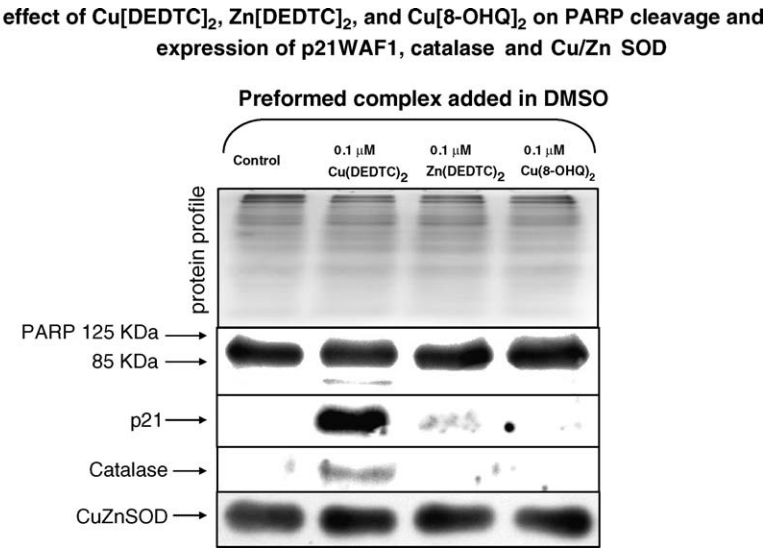

Fig. 4 – Comparison of the nanomolar effect of Cu[DEDTC]$_2$, Zn[DEDTC]$_2$ and Cu[8-OHQ]$_2$ on PARP cleavage and expression of p21WAF1, catalase and Cu/Zn SOD. SKBR3 carcinoma cells were seeded in 9 cm dishes for 20 h followed by a 48 h exposure to the indicated concentrations of the preformed complexes Cu[DEDTC]$_2$, Zn[DEDTC]$_2$ and Cu[8-OHQ]$_2$ dissolved in DMSO, adding the equivalent 0.1% amount of DMSO to control cells. Subsequently, cells were lysed and processed for immune blotting after bi-directional transfer to replicate nitrocellulose membranes [32]. Blots were reacted with antibody to catalase and antibody to p21, followed by addition of either anti-mouse IgG-peroxidase or Protein A-peroxidase, depending on whether primary antibodies were mouse or rabbit antibodies and detection was achieved by Super Signal-mediated chemiluminescence. Subsequently, signals were erased as indicated under Section 2 and reprobing was carried out with antibodies to PARP and antibody to Cu/ZnSOD. Note PARP cleavage concomitant with induction of catalase and p21 only in cells treated with Cu[DEDTC]$_2$. These results are representative of two different experiments.

associated PARP fragmentation [35]. This confirmed that in contrast to Cu–8-OHQ or Zn–DEDTC only the Cu–DEDTC combination induced PARP cleavage, concomitantly with induction of p21WAF1 (Fig. 4). This specific effect of copper–DEDTC was of interest since SKBR3 carcinoma is known to harbor a mutant p53 [20,21] and p21WAF1 is usually activated by a wt p53 [32]. To ask whether copper–DEDTC kills SKBR3 carcinoma by promoting oxidative imbalance requiring induction of catalase to try to overcome induction of peroxide [26], we reprobed the same blots. This showed that neither control cells nor those treated with copper–8-OHQ or Zn–DEDTC showed induction of catalase but this was clearly seen in cells treated with copper–DEDTC, which also show apoptosis-associated PARP cleavage [35] and induction of p21WAF1. No comparable induction was seen in Cu/Zn superoxide dismutase (Fig. 4).

3.4. *Joint induction of MnSOD and catalase without a comparable effect on Cu/Zn SOD by copper–DEDTC*

Cu/Zn superoxide dismutase-1 (SOD1) is an important enzymatic defense against oxidative stress that has been shown to be a potentially useful target for anti-cancer therapy. Because SOD1 uses both copper and zinc as co-factors and we showed that DEDTC forms complexes with both metals (Fig. 2), we postulated that it might have anti-tumor activity by virtue of chelating one or both of these metals and affecting the expression of Cu/Zn superoxide dismutase-1. To enquire about specificity, we also studied the corresponding changes

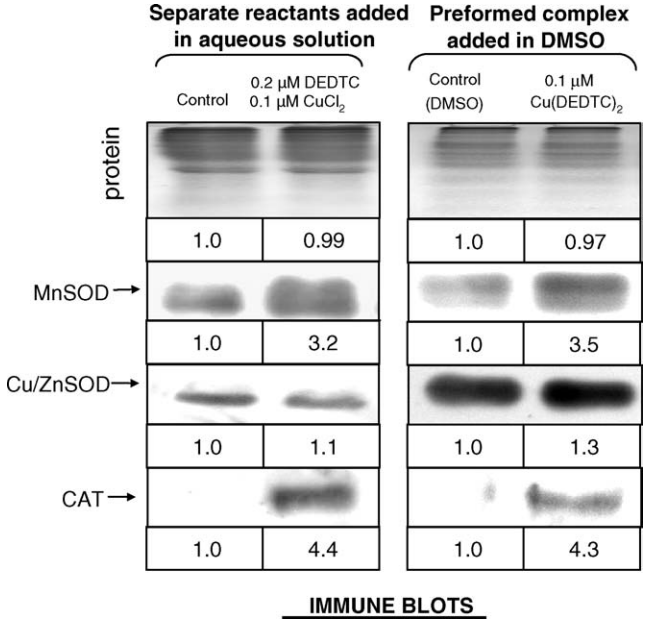

Fig. 5 – Induction of MnSOD and catalase expression is not paralleled by increases in Cu/ZnSOD following treatment with Cu[DEDTC]$_2$. SKBR3 carcinoma cells were seeded in 9 cm dishes for 20 h followed by a 48 h exposure to either DEDTC and copper in a 2:1 ratio or to the preformed complexes Cu[DEDTC]$_2$ at a similar ratio. Subsequently, cells were harvested, lysed and assayed as indicated in the caption for Fig. 4, for immune blots. Note increases in MnSOD and CAT (catalase) in cells treated with Cu[DEDTC]$_2$.

in mitochondrial MnSOD and in catalase. These results revealed no effect of either the preformed $Cu[DEDTC]_2$, complex or the aqueous separate copper–DEDTC reactants on expression of Cu/ZnSOD. In contrast, mitochondrial MnSOD and catalase expression were significantly increased by these treatments (Fig. 5).

3.5. *Anti-oxidant specificity in protection from copper–DEDTC*

Since the induction of anti-oxidant enzymes like catalase and MnSOD suggested that copper–DEDTC was suppressing SKBR3 carcinoma survival by inducing oxidative stress, we investigated whether this was counteracted by either a thiol anti-oxidant like *N*-acetyl cysteine [32] and the hydroxyl ($OH^{\bullet}$) radical scavengers DMSO [36,37] or mannitol [38,39]. Studies measuring relative cytotoxicity by Alamar Blue fluorescence included copper–DEDTC which proved cytotoxic, *N*-acetyl cysteine and reduced glutathione which protected from copper–DEDTC, and mannitol or DMSO which did not protect from copper–DEDTC (Fig. 6A). These findings were essentially confirmed by clonogenicity studies showing that only thiols like *N*-acetyl cysteine protected from copper–DEDTC (Fig. 6B). Immune blotting confirmed that copper–DEDTC induced apoptosis-associated PARP fragmentation, with no comparable effect seen when cells were pretreated for 2 h with *N*-acetyl cysteine (Fig. 6C).

3.6. *ROS increase in apoptosis induced by copper–DEDTC*

Since the prior results (Fig. 6) suggested the mediation of ROS in the initiation of events leading to apoptosis mediated by copper–DEDTC, this was investigated by fluorescence microscopy with living cells at early stages of treatment, using H_2DCFDA [33], as indicated under Section 2 [33]. This revealed very low green fluorescence in control cells (Fig. 7, upper left), in spite of the significant number of cells in this field (Fig. 7, upper right). However, by 4 and 7 h of treatment with copper–DEDTC, cells progressively increased their green fluorescence, indicative of greater ROS production (Fig. 7, lower left and right) [33].

3.7. *Exogenous catalase protects SKBR3 cells from apoptosis induced by copper–DEDTC*

To examine whether exogenous peroxide may be responsible for cytotoxicity induced by either the preformed $Cu[DEDTC]_2$ complex or the aqueous separate copper–DEDTC reactants, we used exogenous catalase which is a dimeric zinc metalloenzyme with total molecular weight of 52.296 g/mol. The presence of catalase in the growth medium has been associated with an

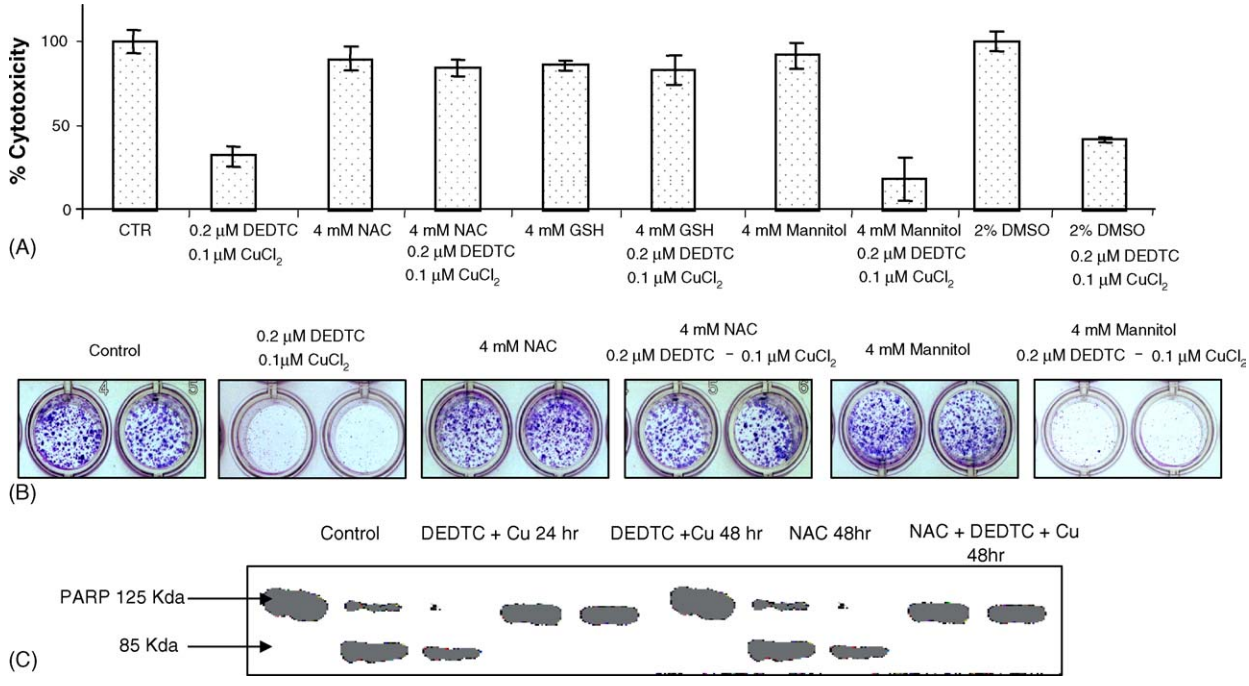

Fig. 6 – Thiol anti-oxidants protect from cytoxicity induced by $Cu[DEDTC]_2$. (A) Alamar Blue assay of SKBR3 carcinoma cells seeded in triplicate 96 well plates for 20 h followed by a 48 h exposure to 0.2 μM DEDTC and to 0.1 μM copper, including thiol anti-oxidants like *N*-acetyl cysteine (NAC) or glutathione (GSH), or the hydroxyl ($OH^{\bullet-}$) radical scavengers DMSO or mannitol, all added at the same 4 mM concentration, 4 h prior treatment with $Cu[DEDTC]_2$. Note that protection from the latter agent is provided only by thiol anti-oxidants. Results shown are representative of six different experiments. (B) Cells from the prior assay were collected and seeded in 3 cm dishes, confirming that clonogenicity is preserved by pre-treatment with thiol anti-oxidants in cells treated with $Cu[DEDTC]_2$. (C) Apoptosis-associated PARP cleavage is partial by 24 h with $Cu[DEDTC]_2$ with total loss of intact PARP (upper band) by 48 h of treatment. However, no similar effect is seen in control cells, in those treated with 4 mM NAC or in those pretreated with NAC and then exposed to $Cu[DEDTC]_2$.

increase in the cumulative number of population doublings in cultured fibroblasts from Down syndrome and normal donors [40]. SKBR3 cultures were pretreated for 1 h with 0.5 mg/mL catalase whenever indicated and subsequently treated for 48 h with either 0.2 μM DEDTC or 0.1 μM Cu(II) in aqueous solution or with an equivalent amount of the preformed complex Alamar Blue fluorescence was used to quantitate relative cytotoxicity (Fig. 8A and B), morphology was used to monitor cell damage (Fig. 8A1 and B1) and clonogenicity was used to assay lack of colony formation as a result of these treatments (Fig. 8A2 and B2). These studies not only confirmed the lethal effects of copper–DEDTC but also showed that catalase pretreatment counteracted these effects, implying that exogenous hydrogen peroxide is partly responsible for the observed toxicity.

3.8. *Nuclear changes induced by copper–DEDTC include loss of NFκB p65 and cyclin A together with changes in Sp1 phosphorylation*

Since responses to copper–DEDTC were now linked to induction of the cyclin-dependent inhibitor p21WAF1 [27] and expression of anti-oxidant enzymes like MnSOD [23,24] or catalase [26] without a comparable change in Cu/ZnSOD [25] and expression of all these molecules has been linked to the Sp1 transcription factor [28], whose transcriptional activity is up-regulated by dephosphorylation [28–30], we also investigated whether copper–DEDTC induced changes in Sp1 phosphorylation and in the expression of *nuclear* NFκB p65, since dithiocarbamates are known as potent inhibitors of nuclear factor B activation in intact cells [7]. Immune blotting and erasing of the same blots for repeated reaction showed that the increase in p21WAF1 occurred reciprocal with a decline in cyclin A, linked to cell cycle progression into S [10,32]. As expected, apoptosis-associated PARP cleavage coincided with down-regulation of nuclear NFκB p65 [7]. These changes were correlated with dephosphorylation of Sp1 [28–30], since control SKBR3 cells with no PARP fragmentation showed comparable levels of a 105 kDA phosphorylated Sp1 and the 95 kDA non-phosphorylated Sp1. In contrast, the same cells undergoing PARP fragmentation in response to copper–DEDTC showed complete loss of the 105 kDA phosphorylated Sp1 expressing only the 95 kDa Sp1 species (Fig. 9).

3.9. *Nanomolar copper–DEDTC is more active than micromolar cis-platin versus SKBR3 carcinoma*

Since the Alamar Blue cytotoxicity assay [31] was validated in a number of prior experiments, we used it again to compare the relative cytotoxicity of DEDTC plus copper against WI-38 normal diploid fibroblasts and SKBR3 carcinoma. This revealed preferential cytotoxicity of the copper–DEDTC combination against SKBR3 carcinoma (Fig. 10A). We also compared the response of SKBR3 carcinoma against the DEDTC–copper combination versus that of a widely used

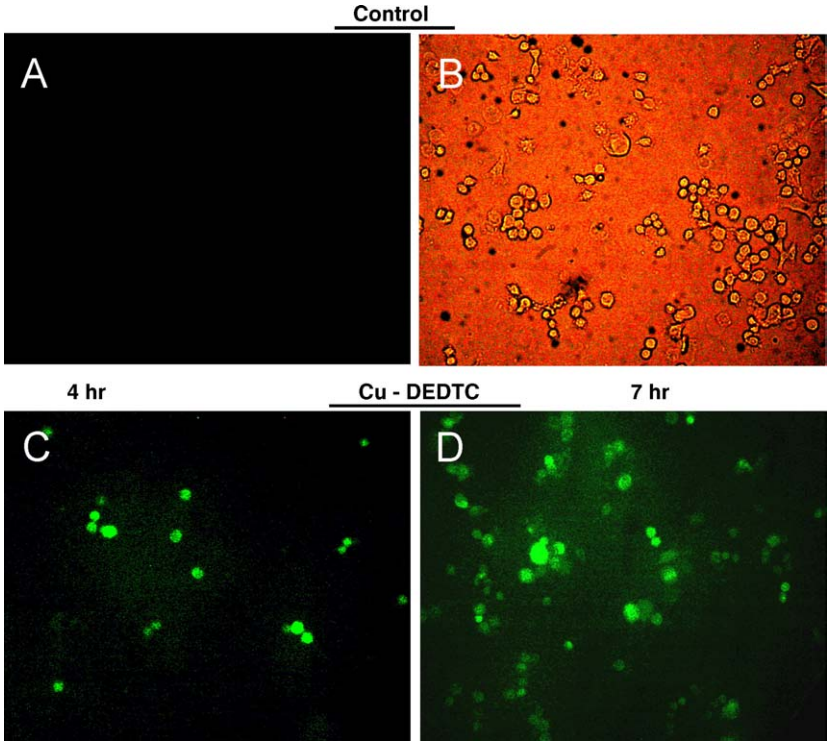

Fig. 7 – Induction of ROS by Cu[DEDTC]$_2$ SKBR3 adherent cells was incubated at different intervals with DEDTC and $CuCl_2$, followed by exposure for 30 min to 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) in DMSO as indicated in Section 2. Note very low green fluorescence in control cells (upper left), in spite of the significant number of cells in this field (upper right) and progressively increased fluorescence in cells treated by 4 and 7 h with copper–DEDTC, indicative of greater ROS production (lower left and right).

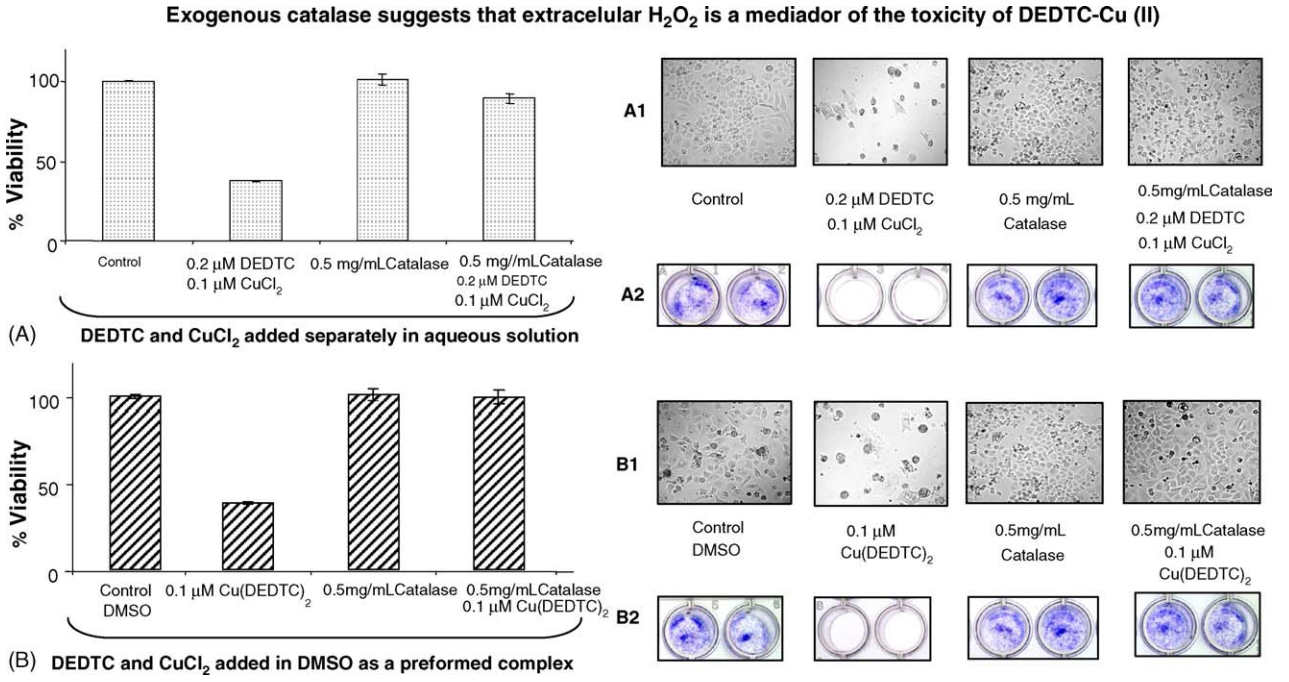

Fig. 8 – Exogenous catalase protect from cytoxicity induced by Cu[DEDTC]$_2$. (A) Alamar Blue assay of SKBR3 carcinoma cells seeded in triplicate 96 well plates for 20 h followed by a 48 h exposure to 0.2 μM DEDTC and 0.1 μM copper, including pre-treatment with 0.5 mg/ml catalase added concentration, 4 h prior treatment with 0.2 μM DEDTC and 0.1 μM copper or Cu[DEDTC]$_2$. Note that protection from the latter agent is provided catalase. (A1 and B1) Morphological changes induced by the complexes, showing protection from cell damage in those pre-treated with catalase prior to adding 0.2 μM DEDTC and 0.1 μM copper or Cu[DEDTC]$_2$, in agreement with the Alamar Blue studies; (B1 and B2), clonogenicity studies showing continued viability in catalase-pretreated cells subjected to subsequent treatment with 0.2 μM DEDTC and 0.1 μM copper or Cu[DEDTC]$_2$. Similar results were obtained in two different experiments.

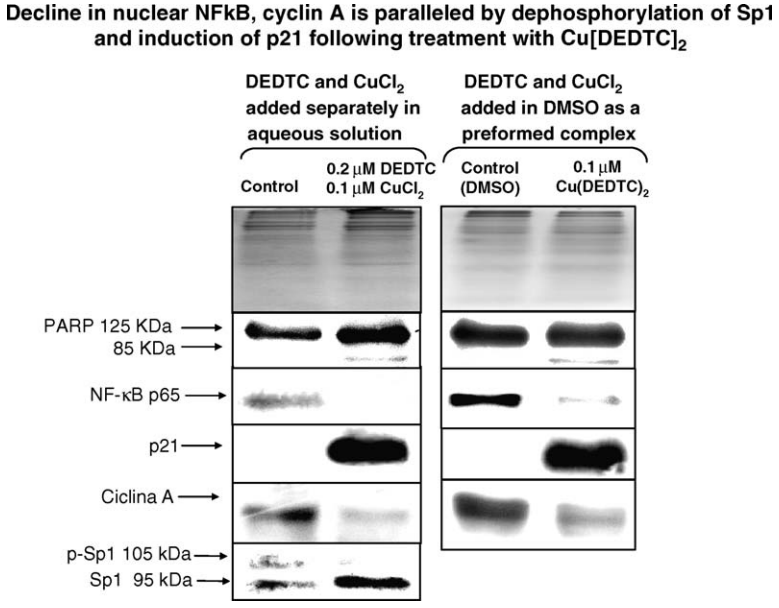

Fig. 9 – Decline in nuclear NFκB, cyclin A is paralleled by dephosphorylation of Sp1 and induction of p21 following treatment with Cu[DEDTC]$_2$. SKBR3 carcinoma cells were seeded in 9 cm dishes for 20 h followed by a 48 h exposure to either DEDTC and copper in a 2:1 ratio or to the preformed complexes Cu[DEDTC]$_2$ at a similar ratio. Subsequently, cells were harvested, *nuclei* were isolated and nuclear proteins were immune blotted as indicated in the caption for Fig. 4. Note that PARP cleavage in treated cells correlates with decreases in NFκB and cyclin A, reciprocal with induction of p21 and an increase in the 95 kDA dephosphorylated form of Sp1 (lower left).

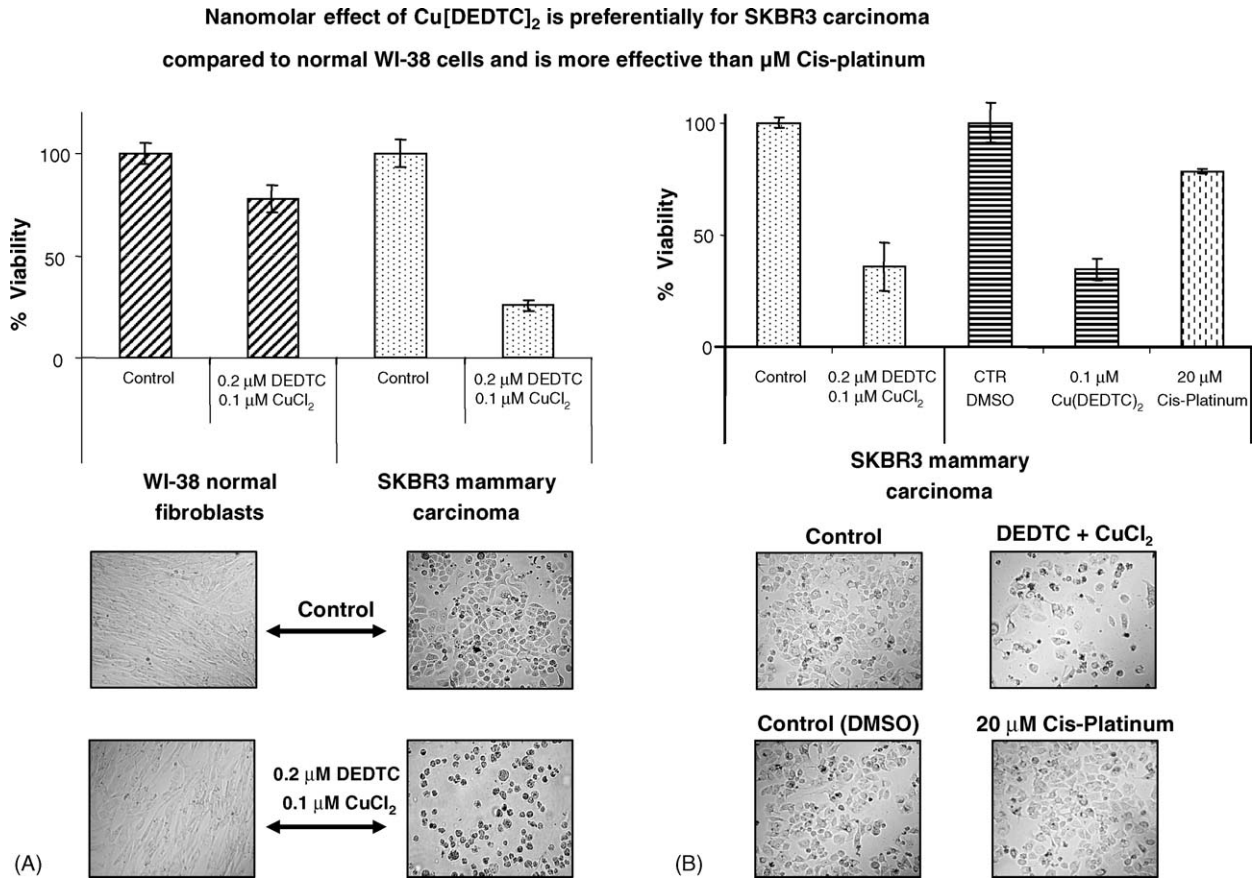

Fig. 10 – Nanomolar effect of Cu[DEDTC]$_2$ is preferentially for SKBR3 carcinoma compared to normal WI-38 cells and is more effective than μM *cis*-platin. (A) Normal diploid WI-38 fibroblasts and SKBR3 carcinoma cells were seeded in triplicate 96 well plates for 20 h followed by a 48 h exposure to aqueous 0.2 μM DEDTC and 0.1 μM copper. Note preferential loss of viability (upper) and greater morphological damage (lower) in SKBR3 cells. (B) Comparison of the effect of aqueous 0.2 μM DEDTC and 0.1 μM copper and its control, with that of DMSO vehicle compared to 20 μM *cis*-platin on the viability and morphology of SKBR3 cells. Note preferential damage in cells treated with aqueous 0.2 μM DEDTC and 0.1 μM copper.

organic metal complex like *cis*-platin. This revealed preferential activity of nanomolar copper–DEDTC versus micromolar levels of *cis*-platin (Fig. 10B).

4. Discussion

In a comparison of the ability of diverse metals to activate the cytotoxic effect of DEDTC, we now demonstrate that only Cu is effective in synergizing with DEDTC to decrease survival of SKBR3 carcinoma. To learn whether the reported effects of copper–DEDTC can be modulated by exogenous copper or the sequential addition of copper and DEDTC, which may be helped by copper transporters, we added up to 20 μM $CuCl_2$ to SKBR3 carcinoma cells, but found no inhibitory effect on these cells. Identical results resembling those of control cells were found when copper was added for 24 h and removed prior to addition of DEDTC (results not shown). This suggests that the cytotoxic effect of copper–DEDTC is strictly dependent on the formation of a Cu[DEDTC]$_2$ complex (Fig. 2). Results showing the cytotoxic effect of DEDTC and Cu were extensively followed in mammary SKBR3 carcinoma. These target cells were chosen because they harbor a dysfunctional tumor suppressor p53 with inactivating point mutations at codon 175 and also overexpress erbB2, parameters contributing to poor prognosis and low therapeutic response [19,20]. A similar cytotoxic ability of copper–DEDTC compared to that of Co, Fe or Zn with DEDTC was also found against other human tumor cells with differing p53 status (Table 1). A comparison of the effect of nanomolar copper–DEDTC toxicity against human WI-38 normal diploid fibroblasts [21] and SKBR3 carcinoma [19,20] revealed preferential susceptibility of the SKBR3 cells (Fig. 10), in agreement with earlier reports showing that the same stoichiometric ratio of this organometallic combination preferentially kills melanoma tumor cells compared to melanocytes [15,16]. Effects were similar when DEDTC and copper were added together in a 2:1 ratio, either as aqueous reactants at nM levels or when the preformed Cu[DEDTC]$_2$ complex in DMSO was added to cells at a comparable concentration. Although others previously demonstrated that DEDTC was active when complexed to copper or zinc as Cu[DEDTC]$_2$ or Zn[DEDTC]$_2$ [9,10] and copper–hydroxyquinoline Cu[8-OHQ]$_2$, was also effective in inducing tumor cell apoptosis [18], no prior study compared in parallel the structure–activity relationship of these different copper–chelator complexes. We now show that neither zinc bound

to DEDTC nor 8-OHQ bound to copper exhibit cytotoxic activity at 0.2 μM of the chelator versus 0.1 μM of the metal, except the $Cu[DEDTC]_2$ complex. Since $Cu[DEDTC]_2$ is predicted to exert its effects partly by augmenting copper or zinc uptake [6,17], it was unexpected to find that this complex primarily induced mitochondrial MnSOD rather than cytosolic Cu/ZnSOD, although overexpression of either molecule is reported to inhibit tumor growth [41–45]. Another unexpected result was that $Cu[DEDTC]_2$ mediated induction of MnSOD occurred in cells harboring a mutant p53 like SKBR3 [19,20], since others have shown that wt p53 up-regulates MnSOD [41–45], resulting in oxidative stress. Since SKBR3 carcinoma have mutation at codon 175 of the p53 tumor suppressor gene, we postulate that dephosphorylation of the Sp1 transcription factor induced by $Cu[DEDTC]_2$ may be partly responsible for induction of MnSOD (SOD2) [22] and catalase [24]. Dephosphorylation of Sp1 is associated with up-regulation of its transcriptional activity [26–28]. Hence, a p53-independent mechanism to explain induction of MnSOD, catalase and the cyclin-dependent kinase inhibitor p21WAF1 is based on independent findings demonstrating that all these genes are induced by Sp1-regulated transcription [22,24,25] activated by dephosphorylation [27]. In the present studies, concomittant increases in MnSOD, catalase and p21WAF1 occurred in parallel with the conversion of Sp1 from 105 to 95 kDa species [28], change linked to dephosphorylation and up-regulation of the Sp1 transcriptional activity [26,27]. Although MnSOD (SOD2) and Cu/ZnSOD (SOD1) can be both induced in a Sp1-dependent manner, MnSOD (SOD2) transcription requires interaction of Sp1 and Sp3 with the basal promoter [22] whereas transcription of Cu/ZnSOD (SOD1) requires Sp1 and C/EBP-related factor, which may not be induced by $Cu[DEDTC]_2$ in SKBR3 cells [23]. Others have reported that Sp1 and AP-2 have opposite roles in regulating transcription of the human MnSOD (SOD2) gene [29] Since MnSOD and catalase are both increased by copper–DEDTC, the cytotoxic effect of the latter may be due to greater superoxide dismutation by MnSOD increasing peroxides, together with induction of intracellular catalase in an attempt to overcome the effect of excess H_2O_2 which can alter the thiol redox balance by reversibly oxidizing specific thiol groups [46]. This possible mechanism is supported by the finding that either supply of exogenous catalase or addition of thiols like reduced glutathione or its precursor NAC, protect from copper–DEDTC toxicity, suggesting that extracellular hydrogen peroxide is produced but not sufficiently destroyed, thereby producing toxicity associated with glutathione oxidation (Fig. 11). Protection against copper–DEDTC toxicity provided by NAC or exogenous catalase suggested the involvement of ROS in such effects. This was confirmed by fluorescence microscopy showing an early ROS induction evidenced by oxidation of H_2DCFDA [33]. Of special interest was the preliminary observation that nanomolar copper–DEDTC is more active than micromolar *cis*-platin against SKBR3 carcinoma, suggesting that this copper complex may eventually become an alternative to the widely used *cis*-platin in cases in which there is no adequate response to the latter drug. A recent report indicated an interesting anti-cancer activity of an 8-hydroxyquinoline analog clioquinol [5-chloro-7-iodo-8-hydroxyquinoline] which resembles DEDTC in that both chelate copper or zinc. Treatment with clioquinol for 72 h reduced viability of some breast cell lines (MCF-7, MDA-MB231), with IC50s ranging from 29.7 to 19.9 μmol/L, respectively. Addition of metals at μmol concentration enhanced the cytotoxicity of 5 μmol/L clioquinol [47]. This report shows that DEDTC and copper added, respectively, at the lower concentrations of 0.2 μM DEDTC and 0.1 μM copper

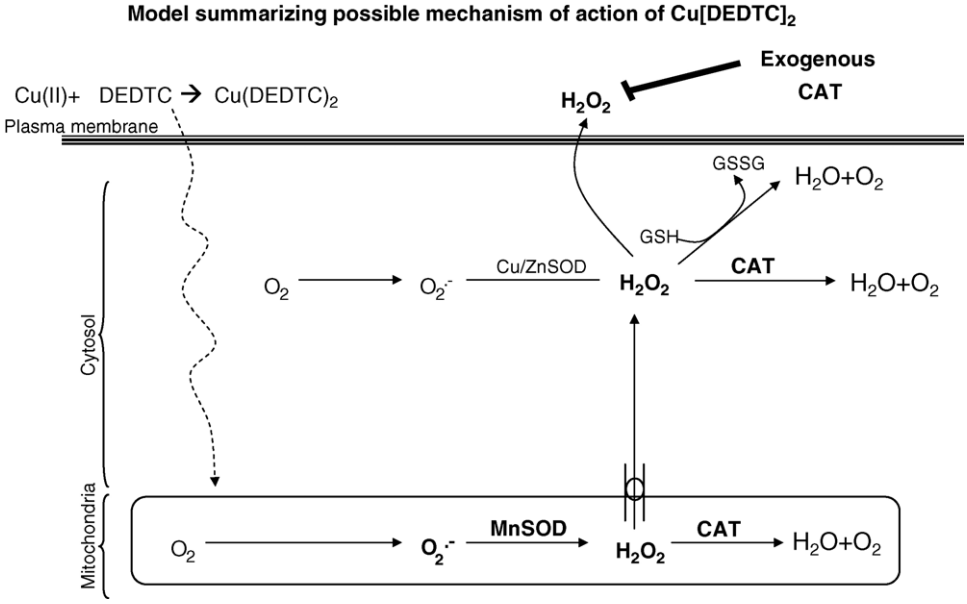

Fig. 11 – Model summarizing possible mechanism of action of $Cu[DEDTC]_2$. Either aqueous DEDTC and copper reactants or the preformed complex show cytotoxic activity at nM levels, affecting primarily mitochondrial MnSOD with no comparable effect on cytosolic Cu/Zn SOD. This could enhance superoxide dismutation to hydrogen peroxide, which induces intracellular catalase, but exogenous peroxide helps to kill SKBR3 cells, unless cells are pretreated with exogenous catalase or supplemented with thiols that prevent loss of reduced glutathione.

are more effective than the combination 8-hydroxyquinoline plus copper against SKBR3 human carcinoma cells, without a comparable toxicity against human normal diploid WI-38 fibroblasts. The clioquinol data [47] and the present results with copper–DEDTC, strongly suggest that organic copper complexes may have an important potential as anti-tumor agents, and they should be further examined in cases in which there is resistance to *cis*-platin.

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