BIOCHEMICAL PHARMACOLOGY 74 (2007) 841–850

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Role of peroxidases, thiols and Bak/Bax in tumor cell susceptibility to Cu[DEDTC]$_2$

Maricela Viola-Rhenals [*], *Mary Strasberg Rieber*, *Manuel Rieber* [1,**]

Instituto Venezolano de Investigaciones Científicas, IVIC, Centre for Microbiology & Cell Biology, Laboratory of Tumor Cell Biology, Apartado 21827, Caracas 1020 A, Venezuela

ARTICLE INFO

Article history:
Received 4 May 2007
Accepted 29 June 2007

Keywords:
Glutathione
Peroxidase
Bak
Bax
Mcl-1
Dithiocarbamate
G2

ABSTRACT

Copper and two molecules of diethyl dithiocarbamate [DEDTC] form the Cu[DEDTC]$_2$ complex, which shows cytotoxicity against melanoma and carcinoma cells, making it a potentially useful anti-cancer agent. The differential response to Cu[DEDTC]$_2$ in susceptible human SKBR3 carcinoma and C8161 melanoma cell variants of moderate and high resistance to this organometallic complex was evaluated in this study. Both cell lines underwent apoptosis-associated PARP cleavage, changes in expression of nuclear NFkB p65, p21WAF1 and cyclin A, with loss of clonogenicity in response to this agent. However, a threefold greater concentration [IC_{50} 0.6 μM DEDTC: 0.3 μM Cu] was required to kill moderately resistant C8161 melanoma compared to highly susceptible SKBR3 cells. Decreased susceptibility to Cu[DEDTC]$_2$ in C8161 melanoma correlated with greater levels of glutathione peroxidase and catalase, and a fourfold lower requirement for *N*-acetyl cysteine (1 mM) to overcome toxicity. Whereas melanoma cells selected for resistance to [0.8 μM DEDTC: 0.4 μM Cu] showed persistent catalase and GPx activity, melanoma cells with moderate susceptibility showed decreased catalase and Gpx when responding to treatment. Cytotoxic response in moderately susceptible C8161 melanoma cells involved an early accumulation of pro-apoptotic Bax in the G2 cell cycle phase, followed by an increased ratio of pro-apoptotic Bak to anti-apoptotic Mcl-1 in mitochondria. Our data suggests that Cu[DEDTC]$_2$ toxicity is mediated through an increase in pro-apoptotic Bak/Bax via disruption of the peroxide and thiol metabolism.

© 2007 Elsevier Inc. All rights reserved.

1. Introduction

The BCL-2 family of proteins regulates apoptosis and proper control of this process is required for preventing disease. Members of the BCL-2 family include anti-apoptotic proteins like Mcl-1 and proapoptotic proteins like Bax and Bak [1]. Bax is a latent monomer that resides in the cytoplasm. In healthy cells, Bak associates with Mcl-1, but upon a cytotoxic signal, Bak is displaced from the Mcl-1 complex and induces cell death [2]. Apoptotic stimuli that activate Bax induce a profound change in its conformation, revealed by its ability to form homodimers and oligomers [3–5] Others have reported that anti-apoptotic Mcl-1 prevents mediated apoptosis through titration and complexing with Bak, unless the latter

* *Corresponding author.*
** *Corresponding author.* Tel.: +58 212 5041494; fax: +58 212 5041382.
E-mail addresses: mviola@ivic.ve (M. Viola-Rhenals), mrieber@ivic.ve (M. Rieber).

[1] Research supported by LOCTI Project 168 and the Terry Fox Run for Cancer Research (CIC-CCA) awarded to mrieber@ivic.ve.

Abbreviations: DEDTC, diethyl dithiocarbamate; $CuCl_2$, copper chloride (II); Px, peroxidase; GPx, glutathione peroxidase

0006-2952/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2007.06.048

is in excess in the mitochondria [2,6]. However, effective apoptosis is helped by the combined functions of Bak and Bax [7,8]. Pro-apoptotic signalling can also be favoured by oxidative imbalance, leading to activation of mitochondrial Mn superoxide dismutase that promotes conversion of superoxide radicals to hydrogen peroxide (H_2O_2). The latter requires the action of peroxidases like catalase, to help detoxify excess H_2O_2 [9–11]. When these peroxide-degrading enzymes fail, excessive hydrogen peroxide may accumulate helping to trigger apoptosis [10]. Since transition metal complexes may trigger apoptosis through oxidative stress, we now investigated some of the mechanisms of action of diethyl dithiocarbamate (DEDTC) bound to copper [12,13]. In recent studies, 0.17 μM disulfiram that originates diethyl dithiocarbamate (DEDTC) at the same concentration, showed highly selective and significant toxicity against A375 and c81–46a melanoma cells but not normal melanocytes, dependent on addition of 1 μM $CuCl_2$ [12]. 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 antimelanoma active species is in fact attributable to the Cu[DEDTC]$_2$ complex. Our laboratory recently reported that 200 nM Cu[DEDTC]$_2$ did not decrease significantly the viability of normal diploid human WI-38 fibroblasts, but was highly cytotoxic against human SKBR3 breast carcinoma [13], which harbour a dysfunctional tumor suppressor p53 with inactivating point mutations at codon 175 [14]. To gain further insight into the mechanism of action of Cu[DEDTC]$_2$ on human melanomas, we now compared the effect of this complex against wt p53 human C8161 melanoma [15] and SKBR3 breast carcinoma [14]. Besides demonstrating that SKBR3 carcinoma are more susceptible to Cu[DEDTC]$_2$ than C8161 melanoma irrespective of their unequal p53 status, we now show that greater susceptibility to this treatment correlates with lower basal levels of glutathione peroxidase and catalase [9,10] and nuclear NFkB p65 [16]. We also demonstrate that C8161 melanoma undergo G2 arrest and induce pro-apoptotic Bak and Bax condensation, in response to the indicated treatment. Our data also support an involvement of hydrogen peroxide in Cu[DEDTC]$_2$ cytotoxicity, since the latter is counteracted by exogenous peroxidase activity or thiol anti-oxidants.

2. Materials and methods

2.1. Cell cultures and treatment

(a) SKBR3 human breast carcinoma harboring mut p53 [13,14] was cultured in DMEM medium supplemented with 10% fetal bovine serum; (b) C8161 human melanoma harbouring wt p53 [16] was cultured in DME:F12 medium supplemented with 10% fetal bovine serum. (c) resistant C8161 melanoma cultures were developed by progressive adaptation and survival in (0.8 μM DEDTC: 0.4 μM Cu). Whenever indicated, subconfluent cultures seeded the previous day, were treated with nanomolar equivalents of $CuCl_2$ and 2X nanomolar equivalents of diethyl dithiocarbamate [DEDTC]2 to give Cu[DEDTC]$_2$. Whenever indicated, experiments included *N*-acetyl cysteine (Sigma, No. A-8199) or glutathione (Sigma, No. G-4251) at 4 mM, and catalase (Sigma, No. C-40) or peroxidase (Sigma, No. P-8375), each added to 250 U/ml.

2.2. Quantitative assesment of cytotoxicity

Relative cell viability/cytotoxicity was estimated with Alamar Blue (resazurin) that measures intracellular redox activity by quantitating the cell-catalyzed conversion of non-fluorescent resazurin to fluorescent resorufin [17]. 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 [13]. As such, this assay is valuable as an endpoint of cytotoxicity, rather than as a kinetic measure for monitoring cell growth [16]. For these experiments, cells (10,000) were allowed to adhere overnight in 96 well TC microtiter dishes. After the corresponding treatments, Alamar Blue (BioSource, Camarillo, CA, USA) was added and fluorescence was measured 4 h later in a Fluoroskan Ascent microplate reader with an excitation of 544 nm and an emission of 590 nm [13,17].

2.3. Colony formation assay

Exponentially growing cells were seeded at 5000 cells per well in 96-well plates and allowed to attach for 18 h. After 48 h of the respective treatments, cells were washed in isotonic phosphate-buffered saline, detached and transferred to 3.5 cm plates with drug-free complete medium added. Cultures were 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 [18].

2.4. Subcellular fractionation of mitochondrial proteins

Cell pellets were resuspended in sucrose-supplemented cell extract buffer (300 mM sucrose, 10 mM Hepes pH 7, 50 mM KCl, 5 mM EGTA, 5 mM MgCl2, 1 mM DTT and protease inhibitor mixture of 1 mM PMSF, 1 mM DTT, 5 μg/mL leupeptin and aprotinin. The cells were homogenized on ice with a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 2000 × *g* for 10 min at 4 °C. The postnuclear supernatant was further spun at 14,000 × *g* for 10 min at 4 °C to yield a mitochondrial pellet. This was solubilized in 0.5% Triton X-100 in 250 mM sucrose, 20 mM Hepes pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 5 μg/mL leupeptin and aprotinin. The supernatant was further centrifuged at 14000 × *g* for 10 min to yield soluble mitochondrial proteins used for immune blotting [19].

2.5. Immune blotting

Cell lysates were prepared and SDS–PAGE and bidirectional immune blots were done as previously described using 70 μg proteins per lane [20] 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. Antibodies used for immune blotting included: PARP (SC-7150), NF-κB p65 (SC-8008), p21 (SC-397), Mcl-1 (SC-819), Cyclin A (SC-751), Bax (SC-7480) and Bak (SC-832) were all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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 [20]. 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. Whenever indicated, western blots were normalized to total protein loadings, in SDS–PAGE gels stained with Coomassie Blue 0.05% after immune blotting. In other experiments, after stripping of the initial signals as indicated above, reprobing of the immune blots with monoclonal antibody to actin (JLA-20; Oncogene Science) was used to normalize protein loadings.

2.6. Activity assay for superoxide dismutase, glutathione peroxidase and catalase

This was carried out in native polyacrylamide gel electrophoresis in which the 5% stacking gel was polymerised with 0.004% riboflavin photoactivated by fluorescent light [21]. Normalization of protein loadings for these non-denaturing gels was carried out by prestaining these gels with the fluorochrome Sypro Ruby (Molecular Probes) prior to enzymatic reactions [22]. Superoxide dismutase activity was demonstrated in 12% native gels by reduction of Nitro Blue Tetrazolium (NBT) by O_2^-, as the basis of assays for superoxide dismutase, which exposes its presence by inhibiting the reduction of NBT [21]. Catalase activity was demonstrated treating the gel with 0.003% H_2O_2 as substrate followed by exposure to 2% ferric chloride and 2% potassium ferricyanide until formation of achromatic bands on a dark blue background. For glutathione peroxidase activity, gels were treated as indicated for catalase, including 1 mM glutathione.

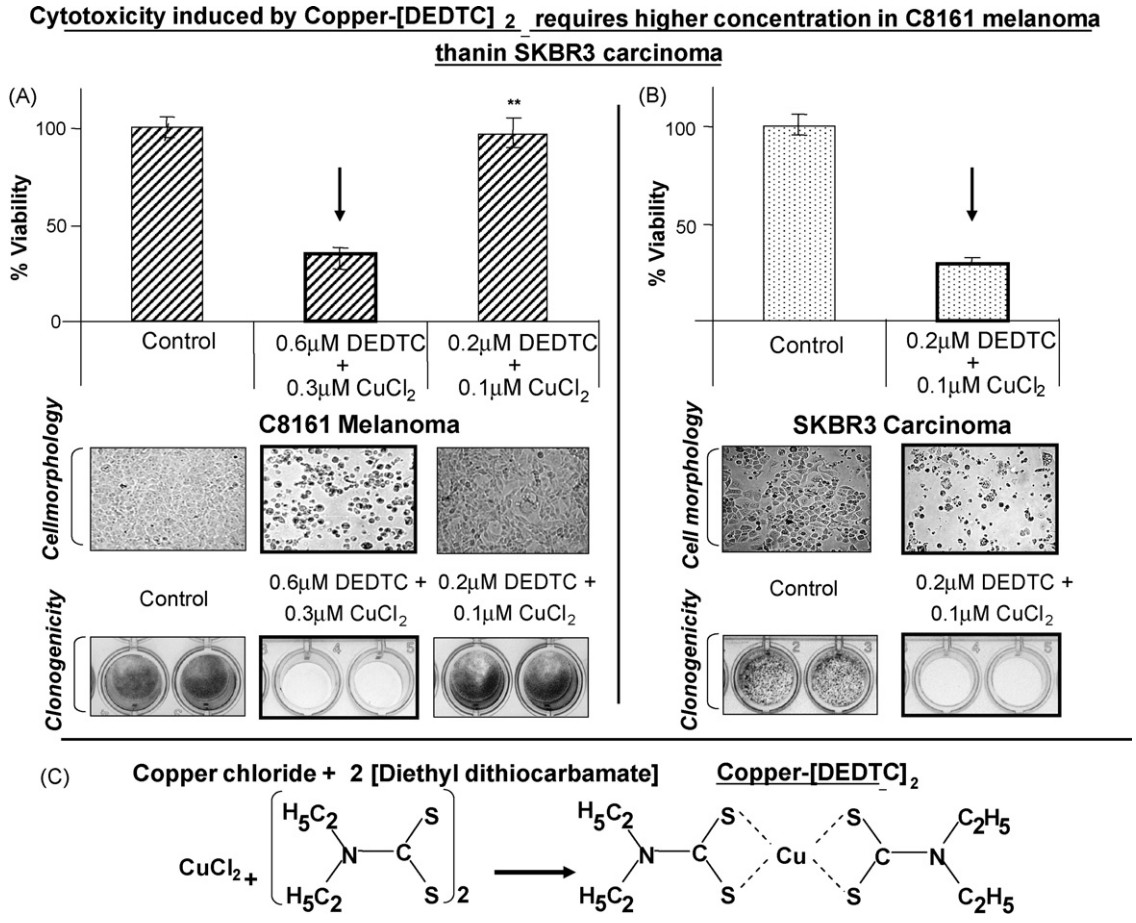

Fig. 1 – Cytotoxicity induced by Cu[DEDTC]$_2$ requires higher concentration in wt p53 C8161 melanoma than mut p53 SKBR3 carcinoma. Cells were seeded in triplicate in 96 well plates for 18 h followed by a 40 h exposure to DEDTC and $CuCl_2$ at the concentrations indicated in each case. Determinations of relative viability by Alamar blue (upper), morphological toxicity (middle) and loss of clonogenicity (lower) reveal that SKBR3 cells (B) are more susceptible to a lower concentration of DEDTC and $CuCl_2$, compared to C8161 melanoma (A). The complex resulting from the interaction of copper chloride with diethyl dithiocarbamate is shown in C (see reference 13). These assays are representative of four different experiments.

2.7. Statistical studies

Standard deviations (S.D.) are shown for every Alamar Blue viability assays, carried out in triplicate and repeated four times. In every assay shown, S.D. results were within ± 5% with a 95% statistical significance ($n = 4$, $^{**}p > 0.0001$ by student t-test).

2.8. Densitometry

Image capture to define 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).

2.9. Laser scanning cytometry (LSC)

An LSC-2 cytometer (Compucyte, Cambridge, MA, USA), which measures fluorescence intensity of individual cells contoured on the basis of nuclear DNA counterstain with propidium iodide, was used. Where indicated, cells attached to LabTek multiwell plates were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by permeabilization with 0.25% Triton X-100, washing in PBS, blocking in 5% albumin (in PBS), then reacted with a mouse monoclonal antibody to Bax (SC-7480) from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative controls were stained with secondary antibody. Detection was achieved by reaction with an anti-mouse secondary antibody conjugated to Oregon Green (Molecular Probes, Eugene, Oregon, USA) for excitation with an Argon laser [19]. Every sample was scanned using identical non-saturating fluorescence settings, to allow quantitative comparisons to be made [20]. To analyze fluorescence changes on individual cells, clustered aggregates were gated out, to include as many individual contoured cells as possible and quantitate integral (total fluorescence within the integral contour) and *maximal pixels*

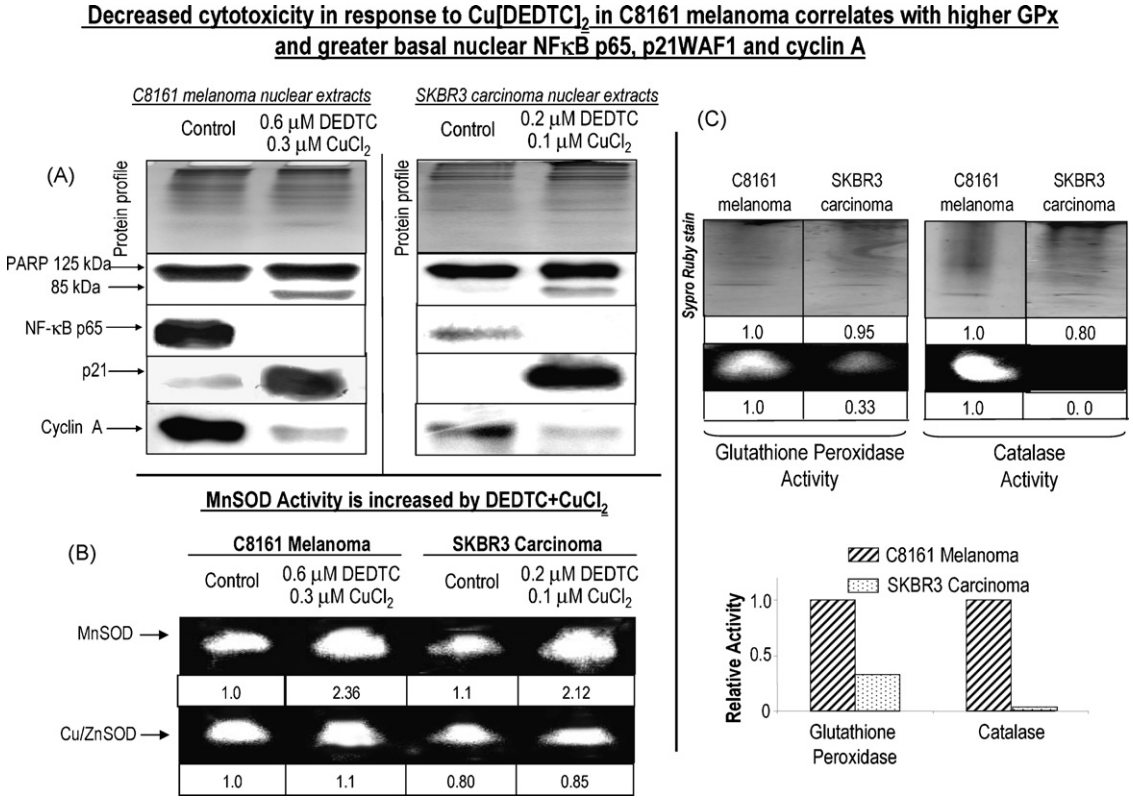

Fig. 2 – Decreased cytotoxicity in response to Cu[DEDTC]$_2$ in C8161 melanoma correlates with higher GPx and greater basal nuclear NFkB p65, p21WAF1 and cyclin A. Nuclear protein extracts were prepared from wt p53 C8161 melanoma and mut p53 SKBR3 carcinoma for immune blots, and activity assays for GPx and catalase were carried out with total cell extracts as indicated in the Section 2. Cells were treated with the indicated concentrations of DEDTC and $CuCl_2$ for comparison with H_2O-treated controls, for 20 h, prior to harvesting and electrophoretic analyses. Note higher nuclear basal levels of NFkB p65, p21WAF1 and cyclin A and greater basal GPx and catalase activity in C8161 melanoma which undergo apoptosis-associated PARP cleavage at a threefold higher concentration of DEDTC and $CuCl_2$, compared to those basal levels in the more susceptible SKBR3 cells. (A, left) PARP assay run under denaturing conditions, results were normalized to Coomassie-blue stained gels. (A, middle and right) Assays run under non-denaturing conditions were normalized to Sypro Ruby fluorometric staining. (B) The ratio of Cu/Zn SOD to Mn SOD in treated cells was obtained in the same gels and were quantified relative to each other and to those of control cells. (C) Results were normalized by prestaining with the Sypro Ruby fluorochrome [22] prior to Glutathione peroxidase assays. These assays are representative of two different experiments.

(highest localized fluorescence within the threshold contour). For quantitation of DNA condensation and cell cycle analysis, diploid and tetraploid populations were selected from propidium iodide-stained cells to determine increases in maximal pixels relative to integral DNA fluorescence. Cell cycle profiles were determined by analyzing the percentages of cells with G1, S and G2 DNA content. To determine Bax induction normalized to DNA, cells were simultaneously stained with anti Bax antibody and the DNA fluorochrome, propidium iodide [20].

3. Results

3.1. C8161 melanoma is more resistant to Cu[DEDTC]$_2$ than SKBR3 carcinoma

In a comparison of the cytotoxic effect of DEDTC–Cu against wt p53 C8161 melanoma and SKBR3 carcinoma, we consistently found by Alamar Blue fluorescence [16] (Fig. 1, upper), morphological analysis (Fig. 1, middle) and by clonogenic survival assays (Fig. 1, lower) that SKBR3 carcinoma cells (Fig. 1B) lost viability at a 0.2 μM of DEDTC and 0.1 μM concentration of $CuCl_2$ which forms the corresponding Cu[DEDTC]$_2$ complex [13] (Fig. 1C). However, C8161 melanoma were not affected by this concentration of the Cu[DEDTC]$_2$ complex, but were killed by a dose of 0.6 μM: 0.3 μM of the complex (Fig. 1A).

3.2. Lower basal catalase and peroxidase activity correlate with greater susceptibility to Cu[DEDTC]$_2$, in spite of common changes in NFkB p65, cyclin A, p21WAF1 and Mn SOD

As expected from cells harbouring a wt p53, C8161 melanoma exhibited greater basal nuclear levels of the cyclin-dependent inhibitor p21WAF1 [16] compared to mut p53 SKBR3 cells [13]. Other important differences between these two tumor cell types was the greater basal nuclear levels of NFkB p65 and cyclin A in wt p53 C8161 melanoma [15]. In spite of these basal differences, when the Cu[DTC]$_2$ complex was used at the corresponding lethal doses (Fig. 1), apoptosis-associated PARP fragmentation [23] was evident in both cell types, accompanied by induction of the cyclin-dependent kinase inhibitor p21WAF1 and reciprocal losses in nuclear NFκB p65 and in proliferation-associated cyclin A (Fig. 2A). In spite of the differences revealed above by immune blotting, a comparison of superoxide dismutase (SOD) activities [21] revealed comparable levels of Cu/Zn SOD which did not change significantly in response to treatment with the [DEDTC]$_2$–Cu in both cell types. In contrast, when normalized to Cu/Zn SOD levels in the same gel, Mn SOD activity essentially doubled in both cell types treated with the copper complex (Fig. 2B). We then asked whether the greater resistance to Cu[DEDTC]$_2$ correlated with levels of basal hydrogen peroxide –degrading enzymes. This revealed a threefold lower level of glutathione peroxidase and negligible catalase activity in the more susceptible SKBR3 cells compared to the more resistant C8161 melanoma (Fig. 2C).

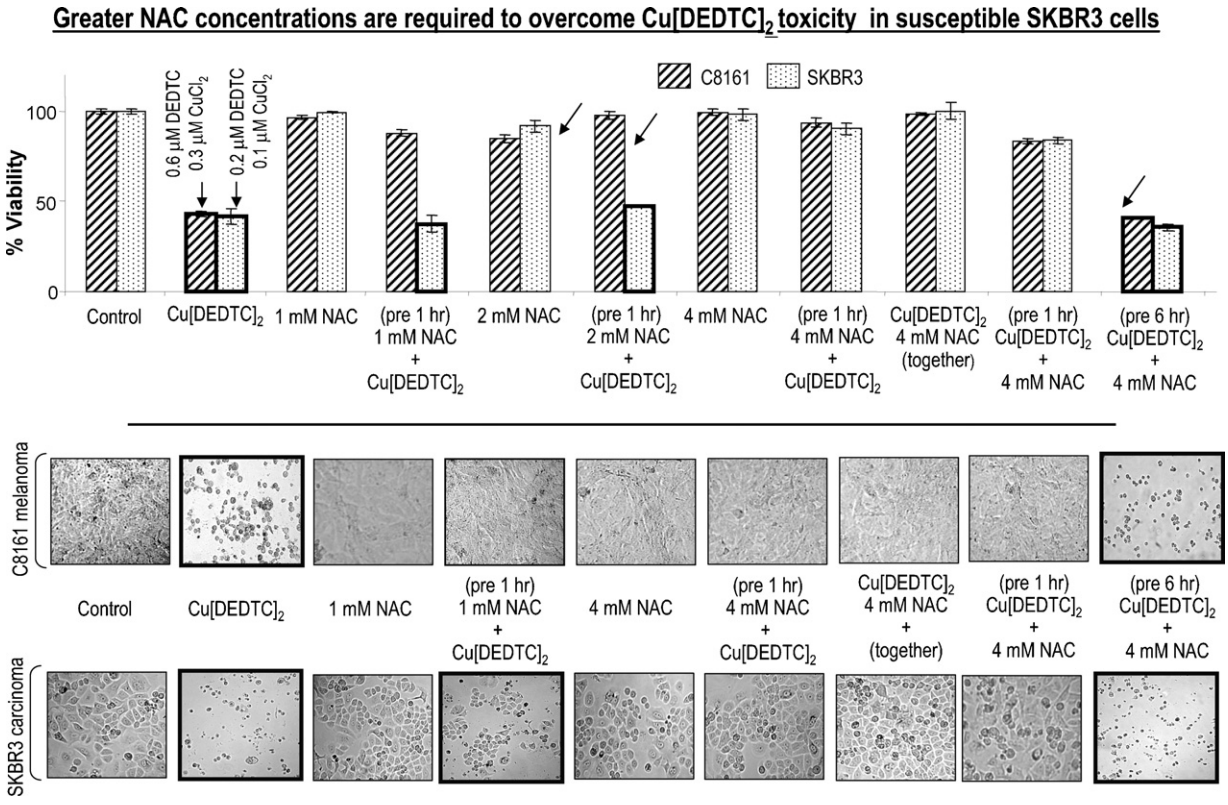

Fig. 3 – Greater NAC concentrations are required to overcome Cu [DEDTC]$_2$ toxicity in susceptible SKBR3 cells. Determinations of relative viability by Alamar blue (upper) and morphological toxicity (lower) reveal that suppression of toxicity induced by a 40 h treatment with DEDTC and $CuCl_2$ was achieved by 1 mM NAC in C8161 melanoma, and by 4 mM NAC in SKBR3 cells. These assays are representative of four different experiments.

3.3. *Greater thiol concentration is required to overcome Cu[DEDTC]$_2$ toxicity*

Since glutathione peroxidase activity was lower in the more susceptible SKBR3 cells, we asked whether this correlated with a higher requirement for the glutathione precursor *N*-acetyl cysteine (NAC) [24], to protect from Cu[DEDTC]$_2$. Even when using 0.2 μM DEDTC plus 0.1 μM $CuCl_2$, SKBR3 cells lost viability unless pre-treated with 4 mM NAC (Fig. 3). In contrast, C8161 melanoma killed by 0.6 μM: 0.3 μM of the complex only required a 1 h pre-treatment with 1 mM NAC, to counteract the toxicity of the complex. However, Fig. 3, upper right showed that no protection was afforded by 4 mM NAC when added several hours after the complex.

3.4. *Peroxidase, catalase and glutathione control response to DEDTC–Cu*

To learn more about the basis for resistance to Cu[DEDTC]$_2$, we developed a C8161 melanoma variant resistant to [0.8 μM DEDTC: 0.4 μM Cu] for comparison with parental C8161 melanoma highly susceptible to [0.6 μM DEDTC: 0.3 μM Cu] . In upper Fig. 4A, left, these cells failed to undergo apoptosis-associated PARP cleavage in response to the complex, in contrast to the moderately susceptible parental C8161 melanoma. The latter cells showed a parallel decline in glutathione peroxidase (GPx) and in catalase activity upon treatment with the complex, in contrast to the resistant variant cells (Fig. 4,A). This led us to ask whether exogenous sources of peroxidase, catalase or glutathione counteracted Cu[DEDTC]$_2$ toxicity. A 60 min pre-treatment with exogenous peroxidase (250 U/ml) or comparable levels of catalase, known to degrade H_2O_2, also protected susceptible SKBR3 and intermediately susceptible C8161 melanoma cells from cytotoxicity (Fig. 4B). A similar pre-treatment with 4 mM of glutathione (GSH) was also sufficient to protect both cell types from cytotoxicity (Fig. 4C). These results suggest that increased production of H_2O_2 and/or a decrease in glutathione are probably involved in the lethality of Cu [DEDTC]$_2$ in SKBR3 and parental C8161melanoma.

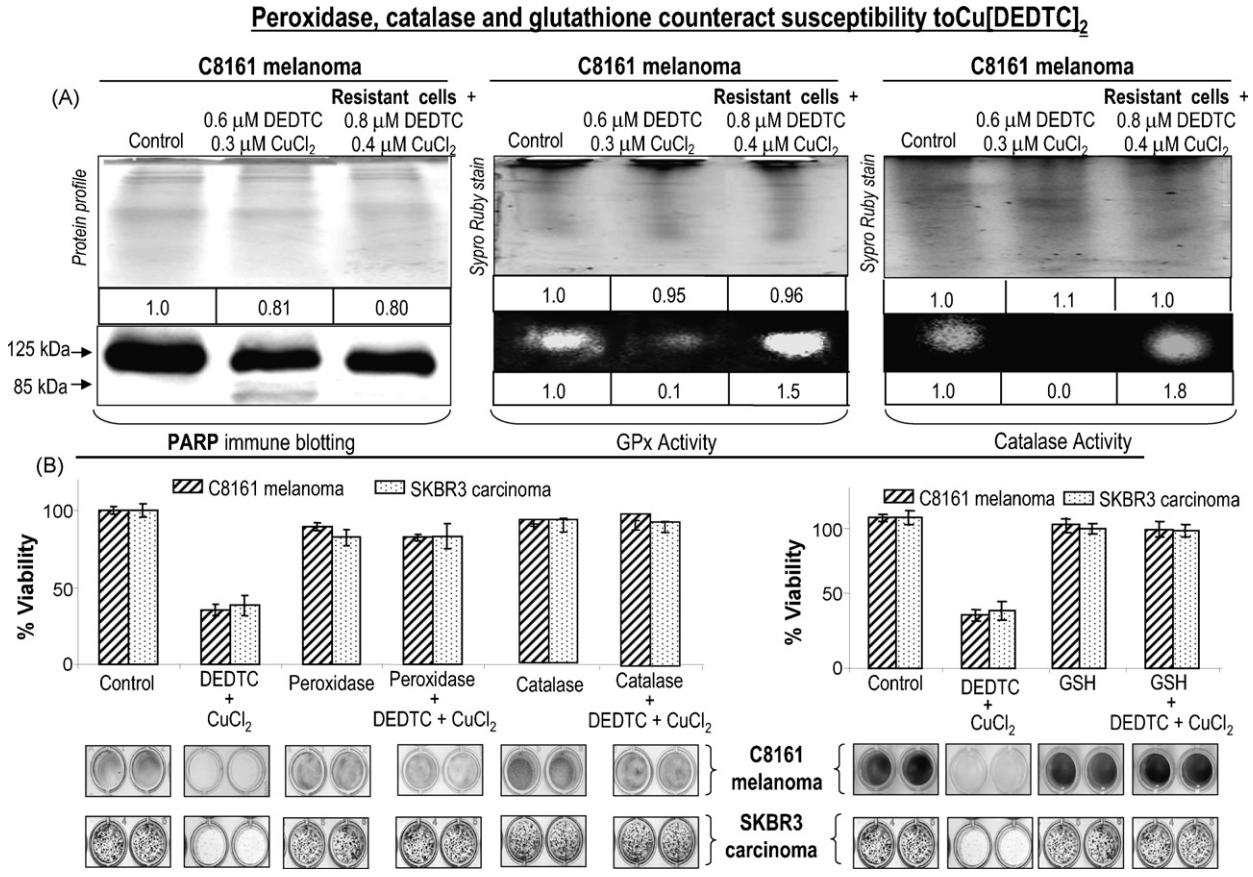

Fig. 4 – Peroxidase, catalase and glutathione counteract susceptibility to Cu[DEDTC]$_2$. (A) Control cultures of C8161 melanoma and those susceptible to 0.6 μM DEDTC and 0.3 μM $CuCl_2$ were compared with C8161 cells selected for resistance to 0.8 μM DEDTC and 0.4 μM $CuCl_2$. Note PARP cleavage and a decline in GPX and catalase only in the susceptible cells exposed for 30 h to 0.6 μM DEDTC and 0.3 μM $CuCl_2$. Results were normalized as indicated in legend to Fig. 2. (B) Toxicity induced by a 30 h treatment with DEDTC + $CuCl_2$ is counteracted by a 60 min pre-treatment with exogenous peroxidase or catalase (left) or by exogenous glutathione (4 mM), irrespective of cell type. Whenever cells were treated with catalase or peroxidase, this was for 1 h prior to treatments with the copper complex as indicated under Section 2. These assays are representative of four different experiments.

3.5. *Cu[DEDTC]$_2$ induces DNA condensation, Bax accumulation and G2 cell cycle arrest*

Chromatin condensation other than that occurring in mitotic populations is one of the most important criteria that are used to identify apoptotic cells [25]. To determine the extent of DNA condensation induced by an 8-h treatment with [DEDTC]$_2$–Cu in C8161 melanoma, we used quantitative laser scanning cytometry (LSC) [20]. This produces a histogram of DNA integral fluorescence within the integral contour [2*n* (diploid) to 4*n* (tetraploid)] plotted versus DNA maximal pixel (highest intensity of fluorescence within the threshold contour) [19]. Assessment of DNA maximal pixel in diploid to tetraploid DNA is an indication of relative DNA condensation. This assay now revealed that [DEDTC]$_2$–Cu increased DNA condensation in control C8161 melanoma from 36.4% to 89.1%. Moreover, the average mean fluorescence per single cell increased from 13,684 in control cells to 14,611 in cells treated with the complex for 12 h (Fig. 5A). This was paralleled by a doubling in condensation of pro-apoptotic Bax preferentially in the G2 cell population in response to the complex (Fig. 5B). Bax induction by Cu[DEDTC]$_2$ was also seen by immune blotting with cells cultured on tissue culture plates, assay in which detached and adherent cells were pooled (lower Fig. 5B). Cell cycle studies also revealed that Cu[DEDTC]$_2$ induced a twofold increase in the G2 cell population since control cells showed 55.2% of cells in G1, 32.1% in S phase and 15.8% in G2 in contrast with 42.9% of cells in G, 26.1% in S phase and 31.5% in G2 in cultures treated for 12 h with Cu[DEDTC]$_2$ (Fig. 5C).

3.6. *Cytotoxic effect of Cu[DEDTC]2 versus C8161 melanoma involves an increase in the mitochondrial Bak/Mcl-1 ratio*

Since pro-apoptotic Bak associates with and is antagonized by anti-apoptotic Mcl-1 in healthy cells, and the ratio of mitochondrial Bak/Mcl-1 is important in apoptosis [1,2,6], we investigated whether the cytotoxic Cu[DEDTC]$_2$-complex influenced the ratio of Bak/Mcl-1 in parental C8161 melanoma. Immune blotting results from bidirectional transfer shown in Fig. 6B revealed high levels of mitochondrial pro-apoptotic Bak and Mcl-1 compared to those seen in control cells by 12 h of cytotoxic treatment and prior to overt morphological damage. However, by 24 h of such treatment, levels of Bak remained

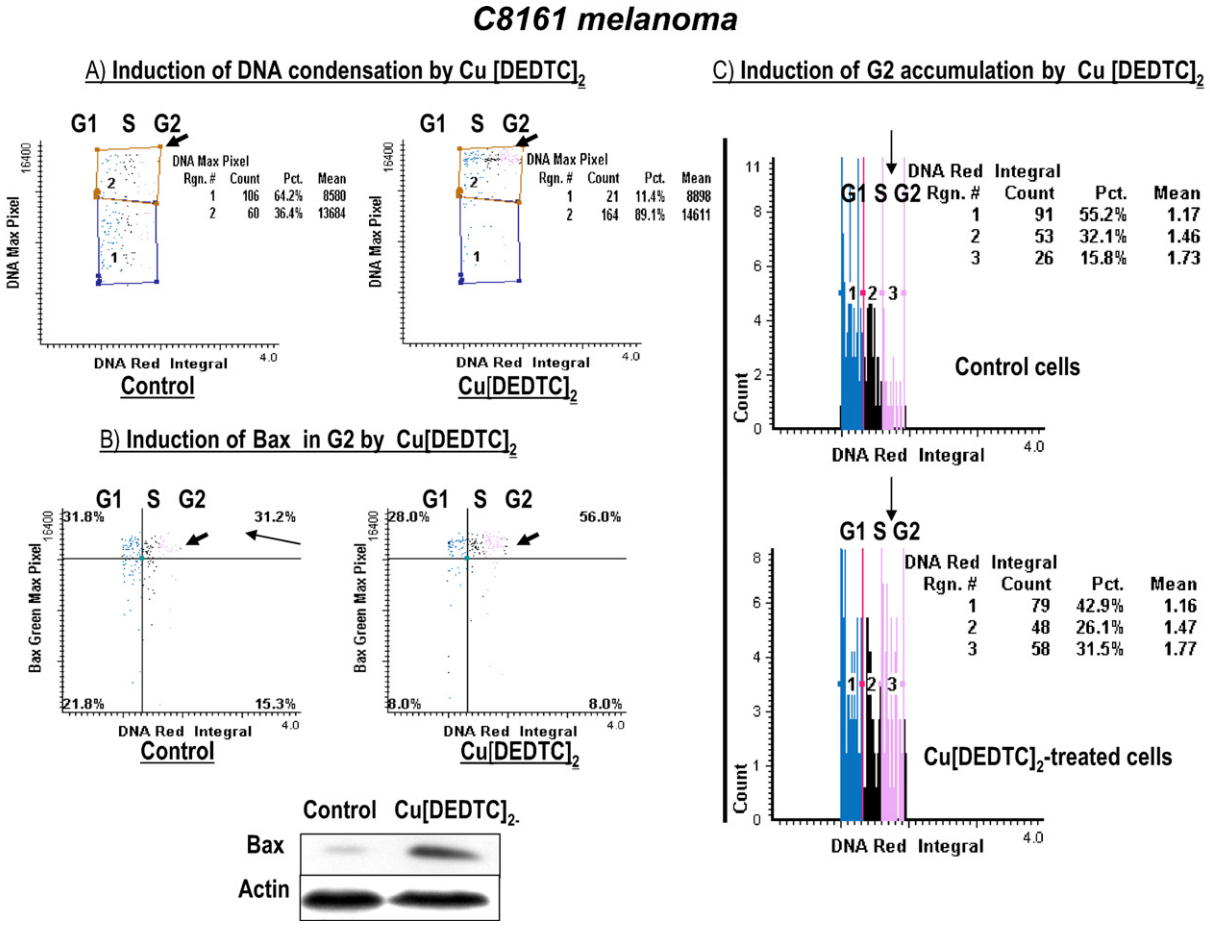

Fig. 5 – Induction of DNA condensation, Bax accumulation and G2 cell cycle arrest in C8161 melanoma by Cu[DEDTC]$_2$. Laser scanning cytometry of cells treated for 8 h with 0.6 μM DEDTC and 0.3 μM $CuCl_2$ was used to assess: (A) differential DNA condensation vs. DNA content in moderately susceptible C8161 melanoma. Note that the percentage of cells with DNA maximal pixel is more than doubled (area 2) in cells treated for 8 h with 0.6 μM DEDTC and 0.3 μM $CuCl_2$. (B) Bax accumulation in G2, from 31% to 56%. (C) Doubling of the G2 population from 15.8% to 31.5% in treated cells. Results shown were representative of three different experiments.

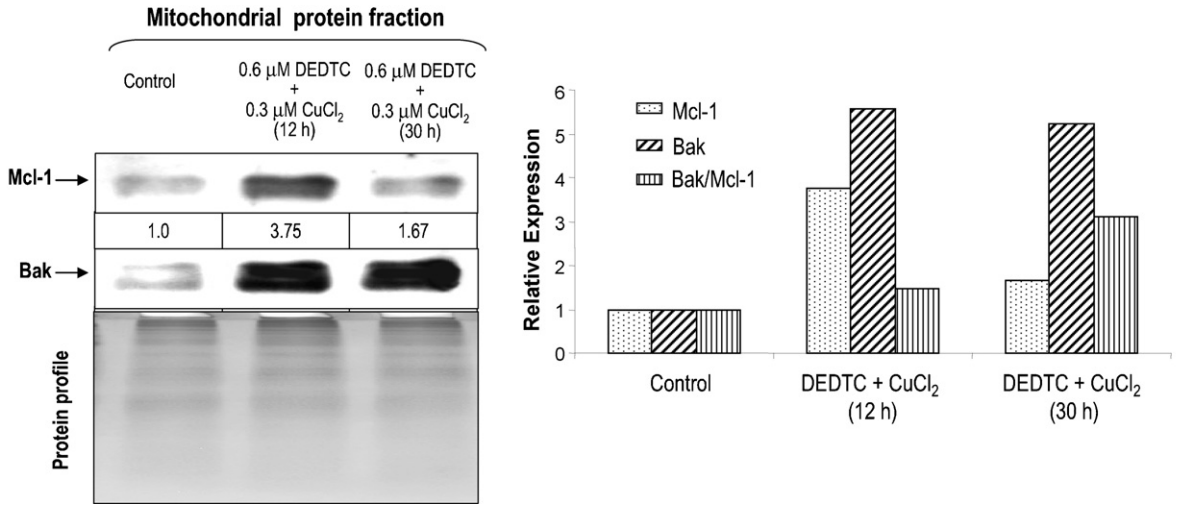

Fig. 6 – Cytotoxic effect of Cu $[DEDTC]_2$ vs. C8161 melanoma involves an increase in the Bak/Mcl-1 ratio in mitochondrial fraction. Mitochondria prepared and lysed as indicated under Materials and Methods was fractionated by SDS–PAGE, followed by bidirectional passive transfer to replicate nitrocellulose membranes and detection of mitochondrial protein Bak and Mcl-1 with monospecific antibodies and their peroxidase-conjugated secondary antibodies, evaluated in each membrane by chemiluminescence. Note the transient increase in Mcl-1 and a stable increase in Bak following treatment with Cu$[DEDTC]_2$, leading to a sequential increase in the Bak/Mcl-1 mitochondrial ratio. Results shown representing the ratio of Bak to Mcl-1 from a bidirectional transfer of the same SDS–PAGE gel, were normalized to comparable protein loading levels.

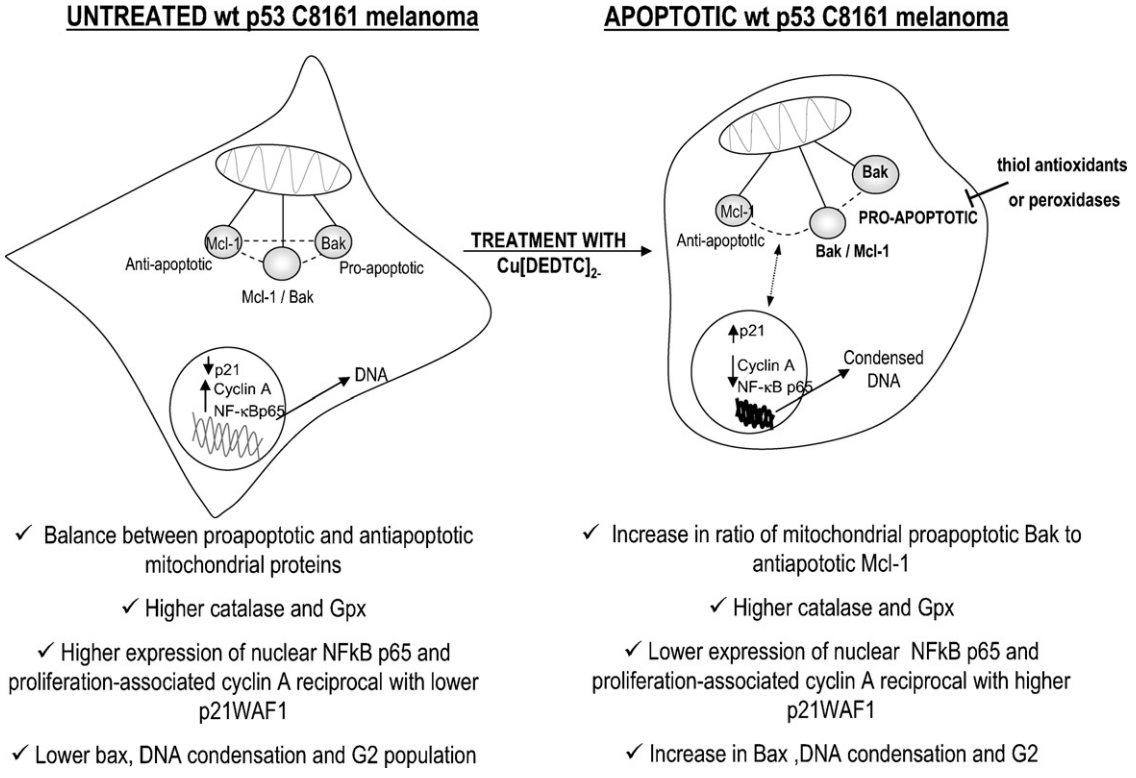

Fig. 7 – Model summarizing possible mechanism of lethality induced by Cu$[DEDTC]_2$ against C8161. In untreated cells, the cyclin-dependent kinase inhibitor p21 is low, proliferation-associated cyclin A NFkBp65 are high, together with catalase and GPx antioxidant activities. After treatment with Cu $[DEDTC]_2$, there is an increase in the ratio of mitochondrial proapoptotic Bak to antiapoptotic Mcl-1 proteins, increases in Bax and p21WAF1, greater nuclear DNA condensation and a decline in cyclin A, NFKBp65, catalase and GPx.

high in contrast to a loss in Mcl-1 (Fig. 6) coinciding with cell rounding and evidence of apoptosis-associated PARP cleavage seen in parental cells (Fig. 4A).

4. Discussion

To learn about determinants of susceptibility to the $Cu[DEDTC]_2$ complex, we now used wt p53 human C8161 melanoma and mutant p53 SKBR3 human breast carcinoma. The latter cells showed a significant susceptibility to this complex at a ratio of 0.2 μM: 0.1 μM of $Cu[DEDTC]_2$. In contrast, this concentration did not affect the proliferation or survival of human C8161 melanoma, which required a threefold higher concentration of $Cu[DEDTC]_2$, to show a cytotoxic response. An activity assay demonstrated that at the respective toxic concentrations, both cell types showed an increase in mitochondrial Mn SOD without a comparable increase in cytosolic Cu/Zn SOD [21] suggesting that a possible increase in the conversion of superoxide to hydrogen peroxide occurs in a p53-independent manner, reason why we investigated whether enzymatic and non-enzymatic anti-oxidants controlled the cytotoxic response. The 3-fold fold higher concentration of $Cu[DEDTC]_2$, needed for a cytotoxic response versus C8161 melanoma correlated with a similar greater basal level of glutathione peroxidase and catalase in these cells, compared to those in the more susceptible SKBR3 carcinoma cells. The importance of these activities in controlling susceptibility to $Cu[DEDTC]_2$ was emphasized by the demonstration that C8161 cells selected for resistance to the complex showed persistent high levels of glutathione peroxidase and catalase in the presence of (0.8 μM DEDTC: 0.4 μM Cu), in contrast to the loss of these activities in parental C8161 cells exposed to only (0.6 μM DEDTC: 0.3 μM Cu) (see Fig. 4). The relevance of hydrogen peroxide-degrading enzymes in susceptibility to $Cu[DEDTC]_2$ was further suggested by the suppression of complex cytotoxicity against SKBR3 and parental C8161 cells by a 1-h pre-treatment with exogenous peroxidase, or catalase. This finding is compatible with others who demonstrated that in spite of the high permeability of H_2O_2, gradients across membranes are indeed formed when a membrane separates the production and consumption sites of H_2O_2 [26]. When cells are exposed to external H_2O_2, the fast consumption of H_2O_2 inside the cells provides the driving force for setting up a gradient across the plasma membrane, with the intracellular concentration of H_2O_2 lower than the extracellular one [26]. Addition of exogenous catalase to cell cultures scavenges hydrogen peroxide diffused from cells, resulting in subsequent depletion of intracellular peroxide [26,27].

Solubility of the $Cu[DEDTC]_2$ complex and its ability to penetrate and accumulate inside cells, does not exclude as a possible mechanism that the H_2O_2 increased inside in response to the complex, forms a gradient across membranes increasing extracellular peroxide and complex lethality [26]. Elimination of the latter by exogenous hydrogen peroxide-degrading enzymes may perhaps explain why these treatments diminish toxicity of $Cu[DEDTC]_2$.

Also, suppression of complex cytotoxicity against SKBR3 and parental C8161 cells by a 1–h pre-treatment with exogenous glutathione or NAC, a glutathione precursor [24]. The significance of the latter was emphasized by results showing that a pre-treatment with 1 mM NAC was enough to protect the moderately susceptible C8161 melanoma, in contrast to 4 mM NAC required to protect the highly susceptible SKBR3 cells from $Cu[DEDTC]_2$.

We also noticed that the anti-apoptotic nuclear NFκB p65 protein was lost and apoptosis-associated PARP cleavage occurred in the susceptible and intermediate cell types within 3 h of treatment with the copper complex, and this may explain why NAC protection is effective only when preceding addition of the complex. Frequently, cell death involves loss of nuclear NFkB p65 in cells harbouring wt. p53 [28,29], but this seems to be occurring also in SKBR3 cells types, irrespective of their mutant p53 status [14]. Although earlier studies demonstrated that $Cu[DEDTC]_2$ shows preferential toxicity versus melanoma compared to melanocytes [12], little was reported about the mechanism of this action. We now show that human metastatic C8161 melanoma [16] undergo G2 accumulation together with DNA condensation and bax induction, together with in mitochondrial pro-apoptotic Bak relative to anti-apoptotic Mcl-1 (See summary, Fig. 7). In a future study, we plan to compare pro-apoptotic and antiapoptotic gene expression in susceptible and resistant C8161 cells. We also demonstrate for the first time that selection for resistance to $Cu[DEDTC]_2$ yields cells with persistently high catalase and glutathione peroxidase activities. The reported lower toxicity of $Cu[DEDTC]_2$ for normal cells [12] and the mechanism of action now reported, suggesting peroxide-mediated killing and mitochondrial pro-apoptotic targets, implies that this complex may be useful as an adjuvant against tumors resistant to traditional genotoxic anti-cancer therapies.

REFERENCES

[1] Wei M, Zong W-X, Cheng E, Lindsten T, Panoutsakopoulou V, Ross A, et al. Proapoptotic Bax and Bak: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727–30.
[2] Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev 2005;19:1294–305.
[3] Adams JM. Ways of dying: multiple pathways to apoptosis. Genes Dev 2003;17:2481–95.
[4] Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004;116:205–19.
[5] Suzuki M, Youle RJ, Tjandra N. Structure of Bax: coregulation of dimer formation and intracellular localization. Cell 2000;103:645–54.
[6] Minet E, Cosse JP, Demazy C, Raes M, Michiels C. Accumulation of the pro-apoptotic factor Bak is controlled by antagonist factor Mcl-1 availability. Apoptosis 2006;11:1039–47.
[7] Lindsten T, Ross AJ, King A, Zong W-X, Rathmell JC, Shiels HA, et al. The combined functions of the proapoptotic Bcl-2 family members, Bak and Bax, are essential for the normal development of multiple tissues. Mol Cell 2000;6:1389–99.
[8] Leu JI, Dumont P, Hafey M, Murphy ME, George DL. Mitochondrial p53 activates Bak and causes disruption of a Bak–Mcl1 complex. Nat Cell Biol 2004;6:443–50.

[9] Rieber M, Strasberg Rieber M. Tumor suppression without differentiation of apoptosis by antisense cyclin D1 gene transfer in K1735 melanoma involves induction of p53, p21WAF1 and superoxide dismutases. Cell Death Diff 1999;6:1209–15.
[10] Hussain SP, Amstad P, He P, Robles A, Lupold S, Kaneko I, et al. p53-induced up-regulation of Mn SOD and GPx but not catalase increases oxidative stress and apoptosis. Cancer Res 2004;64:2350–6.
[11] Gouaze V, Andrieu-Abadie N, Cuvillier O, Malagarie-Cazenave S, Frisach MF, Mirault ME, et al. Glutathione peroxidase-1 protects from CD95-induced apoptosis. J Biol Chem 2002;277:42867–74.
[12] Cen D, Brayton D, Shahandeh B, Meyskens Jr FL, Farmer PJ. Disulfiram facilitates intracellular Cu uptake and induces apoptosis in human melanoma cells. J Med Chem 2004;27:6914–20.
[13] Viola-Rhenals M, Strasberg Rieber M, Rieber M. Suppression of survival in human SKBR3 breast carcinoma in response to metal–chelator complexes is preferential for copper-dithiocarbamate. Biochem Pharmacol 2006;71:722–34.
[14] Geisler S, Lonning PE, Aas T, Johnsen H, Fluge O, Haugen DF, et al. Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer. Cancer Res 2001;61: 2505–12.
[15] Alvarez M, Strasberg Rieber M, Rieber M. Chromosome-6 mediated suppression of metastatic ability increases basal expression of UV-inducible superoxide dismutase and induction of p53. Int J Cancer 1998;77:586–91.
[16] Ryan KM, Ernst MK, Rice NR, Vousden KH. Role of NF-κB in p53-mediated programmed cell death. Nature 2000;404:892–7.
[17] ÓBrien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 2000;267:5421–6.
[18] Freshney RI. Culture of animal cells. a manual of basic technique, 4th ed., USA: Wiley-Liss Inc.; 2002, 332–335.
[19] Deng Y, Wu X. Peg3yPw1 promotes p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria. Proc Natl Acad Sci USA 2000;97:12050–5.
[20] Rieber M, Strasberg Rieber M. Signalling responses linked to betulinic acid-induced apoptosis are antagonized by MEK inhibitor U0126 in adherent or 3D spheroid melanoma irrespective of p53 status. Int J Cancer 2006;118:1135–43.
[21] Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971;44:276–87.
[22] Gomez-Sarosi LA, Strasberg-Rieber M, Rieber M. Hydrogen peroxide increases a 55-kDa tyrosinase concomitantly with induction of p53-dependent p21 waf1 expression and a greater Bax/Bcl-2 ratio in pigmented melanoma. Biochem Biophys Res Commun 2003;312:355–9.
[23] Soldani C, Lazze MC, Bottone MG, Tognon G, Biggiogera M, Pellicciari CE, et al. Poly(ADP-ribose) polymerase cleavage during apoptosis: when and where? Exp Cell Res 2001;269:193–201.
[24] Moran LK, Gutteridge JMC, Quinlan GJ. Thiols in cellular redox signalling and control. Curr Med Chem 2001;8:763–72.
[25] Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R, Pavelka M. Chromatin condensation during apoptosis is accompanied by degradation of lamin A + B, without enhanced activation of cdc2 kinase. J Cell Biol 1994;126: 827–37.
[26] Antunes F, Cadenas E. Estimation of H_2O_2 gradients across biomembranes. FEBS Lett 2000;475:121–6.
[27] Pincheira J, Romero P, Marcelain K, Salazar L, de la Torre C. G2 checkpoint-dependent DNA repair and its response to catalase in Down syndrome and control lymphocyte cultures. Cell Biol Int 2007;31:135–40.
[28] Ravi R, Mookerjee B, van Hensbergen Y, Bedi GC, Giordano A, El-Deiry WS, et al. p53-mediated repression of nuclear factor-kappaB RelA via the transcriptional integrator p300. Cancer Res 1998;58:4531–6.
[29] Webster GA, Perkins ND. Transcriptional cross talk between NF-kappa B and p53. Mol Cell Biol 1999;19:3485–95.