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Copper enriches efficacy of Dp44mT in breast cancer cells

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Abstract: Neoplastic cells need essential metals, such as iron and copper, for cellular functions and rapid growth. Metal chelation and changes in their redox cycle in favor of oxidative stress may be critical for making these cells vulnerable to cell death. The HER2-overexpressing breast cancer cell line, MCF7-HER2, and its vehicle control, MCF7-vec cells, were treated with $CuCl_2$, $FeSO_4$, and Dp44mT. Reactive oxygen species (ROS) production in response to these chemicals was detected with flow cytometry, and cell viability was measured by MTT assay in the cells. ROS levels were relatively increased by Dp44mT in the cells, and this was reversed by a combination of iron, while a copper combination further induced ROS. Parallel changes were observed in the inhibition of cell growth by Dp44mT, and NAC partially rescued the inhibition. Additionally, copper decreased the CSC population by half relative to the control in MCF7-HER2 cells. Copper supplement enriches ROS production mediated by Dp44mT in MCF7 cells. However, iron addition recovers Dp44mT-depleted iron levels, yet has no effect on ROS generation. Copper treatment also decreases the proportion of CSCs. In this manner, Dp44mT depletes iron and binds copper to form a redox active complex, which leads to oxidative stress. This dual cytotoxic case is significant for the survival of cancer cells.

Key words: Copper, iron, Dp44mT, metal chelation, reactive oxygen species, HER2

1. Introduction

Cancer cells need greater levels of iron than normal cells in order to achieve higher rates of proliferation and cellular functions (Yu et al., 2006). This occurs via elevated iron uptake at an accelerated rate, making cancer cells vulnerable to iron chelation (Trinder et al., 1996). In addition to iron, these cells strongly require copper relative to their normal counterparts, because it is essentially used in the formation of new blood vessels, termed as angiogenesis, and following tumor growth invasion and metastasis (Gourley and Williamson, 2000; Fox et al., 2001; Finney et al., 2007; Gupte and Mumper, 2009). These essential transition metals, iron and copper, play a significant role in the activity of many enzymes that are critical for growth, energy supply, and development (Buss et al., 2004; Cai et al., 2005; Kalinowski and Richardson, 2005; Denoyer et al., 2015). By acting as cofactors within enzyme active sites, both iron and copper are able to manage the redox cycle (Kalinowski and Richardson, 2005; Denoyer et al., 2015). During the cycle, transferal of electrons to oxygen causes generation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$). This leads to oxidative stress and is followed by cytotoxic damage in proteins, lipids, and DNA (Kalinowski and Richardson, 2005; De Domenico et al., 2008; Yu et al., 2009; Jomova and Valko, 2011). As a result, an intrinsic balance between ROS formation and biological usage is tightly regulated by the concentration of these metals (Grubman and White, 2014; Lane et al., 2015).

Remarkably, neoplastic cells exhibit alterations in the homeostasis and metabolism of iron and copper (Turski and Thiele, 2009; Torti and Torti, 2013), suggesting a link between the development of malignancies and these metal levels (Toyokuni, 2009). This reveals that cancer cells are potentially sensitive to metal chelation, and that targeting these metals has become a promising anticancer strategy (Kalinowski and Richardson, 2005; Pahl and Horwitz, 2005; Yu et al., 2006; Merlot et al., 2013). One of the most effective iron chelators is di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone (Dp44mT), which markedly indicates anticancer activity (Yuan et al., 2004; Whitnall et al., 2006; Rao et al., 2009; Tian et al., 2010). It acts as an iron chelator and redox cycles of its iron complex in order to generate ROS (Yuan et al., 2004; Richardson et al., 2006; Jansson et al., 2010a). Dp44mT binds both iron and copper, and these drug–metal complexes with redox

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activity lead to an increase in their massive cytotoxicity (Jansson et al., 2010b).

Breast cancer tissues contain high levels of copper (Huang et al., 1999). Parallel changes have been observed in tumorigenesis, metastasis, the proportion of stem cell-like cancer cells (CSCs), and human epidermal growth factor receptor 2 (HER2), which is overexpressed in approximately 30% of patients with breast cancer (Korkaya et al., 2008; Magnifico et al., 2009; Lo et al., 2012). If copper plays an integral role in tumorigenesis and metastasis, there may be a link between copper and HER2. In this study, ROS formation in HER2 overexpression and treatments of Dp44mT, copper(II) chloride ($CuCl_2$), and iron(II) sulfate ($FeSO_4$) was examined in a breast cancer cell line, MCF7. Cell viability was then determined in a combination of iron, copper, and antioxidant N-acetylcysteine (NAC) with Dp44mT in the cells. Finally, the impact of copper on Dp44mT-inhibited cell growth and the proportions of CSCs were identified.

2. Materials and methods

2.1. Cell culture

The MCF7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 5 μg/mL insulin (Sigma, St. Louis, MO, USA), and 1% penicillin/streptomycin solution (Invitrogen, Gaithersburg, MD, USA). These were grown in 10-cm^2 tissue culture dishes (Corning, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2. Subconfluent cells (70%–80% confluency) were subcultured following trypsinization (0.05% trypsin-EDTA, Invitrogen) every 3–4 days.

2.2. Flow cytometry for stem cell-like cells

Antihuman CD44-fluorescein (FITC, clone G44-26) and antihuman CD24-phycoerythrin (PE, clone ML5) (BD Biosciences, Franklin Lakes, NJ, USA) were used for the analysis. Cells were digested with 0.25% trypsin to produce a single cell suspension and were washed twice with a staining buffer (PBS solution containing 0.1% FBS). The cell concentration was adjusted to 1×10^6 cells in 100 μL of buffer. Antibodies were added to the cell suspension at concentrations recommended by the manufacturer and staining was performed in the dark at 4 °C for 30 min, followed by two washes with the same buffer. Samples were acquired with a BD Accuri C6 (Becton Dickinson, San Jose, CA, USA) and analysis was performed with the manufacturer's software (BD Accuri C6 software). The CSC population represents $CD44^+/CD24^-$ cells on histograms.

2.3. Detection of ROS by flow cytometry

Cells were collected with 0.25% trypsin and were washed twice with PBS buffer. They were suspended with serum-free medium containing 0.5 μM 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes, Eugene, OR, USA) as a specific dye probe, which fluoresces on oxidation by reactive oxygen species to 2′, 7′-dichlorofluorescein (DCF). Samples were incubated in the dark at 37 °C for 30 min, followed by two washes with the buffer. They were suspended in a PBS buffer and were acquired with a BD Accuri C6 (Becton Dickinson). Mean fluorescence for each treatment was obtained and analysis was performed with the manufacturer's software (BD Accuri C6 software).

2.4. MTT cell proliferation assay

A total of 6000 cells/well were seeded in a 96-well plate and incubated in growth media with varying concentrations of Dp44mT and its combinations with 2 mM NAC, 1 mM $FeSO_4$, and 100 μM $CuCl_2$. After 4 days, the medium was removed, and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in PBS) was added to each well along with 90 μL of fresh medium. The plate was incubated for 4 h at 37 °C and the medium was aspirated and washed with 1X PBS. We added 150 mL of dimethyl sulfoxide to each well, and the plates were placed on an orbital shaker for 5 min at maximum speed. The plate was read at a wavelength of 590 nm using a microplate reader (BioTek Epoch, Winooski, VT, USA). Triplicate wells were used for each treatment, and the experiments were repeated three times.

2.5. Tumorsphere formation assay

Adherent MCF7 cells were dissociated by 0.25% trypsin-EDTA (Invitrogen) and suspended in serum-free DMEM/F12 medium (Invitrogen), supplemented with 20 ng/mL epidermal growth factor (EGF, Sigma), 10 ng/mL basic fibroblast growth factor (bFGF, Sigma), 5 μg/mL insulin (Sigma), 1X B27 supplement (Invitrogen, NY, USA), and 0.4% bovine serum albumin (BSA, Sigma). Cells were plated in ultralow attachment 6-well plates (Corning) at a density of 10,000 cells/mL and were then incubated under 5% CO_2 at 37 °C for 1 week (Gu et al., 2011; Lo et al., 2012).

3. Results

3.1. Copper enhances Dp44mT-induced ROS formation

Patients with breast cancer have approximately 30% overexpression of HER2, which is correlated with aggressive phenotypes and poor prognosis (Korkaya et al., 2008; Magnifico et al., 2009; Lo et al., 2012). In order to determine whether this overexpression results in ROS generation or not, basal ROS levels in MCF7 cells carrying only the vector (MCF7-vec) and its HER2-overexpressed line (MCF7-HER2) were measured by fluorescent probe H_2DCFDA staining. With a shift to the right in DCF fluorescence, an increase in ROS levels were observed in MCF7-HER2 relative to MCF7-vec cells (Figure 1a). To test the effect of Dp44mT on ROS levels in these cells, they were exposed to increasing concentrations (0.1, 1, and 10 μM) of Dp44mT for 4 days and relative ROS levels were

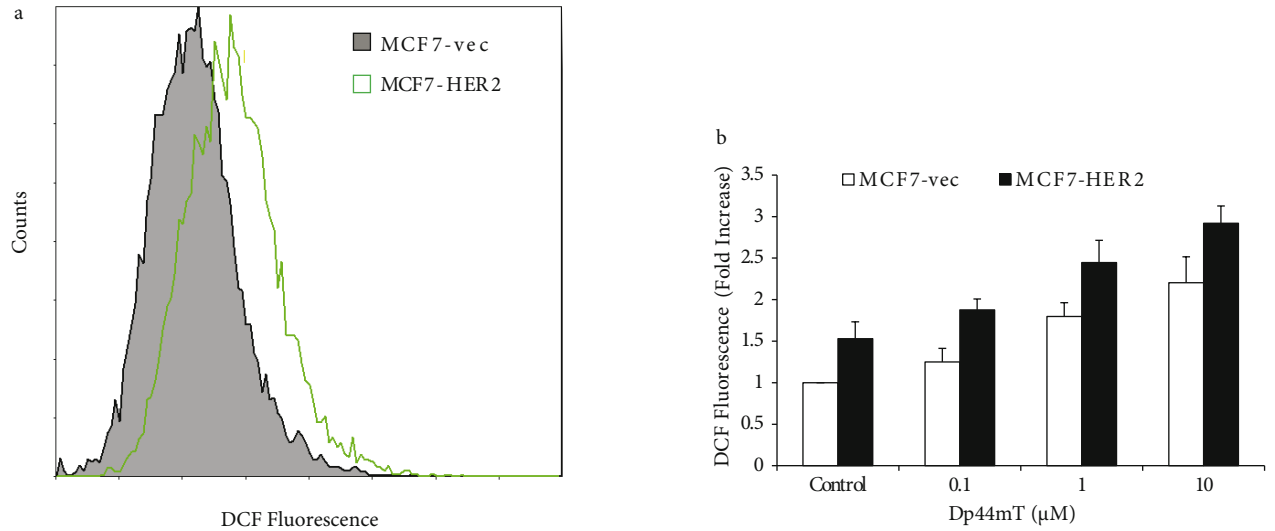

Figure 1. ROS formation in response to HER2 overexpression and Dp44mT. a) Basal ROS levels were measured in MCF7-vec and MCF7-HER2 cells utilizing fluorescent agent H_2DCFDA. b) Cells were treated with 0.1, 1, and 10 μM Dp44mT for 24 h and relative ROS levels were measured.

detected. MCF7-vec control treatment was set as 1, and fold increases over the control were calculated for each treatment. It was observed that ROS levels were relatively induced by Dp44mT up to 2-fold (Figure 1b).

Iron and copper are involved in the redox cycle and in the transfer of electrons to oxygen, leading to ROS formation following oxidative stress (Kalinowski and Richardson, 2005; Denoyer et al., 2015). In order to examine whether these metals alter ROS levels or not, MCF7-vec and MCF7-HER2 cells were treated with 1 mM $FeSO_4$ and 1 μM $CuCl_2$. They slightly increased ROS levels in both cells (Figures 2a and 2b). To assess the impact of the metals on Dp44mT-mediated ROS production, they were cotreated to cells. Iron addition rescued the drug-induced ROS generation, whereas copper enhanced it (Figures 2a and 2b).

3.2. Copper elevates inhibition of cell growth induced by Dp44mT

Iron and copper are essential metals for the activity of many enzymes involved in cell growth, energy supply, and development (Buss et al., 2004; Cai et al., 2005; Kalinowski and Richardson, 2005; Denoyer et al., 2015). Higher requirement for these metals in neoplastic cells makes these cells vulnerable to metal chelation (Kalinowski and Richardson, 2005; Yu et al., 2006; Merlot et al., 2013). To determine whether there is sensitivity of breast cancer

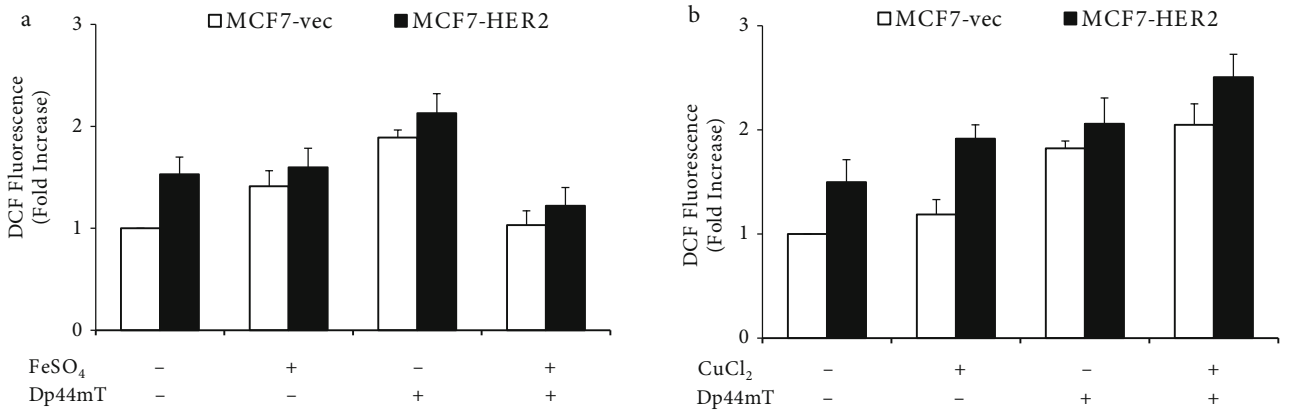

Figure 2. Effects of iron and copper on Dp44mT-induced ROS. a, b) Cells were treated with ±1 mM $FeSO_4$ and 1 μM $CuCl_2$, and their combinations with 1 μM Dp44mT for 24 h. ROS levels in response to chemicals were measured. Bars represent fold increase of the mean fluorescence ± SEM from 3 experiments.

cells to the chelation of these metals, MCF7-vec and MCF-HER2 cells were treated with increasing concentrations (1–1000 nM) of Dp44mT and its combinations with the metals for 4 days, and cell viability was measured. Single treatments were set as 100% and the percentage of viability was calculated only in the Dp44mT control and the combination treatments. In the highest Dp44mT concentration, iron addition markedly recovered drug-mediated growth inhibition in MCF7-vec cells, from 5% to 75%, and MCF7-HER2 cells, from 23% to 81% (Figures 3a and 3b). On the other hand, copper addition significantly abrogates the growth inhibition in MCF7-vec, from IC_{50} ~80 nM to IC_{50} ~18 nM, and in MCF7-HER2, from IC_{50} ~200 nM to IC_{50} ~22 nM (Figures 3a and 3b).

In order to evaluate if this growth inhibition originates from drug-increased ROS production, NAC was added to Dp44mT treatments. Dp44mT-induced growth inhibition was partially rescued by NAC supplement (Figures 3a and 3b).

3.3. Copper reduces the proportion of CSCs

Increase in HER2 expression is associated with maintenance of CSCs (Korkaya et al., 2008; Magnifico et al., 2009). To indicate the ability of copper to diminish the proportion of CSCs through the $CD44^+/CD24^-$ antigenic phenotype (Al-Hajj et al., 2003) in breast cancer cells, MCF7-HER2 cells were treated with 1 μM $CuCl_2$ for 5 days. The proportion of CSCs was reduced by half in copper treatment relative to the control (Figure 4a). In order to validate this, the cells were grown in a specific medium that allows only growth of CSCs, finally forming tumorspheres. Pictures representing the whole dish were taken under a light microscope (Figure 4b). Spheres between 30 μm and 80

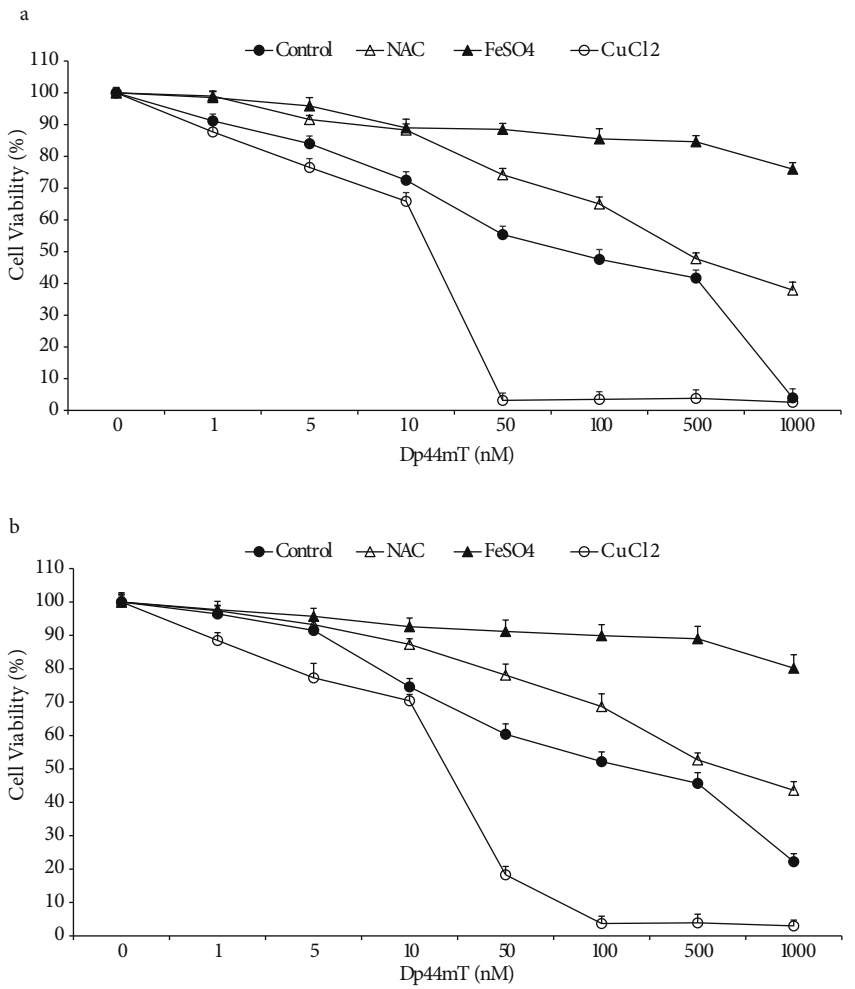

Figure 3. Effects of iron, copper, and NAC on Dp44mT-induced growth inhibition. a) MCF7-vec cells were treated with increasing concentrations of Dp44mT as indicated and its combinations with 1 mM $FeSO_4$, 100 μM $CuCl_2$, and 2 mM NAC for 4 days, and the cell viability (MTT) assay was performed. b) MCF7-HER2 cells were exposed to similar treatments and the MTT assay was performed. Experiments were repeated 3 times and the percentage of viable cells was calculated (±SEM).

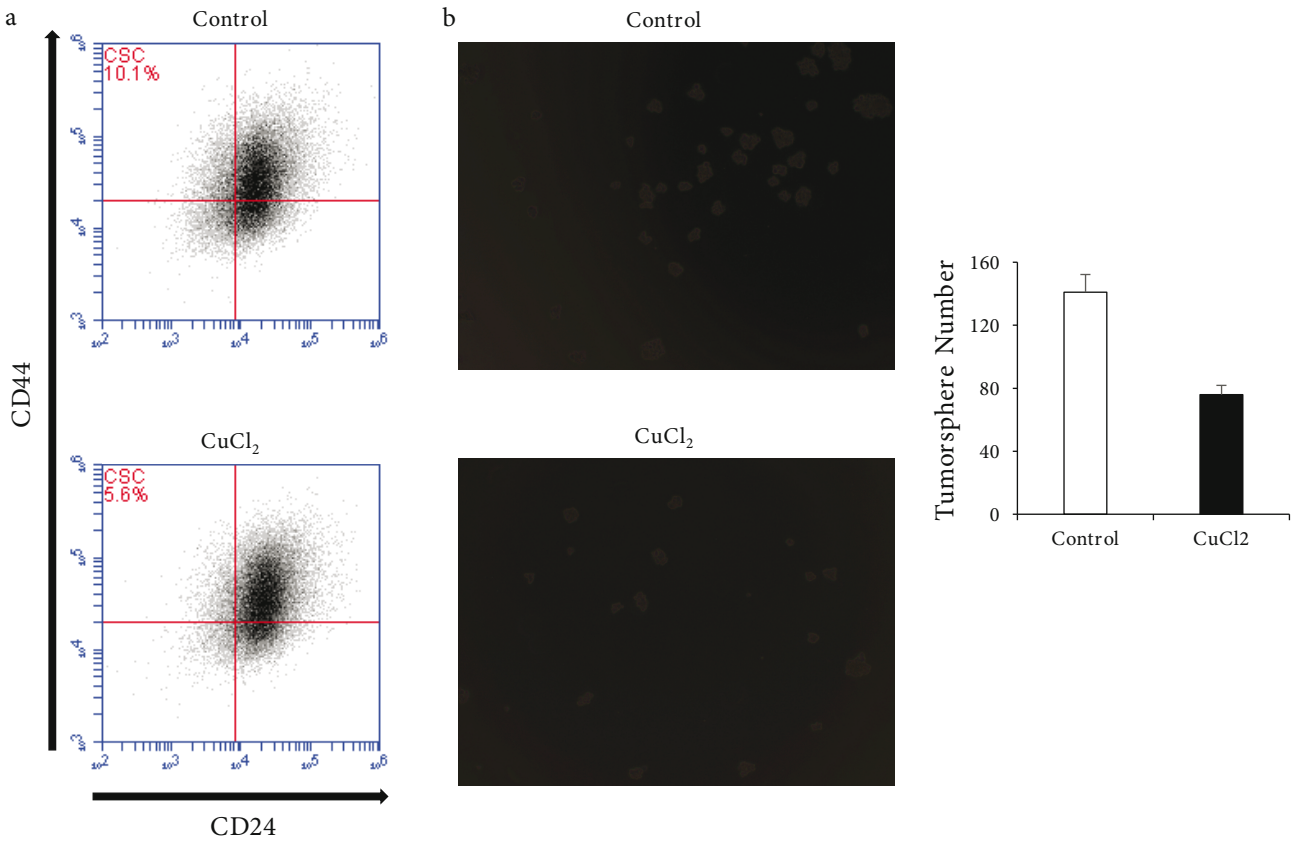

Figure 4. CSC population and tumorsphere formation in response to $CuCl_2$ in MCF7-HER2 cells. a) Cells were treated with 1 µM $CuCl_2$ for 24 h and the proportion of CSCs was assayed by flow cytometry. b) After copper treatment, cells were transferred to sphere growth media for 7 days and tumorspheres were counted and pictured. The results represent 3 separately repeated experiments (±SEM).

µm were counted from the pictures and plotted. Similar to the $CD44^+/CD24^-$ flow cytometry assay, copper decreased the formation of tumorspheres (Figure 4b).

4. Discussion

Copper is highly required by cancer cells for angiogenesis, tumor growth, and eventually metastasis (Gourley and Williamson, 2000; Fox et al., 2001; Finney et al., 2007; Gupte and Mumper, 2009). Cancer cells also need iron to keep up with rapid proliferation and cellular functions (Yuan et al., 2006). These metals act critically not only on cell growth and development, but also on the redox cycle that leads to ROS production through the transfer of electrons to oxygen (Buss et al., 2004; Cai et al., 2005; Kalinowski and Richardson, 2005; Denoyer et al., 2015). Thus, they tightly organize a balance between their biological utilization and ROS generation in neoplastic cells (Grubman and White, 2014; Lane et al., 2015), indicating the significance of the metals in tumor development (Toyokuni, 2009; Turski and Thiele, 2009; Torti and Torti, 2013). As a result, sensitizing cancer cells to metal chelation has become an attractive strategy in the development of anticancer drugs (Kalinowski and Richardson, 2005; Merlot et al., 2013). Dp44mT is a well-known chelator that binds both iron and copper, thereby causing high cytotoxicity (Yuan et al., 2004; Whitnall et al., 2006; Rao et al., 2009; Jansson et al., 2010b; Tian et al., 2010). In breast cancer cells, correlative increases have been seen in levels of copper (Huang et al., 1999) and HER2 expression, followed by a gradual increase in the proportion of CSCs (Korkaya et al., 2008; Magnifico et al., 2009; Lo et al., 2012), suggesting that there may be a link between these increases.

In the current study, the combination of the metals iron and copper with Dp44mT-mediated ROS generation and cytotoxicity was investigated in breast cancer cells. This was performed on HER2-overexpressing MCF7-HER2 and its control MCF7-vec cells. Basal ROS levels were higher in MCF7-HER2 cells compared to MCF7-vec cells (Figure 1a). This may be due to adaptation to oxidative stress and aggressive growth as a result of HER2 overexpression. Then it was shown that Dp44mT relatively increased ROS production in MCF7-vec and MCF7-HER2 cells (Figure 1b). This production was rescued by exogenously supplementing iron while it was

being boosted with the addition of copper, suggesting that Dp44mT forms a preferentially redox complex with copper rather than iron (Figures 2a and 2b).

Dp44mT results in the inhibition of growth because of iron and copper depletion in both MCF-vec and MCF-HER2 cells, and this inhibition was greatly rescued by iron addition (Figures 3a and 3b). In contrast to iron, copper addition tremendously enriched Dp44mT-mediated growth inhibition in both cells, indicating the oxidative effect of the Dp44mT–copper complex on cell death. Partially rescuing drug-induced inhibition by NAC confirms the contribution of oxidative stress to the inhibition (Figure 3). In addition to the enhancement of growth inhibition, copper reduced the proportion of CSCs in MCF7-HER2 cells (Figure 4). These findings show that increases in Dp44mT-copper redox active complexes advance the anticancer activity of the drug due to the dual effect of metal chelation and oxidative stress. Targeting selectively vulnerable factors of cancer cells will bring new insights into treatment. Thus, an optimal understanding of copper facts in cancer metabolism may further our understanding of the efficacy of cancer chemotherapeutics.

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