D-penicillamine combined with inhibitors of hydroperoxide metabolism enhances lung and breast cancer cell responses to radiation and carboplatin via H_2O_2-mediated oxidative stress

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Abstract

D-penicillamine (DPEN), a copper chelator, has been used in the treatment of Wilson's disease, cystinuria, and rheumatoid arthritis. Recent evidence suggests that DPEN in combination with biologically relevant copper (Cu) concentrations generates H_2O_2 in cancer cell cultures, but the effects of this on cancer cell responses to ionizing radiation and chemotherapy are unknown. Increased steady-state levels of H_2O_2 were detected in MB231 breast and H1299 lung cancer cells following treatment with DPEN (100 μM) and copper sulfate (15 μM). Clonogenic survival demonstrated that DPEN-induced cancer cell toxicity was dependent on Cu and was significantly enhanced by depletion of glutathione [using buthionine sulfoximine (BSO)] as well as inhibition of thioredoxin reductase [using Auranofin (Au)] prior to exposure. Treatment with catalase inhibited DPEN toxicity confirming H_2O_2 as the toxic species. Furthermore, pretreating cancer cells with iron sucrose enhanced DPEN toxicity while treating with deferoxamine, an Fe chelator that inhibits redox cycling, inhibited DPEN toxicity. Importantly, DPEN also demonstrated selective toxicity in human breast and lung cancer cells, relative to normal untransformed human lung or mammary epithelial cells and enhanced cancer cell killing when combined with ionizing radiation or carboplatin. Consistent with the selective cancer cell toxicity, normal untransformed human lung epithelial cells had significantly lower labile iron pools than lung cancer cells. These results support the hypothesis that DPEN mediates selective cancer cell killing as well as radio-chemo-sensitization by a mechanism involving metal ion catalyzed H_2O_2-mediated oxidative stress and suggest that DPEN could be repurposed as an adjuvant in conventional cancer therapy.

Graphical abstract

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Increasing Oxidative Stress in a Cancer Cell
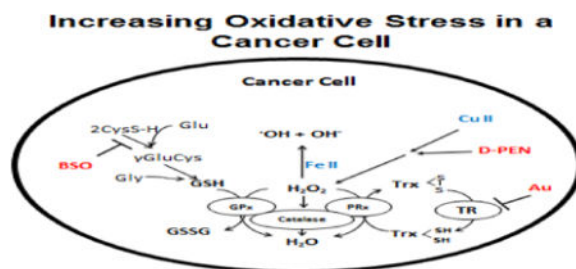
Keywords

D-penicillamine; transition metal ions; breast cancer; lung cancer; oxidative metabolism; hydrogen peroxide

Introduction

The most successful adjuvants to cancer therapy are selectively cytotoxic to malignant cells while sparing normal tissues. Recently there has been a great deal of interest in repurposing redox active drugs that have been used in humans as adjuvants to cancer therapy based on fundamental differences in oxidative metabolism between normal and cancer cells leading to increased steady-state levels of $O_2^{\bullet-}$ and H_2O_2 [1-3]. This paradigm of drug repurposing is based on the in depth assessment of biochemical differences between tumor and normal cell redox metabolism targetable for therapy development. Recent evidence continues to support the hypothesis that cancer cells have aberrant mitochondrial function, leading to increased steady state levels of $O_2^{\bullet-}$ and H_2O_2, compared to normal cells [4-6]. Because of the elevated baseline levels of reactive oxygen species (ROS) in cancer cells, it has also been proposed that using agents that further increase ROS levels will lead to selective cell death in cancer cells while leaving normal cells, relatively unaffected [1, 6-8].

Recent data also suggests that metabolism of redox active metal ions such as Cu and Fe is also dysregulated in tumor versus normal cells. For example, breast and lung cancer patients contain elevated serum copper levels when compared to cancer-free patients [8]. Breast and lung tumors also contain markedly increased copper when compared to their matched normal tissue [9-11]. Labile iron concentrations are also increased in both serum and tumor tissue from cancer patients [12]. Currently, there is also promising research focusing on cancer treatment strategies based on copper and iron chelation [13, 14].

D-penicillamine (DPEN) is an aminothiol and copper chelator used in the treatment of Wilson's disease, cystinuria, and rheumatoid arthritis. When DPEN chelates Cu(II) in either the labile or ceruloplasmin-bound form, it is reduced to Cu(I) and can generate ROS via one electron reductions of O_2 to form $O_2^{\bullet-}$ that rapidly dismutes to form H_2O_2 [15] [16]. In addition, increased levels of $O_2^{\bullet-}$ in cancer cells could lead to increased labile Fe(II) that could react with H_2O_2 to form $^{\bullet}OH$, creating a selectively cytotoxic environment in cancer cells treated with DPEN [17].

In the current report increased steady-state levels of H_2O_2 were quantified in lung and breast cancer cells *in vitro* when treated with DPEN and physiologically relevant concentrations of

copper. DPEN also induced clonogenic cell killing in cancer cells that was inhibited by catalase demonstrating the H_2O_2-dependence of this biological response. DPEN-induced clonogenic cell killing was further enhanced via inhibition hydroperoxide metabolism in both lung and breast cancer cells using the thioredoxin reductase inhibitor auranofin (Au) and the glutathione synthesis inhibitor buthionine sulfoximine (BSO). In addition, the importance of redox active Fe in DPEN-induced effects was apparent when cancer cells were sensitized to DPEN-induced killing by pre-treatment with Fe-sucrose and protected by deferoxamine. Importantly the toxicity of DPEN was significantly greater in human breast and lung cancer cells as compared to non-immortalized primary human mammary and bronchial epithelial cells possibly because of the higher baseline labile iron pools in cancer cells compared to normal cells. DPEN also enhanced the cytotoxicity of carboplatin and radiation in human cancer cells. These results support the hypothesis that DPEN-induced cytotoxicity and radio-chemosensitization are mediated by H_2O_2 and redox active metals as well as supporting the speculation that DPEN could be repurposed as an adjuvant in cancer therapy.

Methods

Cells and Culture Conditions

MB231 human breast carcinoma cells, H1299 human lung carcinoma cells and H292 human lung carcinoma cells were obtained from ATCC and maintained in RPMI 1640 (Mediatec) with 10% fetal bovine serum (FBS; HyClone). Non-immortalized primary human bronchial epithelial cells (HBEpC) were obtained and maintained as suggested in bronchial/tracheal epithelial cell growth media from Cell Applications, Inc. Non-immortalized primary human mammary epithelial cells (HMEC) were obtained and maintained in mammary epithelial growth media as suggested by the vendor (Lonza). Experiments with non-transformed cells (HBEpC and HMEC) were performed between 3-10 population doublings from receipt of the cells from the company. In our experience, the doubling time of the cells did not change until after 10 population doublings. These cells maintained a healthy replicative lifespan in culture which was verified in each experiment by measuring plating efficiency.

Clonogenic Cell Survival Assay

120,000 H1299 cells, 125,000 MB231 or 150,000 H292 cells were plated in 60-mm dishes in 21% O_2 and 37°C and treated during exponential growth using D-penicillamine (DPEN; Sigma-Aldrich; 100 μM), auranofin (Au; Enzo Life Science; 0.5 μM), buthionine sulfoximine (BSO; Sigma-Aldrich; 1000 μM) bovine catalase (cat; Sigma-Aldrich; 50 U/mL), and/or carboplatin (Hospira, Inc.) for 24 hours. Unless otherwise stated, all cell groups were dosed with copper (II) sulfate pentahydrate ($CuSO_4$; Sigma-Aldrich; 15 μM) at t_0. In experiments using deferoxamine (DFO; Sigma-Aldrich; 40 μM) and iron sucrose (Fe sucrose; Venofer; 250 μM) indicated dishes were pretreated for 2 hours. Dishes were washed twice with media to remove extracellular DFO and iron sucrose, and $CuSO_4$ was reapplied to dishes after washing prior to DPEN exposure for 2 hours. In experiments with radiation, cells were irradiated with a dose of 1 Gy, 2 Gy, or 4 Gy (dose rate, 0.365 Gy/min), using a ^{37}Cs source (JL Shepherd, San Fernando, CA). Cells were returned to the incubator for 2 hours followed by analysis with the clonogenic survival assay as described [18]. Media

containing any floating cells was removed from the treatment dish. Attached cells were trypsinized with 0.25% trypsin-EDTA and trypsin was inactivated by re-combining cells with the media from the same treatment dish containing 10% FBS. Samples were centrifuged, re-suspended in fresh media and the resulting total cell population counted using a Beckman Coulter Counter. The cells were then plated 60-mm dishes at a variety of densities ranging from 200-2000 cells per dish. Clones were grown for 10-12 days in complete media with 0.1% gentamycin. Cells were fixed with 70% ethanol, stained with Coomassie blue, and colonies containing > 50 cells were counted. The treatment groups for each cell line were normalized to the control group. The survival analysis was done using a minimum of 3 cloning dishes per experimental condition, and the experiments were repeated a minimum of 3 times on separate occasions.

Calcein Labile Iron Detection Assay

To visualize the intracellular labile iron pool (LIP), the fluorescent dye Calcein-AM (Molecular Probes) was used as described previously with modifications [19]. The method for assessing LIP was based on the measurement of Calcein florescence in cells in the presence and absence of the iron chelator, 100 μM $2',2'$-bipyridyl (BIP-Sigma) [19]. 150,000 H292 and 250,000 HBEpC cells were plated in 60 mm dishes in their respective media and grown for 3 days at 4% O_2. Media was changed to HBEpC media in all dishes then treated with either 250 μM Fe sucrose (positive control) or 250 μM DFO (negative control) for 4 hours, washed with PBS, trypsinized, counted and 10^6 cells each placed in 15 mL tubes. After centrifuging at 1200 rpm for 5 minutes, cells were resuspended in PBS containing 500 nM Calcein-AM incubate at 37°C for 15 minutes. A background sample was created by adding only PBS. Calcein-AM was then removed by centrifugation, and cells were resuspended in 1 mL PBS and each sample was split into two flow tubes and 2,2 bipyridyl was added to one sample in each treatment group. BIP-treated samples were run at least 15 minutes after the addition to ensure adequate time for iron chelation. Cells were analyzed using FACS on LSR Violet (Becton Dickinson LSR II) with 515/15 emission filter.

Tumor versus Normal cells Clonogenic Survival Assays

100,000 H1299T cells and 250,000 HBEC cells or 125,000 MB231 cells and 300,000 HMEC cells were plated on 60-mm dishes as before for 72 hours at 4% O_2 and 37°C. All cells were then washed with warm PBS and placed in either complete Bronchial/Tracheal Epithelial Cell Basal Media (H1299T and HBEC cells) or Mammary Epithelial Cell Basal Medium (HMEC and MB231 cells)(Cell Applications, Inc.). Cells were then treated with 15 μM $CuSO_4$ and 100 μM DPEN for 3 hours, 50 μM H_2O_2 for 3 hours, or 10 μM carboplatin for 24. A clonogenic cell survival assay was performed as above with the following exceptions. All cells were trypsinized using trypsin/EDTA followed by trypsin neutralizing solution (Cell Applications, Inc) instead of FBS containing media. HBEC cloning dishes contained 100,000 lethally irradiated (30 Gy) H1299T feeder cells to encourage colony formation. Lethally irradiated feeder cells were also plated in dishes without test cells to confirm the lack of colony formation.

Peroxy Orange-1 H_2O_2 Production Flow Cytometry Assay

Exponentially growing MB231 cells were assayed for peroxide content using PeroxyOrange-1 (PO-1; BD Biosciences) as previously described [20]. Briefly, dishes were washed with warm phosphate buffer solution (PBS) in dimly lit conditions. 10 μM PO-1 probe was added. The dishes were then incubated at 37°C for 1 hour, washed with warm PBS, and dosed with 2 mL of cell media containing 15 μM $CuSO_4$, 100 or 500 μM DPEN, and/or 100 U/mL catalase as a negative control. A positive control was generated by adding H_2O_2 every 30 minutes starting at t_0 to reach a steady state of 100 μM. The treated cells were allowed to incubate at 37°C for 3 hours. All subsequent steps utilizing the PO-1 probe were done on ice to preserve the analytical capabilities of the probe. The dishes were then washed with PBS and trypsinized with 0.5% trypsin for 10-15 minutes. The trypsin was then neutralized by re-suspending cells in PBS containing 10% FBS. Cells were centrifuged at 4°C, 1200 rpm for 5 minutes, resuspended in 300 μL PBS, and analyzed using flow cytometry (Becton Dickinson LSR II) with a 561 nm laser, 585/20 collection.

Hydrogen Peroxide Quantification

To quantify differences in steady-state levels of intracellular H_2O_2 during exposure to DPEN, we utilized a method based on the stoichiometric inactivation of catalase by 3-amino-1,2,4-triazole (3AT) in the presence of H_2O_2 as previously described [5, 21, 22]. One catalase active site specifically reacts with one molecule of H_2O_2 leading to the formation of one molecule of the Compound I intermediate [22]. Compound I can then covalently interact with one molecule of 3AT leading to the irreversible inhibition of catalase activity. The pseudo first-order rate constant for the 3AT-mediated inactivation of catalase (k_{AT}) is then derived through kinetic analysis. Intracellular steady-state H_2O_2 concentrations are determined by: $[H_2O_2] = k_{AT}/k_1$, where $k_1 = 1.7 \times 10^7$ M^{-1} s^{-1} [21-23]. Because endogenous catalase activity in H1299T lung cancer cells is relatively low, the cells were first transduced with replication incompetent adenoviral vector AdCMV-Catalase (Viraquest). Cells were first counted to determine the quantity of virus needed. Media was changed to include 2% FBS, 25 MOI adenovirus was added, and cells incubated for 6 hours at 37°C at 21% O_2. The media was then changed to 10% FBS and cells were allowed to recover for 48 hours in the absence of virus. After 48 hours, the cells were rinsed in PBS and FBS-free media was added. 50 mM 3AT was added to the cultures for 5 minutes before 15 μM $CuSO_4$ and 100 μM DPEN was added. A positive control was generated by treating cells with 100 μM H_2O_2. Cells were harvested by rinsing twice in ice cold PBS, scraping into catalase buffer (50 mM K Phosphate pH 7.0) and frozen until analysis of remaining catalase activity. Control cells were harvested at 0, 15, 30, 45 and 60 minutes while the DPEN + $CuSO_4$ and H_2O_2 treated dishes were harvested at 0, 5 10 and 15 minutes. Two dishes were assayed at each time point and the experiment was repeated on three separate occasions.

Catalase Activity Assay

Samples were thawed, homogenized by sonication and the proteins quantified using the Lowry assay. Catalase activity was determined on the soluble portion of the homogenates by measuring the disappearance of 10 mm hydrogen peroxide ($\varepsilon_{240} = 39.4$ m^{-1} cm^{-1}) in 50

mM potassium phosphate, pH 7.0, monitored at 240 nm and the units were expressed as milli-k units/mg of protein as described [24].

Xenograft Tumor Growth

Female athymic nude mice were purchased from Envigo and housed according to Office of The Institutional Animal Care and Use Committee. 10^6 H292 cells were injected into the right flank and when tumor volumes averaged 100-300 mm^3, mice were randomized into treatment or control groups (N=6 mice per group). DPEN (150 mg/kg) was administered by IP injection twice daily in normal saline for injection. Control mice were injected with vehicle. Tumor volumes were measured using Vernier calipers, and both tumor volumes and mouse weights were measured daily.

Statistical Analysis

Unless otherwise noted in figure legend, data was analyzed for significance using unpaired t-tests in GraphPad Prism.

Results

DPEN combined with copper results in increased steady-state levels of hydrogen peroxide in cancer cells

To verify previous reports [25] of increased H_2O_2 in cancer cells treated with DPEN and $CuSO_4$, the PO1 oxidation assay was used in MB231 breast cancer cells. Intracellular oxidation of the PO1 probe as detected by flow cytometry increased significantly 1.3-fold following treatment with 100 μM DPEN + 15 μM $CuSO_4$ and 5-fold following treatment with 500 μM DPEN + 15 μM $CuSO_4$ (Figure 1A). The highest DPEN dose resulted in a PO1 oxidation mean fluorescent intensity similar to the 100 μM H_2O_2 control. Pretreatment of the MB231 cells with 100 U/mL catalase prior to treatment with DPEN and $CuSO_4$ completely inhibited the oxidation of PO1, confirming that the probe oxidation was mediated by H_2O_2.

To quantify the increased steady-state levels of H_2O_2 in H1299T lung cancer cells during exposure to DPEN and $CuSO_4$, a kinetic analysis was used based on the stoichiometric inactivation of catalase by 3-amino-1,2,4-triazole (3AT) in the presence of H_2O_2 (Figure 1B). 100 μM DPEN was chosen because it reflects a pharmacologically relevant level in humans [26] and 15 μM $CuSO_4$ was used because this represents a physiological relevant tissue concentration in humans. [8]. Addition of $CuSO_4$ did not significantly increase intracellular H_2O_2 from baseline (17 pM vs. 19 pM). However, the addition of the combination of DPEN and $CuSO_4$ significantly increased the intracellular H_2O_2 to 127 pM, similar to the levels seen upon the addition of 100 μM genuine exogenous H_2O_2 (142 pM) (Figure 1C). Together this data strongly supports the hypothesis that treatment of cancer cells with physiologically relevant concentrations of DPEN and $CuSO_4$ results in significant increases in steady-state $[H_2O_2]$.

DPEN decreases breast and lung cancer cell clonogenic survival which is enhanced with Au and BSO

The lack of toxic effects of DPEN or $CuSO_4$ individually was confirmed using clonogenic survival by treating H292 lung and MB231 breast cancer cells 24 hour with 15 μM $CuSO_4$ or 100 μM DPEN or the combination (Figure 2A). Major cellular detoxification pathways for H_2O_2 include the glutathione and thioredoxin dependent cellular peroxidases. Because DPEN + $CuSO_4$ resulted in H_2O_2 production, we combined DPEN with Au, a thioredoxin reductase inhibitor, and BSO, an inhibitor of glutathione synthesis. When treated with 100 μM DPEN clonogenic survival of MB231 dropped to 64%, while 1 mM BSO reduced survival to 91% (Figure 2B). When DPEN was combined with BSO significantly greater killing was noted and survival dropped to 48% (Figure 2B). Additionally, 0.5 μM Au reduced survival to 83% and when DPEN was combined with Au resulted survival dropped significantly to 42% (Figure 2B). When 50 U/mL catalase was added immediately before drug treatment with DPEN and Au, toxicity was inhibited demonstrating the H_2O_2 dependence of DPEN mediated cell killing (Figure 2B). When these experiments were repeated in H1299 and H292 lung cancer cells (Figure 2C) similar results were obtained showing the generality of the results with DPEN in three different human cancer cell lines. These results suggest that increased toxicity associated with inhibitors of thioredoxin and GSH metabolism are additive when combined with DPEN + $CuSO_4$.

Labile iron contributes to DPEN+ $CuSO_4$ toxicity in breast and lung cancer cells

To determine if labile iron played a role in DPEN effects on clonogenic cell survival, MB231 and H292 cells were treated with 250 μM Fe sucrose or 40 μM DFO for 2 hours, then the cells were rinsed, and treated with DPEN + $CuSO_4$ (Figure 3). DFO was selected for iron chelation because it has a binding constant for Fe^{3+} that is more than twice that for Cu^{2+} (pKs 30.6 and 14.1 respectively) [27] as well as inhibiting the redox cycling of Fe [28]. Fe sucrose was chosen because it is a clinically available source of supplemental iron that can be given to patients [29]. DFO and Fe sucrose did not cause clonogenic cell killing when used a single agents indicating these treatments were non-toxic (Figure 3AB). Interestingly pretreatment with Fe sucrose prior to DPEN + $CuSO_4$ significantly increased clonogenic cell killing in breast (Fig 3A) and lung (Fig 3B) cancer cell lines when compared to DPEN + $CuSO_4$ alone. Following treatment with DFO, DPEN toxicity was significantly inhibited in both breast and lung cancer cells despite the continued presence of 15 μM $CuSO_4$ during DPEN exposure when the DFO had already been washed off (Figure 3AB). These results support the conclusion that redox active Fe as well as Cu play a significant role in the mechanism of H_2O_2 mediated cell killing induced by exposure to DPEN.

DPEN + $CuSO_4$ selectively induce clonogenic cell killing in breast and lung cancer cells compared to normal breast and lung cells

When clonogenic cell survival was measured in H1299T lung cancer cells treated with DPEN and compared to HBEpC non-transformed primary lung epithelial cells, DPEN was found to be selectively cytotoxic to the lung cancer cells (Figure 4A). Likewise when similar experiments were performed in MB231 breast cancer cells and compared to non-transformed normal HMEC breast epithelial cells, DPEN was found to be selectively

cytotoxic to the breast cancer cells (Figure 4B). DPEN oxidation is accompanied by H_2O_2 production with a molar ratio of approximately 2:1 [16]. As expected the direct clonogenic toxic effects of 50 μM H_2O_2 on HMEC and MB231 cells exhibited similar results as treatment with 100 μM DPEN + Cu (Figure 4C).

To further confirm labile Fe plays a key role in the cytotoxic effect observed with DPEN + $CuSO_4$, intracellular LIPs were measured in H292 lung cancer cells and normal lung epithelial cells HBEpC using Calcein-AM as previously described [30] (Figure 4D). This probe binds Fe(II) rapidly, stoichiometrically, and reversibly while forming fluorescence-quenched Calcein-Fe complexes. Intracellular LIP is assessed from the relative rise in fluorescence elicited by addition of the highly permeant and high-affinity binding chelator, BIP. The results in Figure 4D demonstrate that the relative LIP of H292 lung cancer cells is 2.8 fold greater than the LIP of HBEpC. In addition pretreatment of cells with 250 μM Fe sucrose resulted in an approximate twice as much LIP in H292 cancer cells as compared to HBEpC normal cells. As expected, pretreatment with the iron chelator, DFO, resulted in a LIP below the detectible limit (data not shown). These results support the conclusion that fundamental differences in oxidative metabolism govern the selective toxicity of DPEN in cancer versus normal cells.

DPEN + CuSO$_4$ decrease clonogenic cell survival when combined with radiation or carboplatin

Both breast and lung cancer patients are treated with radiation and/or carboplatin. To test if DPEN + $CuSO_4$ could be combined with these standard cancer therapy modalities MB231 and H292 cancer cells with DPEN + $CuSO_4$ followed by 2 Gy IR (Figure 5A). In both cell lines the combination of DPEN with IR resulted in significantly more cell killing than either treatment alone. These results were also recapitulated with 1 and 4 Gy IR doses with similar results (data not shown). Catalase reversed the toxicity resulting from DPEN or IR + DPEN but not IR alone in both MB231 and H292 cell lines showing that H_2O_2 was responsible for the effects of DPEN (Figure 5A). Similarly DPEN significantly increased clonogenic cell killing when combined with 5, 10, and 20 μM carboplatin compared to either agent alone (Figure 5B). Non-transformed human bronchial epithelial cells (HBEpC) along with H292 and H1299 lung cancer cells were treated with carboplatin, DPEN + Cu and the combination followed by clonogenic survival. The resulting data in Figure 5C continues to support the hypothesis that DPEN + Cu differentially sensitize lung cancer cells to carboplatin, relative to normal cells.

DPEN decreases tumor growth of H292 lung cancer xenographs

Results of *in vivo* studies showed that DPEN effectively decreased H292 tumor growth in mouse xenografts (Supplemental Figure 1A). Tumors were measured daily and each tumor's beginning volume was measured relative to the average volumes of tumor on the first day of treatment with DPEN (150 mg/kg 2×/day). Analysis was discontinued on the day the first mouse reached criteria for euthanasia (tumor diameter of 1.5 mm). Compared to control, DPEN alone showed a statistically significant decrease in tumor growth rate at day 9 (Supplemental Figure 1A). DPEN treatment did not result in a significant difference in mouse weight or any other observable evidence of toxicity. This *in vivo* data shows promise

for the future exploration of DPEN in combination with standard of care chemotherapy or radiation.

Discussion

When developing cancer therapies, exploiting the fundamental biochemical differences between cancer and normal cells is essential. One such difference is alterations in oxidative metabolism that have been noted in cancer versus normal cells [4, 31, 32] as well as cancer stem cells [33]. We have recently published data demonstrating that disruption of redox balance radiosensitizes breast cancer stem cells [34]. Cancer cells appear to demonstrate both increased steady state levels of ROS ($O_2^{\bullet-}$ and H_2O_2) and abnormal metabolism of transition metals when compared to normal cells [4, 35]. It has been shown that free and bound copper and iron concentrations are higher in breast compared to the normal surrounding tissue [9, 10, 36]. These metal ions participate in many critical biological functions however the same properties that allow for the gain or loss of electrons also results in cytotoxicity through the donation of electrons to oxygen, leading to the generation of highly toxic hydroxyl radicals and other ROS. Recently, the importance of ferrous iron [Fe(II)] as the critical initiator of the Fenton reaction and the accompanying pathology *in situ* in carcinogenesis models was clearly demonstrated [37, 38]. Others have demonstrated that H_2O_2, generated via plasma activated media, induces apoptosis in lung cancer cells compared to normal cells in a Fe(II) dependent mechanism [39]. Based on alterations in oxidative metabolism as well as differences in redox active metal ions in cancer cells, agents that generate H_2O_2 represent new therapeutic tools for selectively killing cancer [25, 30].

D-penicillamine, a potent copper chelator, has been used in patients with rheumatic arthritis and Wilson's disease for many years. DPEN is tolerated well in humans, and when dosed at 750 mg per day, steady-state plasma levels reach 100 μM [26]. However proteinuria, thrombocytopenia, mucocutaneous lesions, and neutropenia have been reported in patients on DPEN therapy which limits its chronic use. The majority of *in vivo* copper is bound to either ceruloplasmin or albumin with a much smaller fraction being labile copper. Each ceruloplasmin protein has the ability to bind 8 copper atoms, however it is known that only 6 are tightly bound at physiologic conditions and at least one is exchangeable and available for chemical reaction [40]. Previous studies have shown the generation of H_2O_2 by DPEN in the presence of physiologically relevant concentrations of ceruloplasmin [15]. Additionally, albumin binds copper even less tightly than ceruloplasmin explaining why DPEN is effective at removing excess albumin-bound and free copper in Wilson's disease patients [25]. The resulting copper chelation and reduction produces H_2O_2 as previously described [15]. It has been reported that the H_2O_2 production from DPEN is concentration dependent up to 4 μM of copper intimating the differences between the tumor tissue and the normal surrounding tissue (1.57 μM vs 0.57 μM respectively) could result in a significantly different amount of H_2O_2 production in tumor tissue *in vivo* [10, 41]. Using PeroxyOrange-1, we have demonstrated that intracellular H_2O_2 increases with increasing DPEN concentration in breast cancer cells. Additionally, using the 3-amiotriazole mediated inhibition of catalase activity we quantified the intracellular H_2O_2 concentrations in lung cancer cells after treatment with biologically relevant concentrations of DPEN. Within minutes DPEN + $CuSO_4$ significantly increased the intracellular [H_2O_2] similar to the levels seen upon

exposure to 50 μM exogenous H_2O_2. Using the clonogenic survival assay to measure cancer cell reproductive integrity, we demonstrated that agents that inhibit H_2O_2 metabolism (Au and BSO) enhance cell killing when combined with DPEN + Cu, while catalase inhibited DPEN-induced cell death, clearly demonstrating that H_2O_2 is responsible for cell death.

To determine the role of redox active iron in DPEN-induced cancer cell toxicity, we chelated Fe using DFO and demonstrated that DPEN toxicity was inhibited. Consistent with this finding when cells were preloaded with Fe sucrose prior to treatment with DPEN + $CuSO_4$ cancer cell death was significantly enhanced. Furthermore, the Calcein assay confirmed that the chelation and addition of Fe lead to a significant decrease and increase of the labile iron pool respectively. Previous reports have demonstrated the iron catalyzed oxidation rates of DPEN are minor at physiologic pH [25]. Although iron does not oxidize DPEN directly, we have demonstrated that its presence inside the cell is important for toxicity possibly by converting the H_2O_2 to more highly toxic oxidants such as $^\bullet OH$ via the Fenton reaction. Finally we observed that DPEN was selectively toxic to breast and lung cancer cells vs normal breast and lung epithelial cells which could be the result of fundamental differences in oxidative metabolism involving either the removal of H_2O_2 or the formation of $^\bullet OH$ by metal catalyzed reactions in cancer versus normal cells.

DPEN has been investigated in a phase 2 trial of 40 glioblastoma patients in combination with a low copper diet and radiation. Doses of DPEN of 2000 mg/day for several months resulted minimal side effects (mainly leukopenia and thrombocytopenia) as previously described for use in rheumatoid arthritis. DPEN did not improve patient survival; however this could be because it was combined with a low copper diet as part of an antiangiogenic strategy or issues related to crossing the blood brain barrier [42]. DPEN has safely and effectively been given alone and combined with other standard-of-care chemotherapeutics in xenograft mouse models in the treatment of multiple cancers [43-45]. Our *in vitro* data demonstrates that DPEN + $CuSO_4$ contribute to enhancement of both radiation and carboplatin cell death which are considered standard of care agents for both lung and breast cancers. In aggregate, the data gathered in the current study supports the hypothesis that DPEN + Cu selectively kills cancer cells via the production of H_2O_2 which is also selectively enhanced in cancer cells via labile iron pools. This data supports further investigation of the combination of DPEN + Cu as an adjuvant to cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- DPEN + Cu at physiologic concentrations increase H_2O_2 levels in cancer cells.

- DPEN's clonogenic toxicity is enhanced using auranofin and buthionine sulfoximine.

- DPEN + Cu treatment is more toxic to cancer cells than to normal epithelial cells.

- DPEN toxicity in correlated with intracellular labile iron pools.

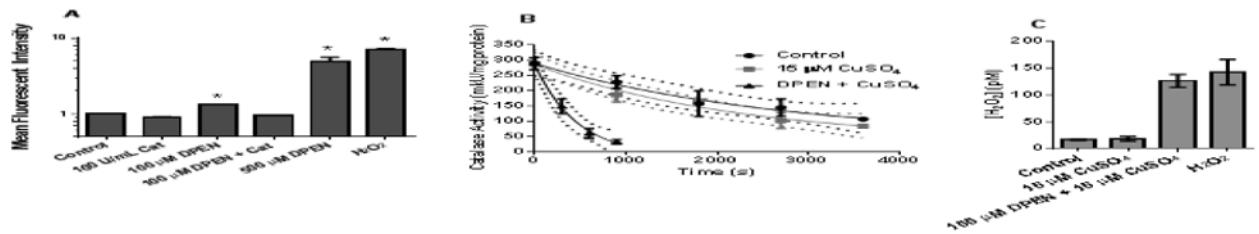- Labile iron pools are higher in cancer cells verses normal lung epithelial cells.

Figure 1. Generation of H$_2$O$_2$ by DPEN and CuSO$_4$ in breast and lung cancer cell cultures
(A) MB231 cells were pre-incubated with PeroxyOrange-1 (PO-1) probe for one hour then treated with 100 μM DPEN, catalase (100 U/mL), or genuine H$_2$O$_2$ (100 μM) for 3 hours followed by analysis by flow cytometry. 15 μM CuSO$_4$ was added to all groups at the time of drug treatment. *Significantly different than control; $p < 0.05$; n=4. (B) The rate of catalase inactivation in the presence of 3-aminotriazole was measured in H1299 lung cancer cells treated with 15 μM CuSO$_4$ and 100 μM DPEN at various time points and (C) intracellular [H$_2$O$_2$] was calculated using the rates of catalase activity disappearance as previously described [23]. $p < 0.05$; n=3. Errors represent ± 1SEM.
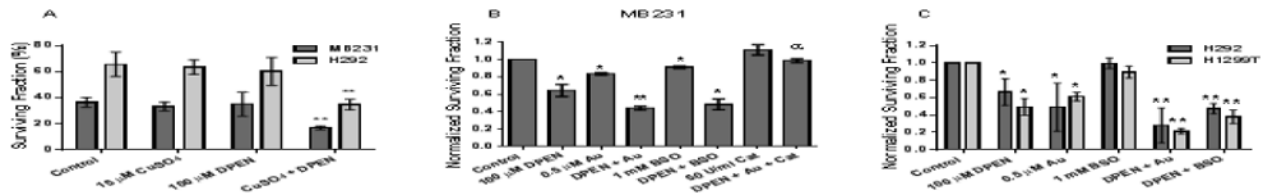
Figure 2. Clonogenic cell killing by DPEN is dependent on CuSO$_4$ and enhanced by inhibitors of glutathione (BSO) and thioredoxin-dependent (Au) metabolism in human lung and breast cancer cells

Clonogenic survival assays were performed with lung cancer cells or breast cancer cells. (A) MB231 breast and H292 lung cancer cells were treated with 15 µM CuSO$_4$ and/ or 100 µM DPEN for 24 hours. (B) MB231 cells (C) H1299T and H292 cells were treated with the indicated concentrations of DPEN, Au, and/or BSO for 24 hours, 15 µM CuSO$_4$ was added to all dishes at the time of treatment. 50 U/mL bovine catalase was added immediately prior to drug treatment. *Significantly different than control. **Significantly different than either drug alone. α significantly different than drugs without catalase. p< 0.05; n=3. Errors represent ± 1SEM.

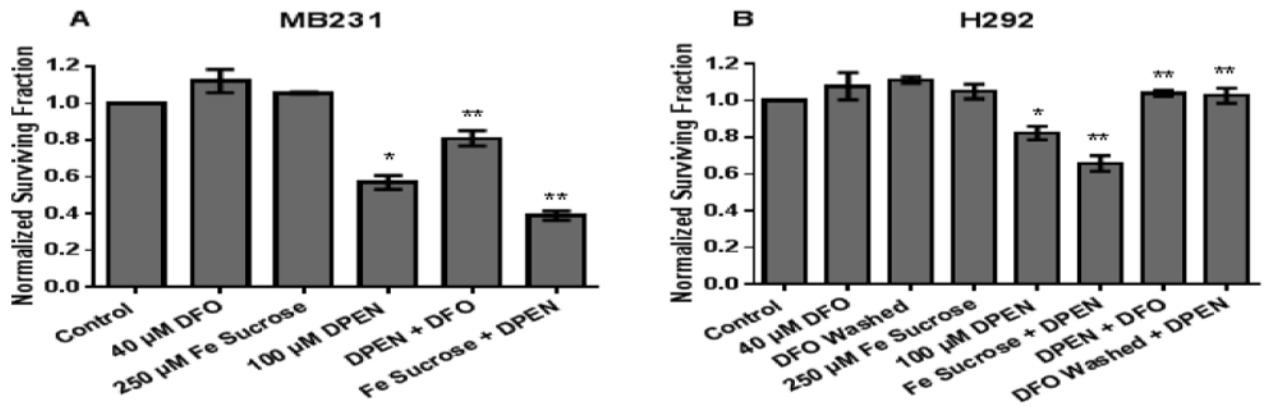**Figure 3. Clonogenic cell killing in cancer cells by DPEN + CuSO$_4$ is enhanced with Fe sucrose and inhibited with Fe chelation**

Iron chelation using 40 μM DFO for 2 hours prior to 100 μM DPEN + CuSO$_4$ decreases clonogenic killing in MB231 cancer cells (A) and H292 cancer cells (B). In contrast, pretreatment with 250 μM Fe sucrose for 2 hours prior to addition of DPEN + CuSO$_4$ enhances clonogenic cell death (A,B). 15 μM CuSO$_4$ was added to all groups at time of DPEN treatment. *Significantly different than control; p< 0.05. **Significantly different than DPEN. p<0.05; n= 3. Errors represent ± 1 SEM.
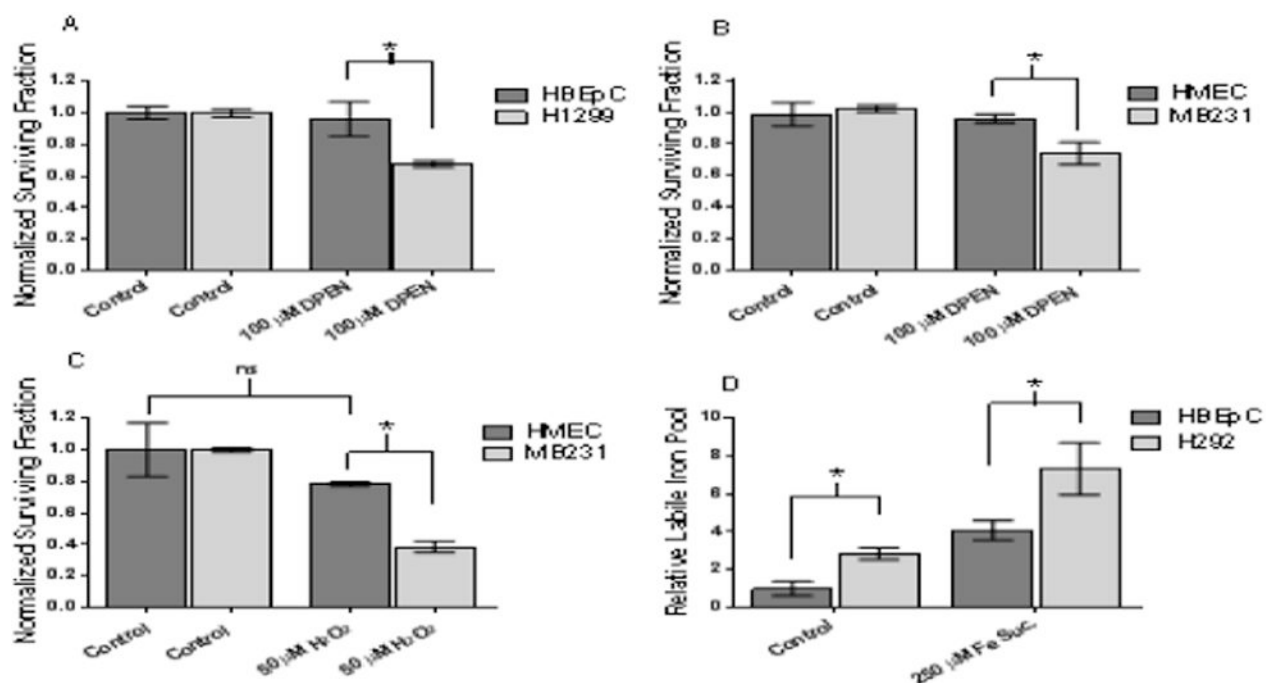
Figure 4. DPEN + CuSO$_4$ selectively induced clonogenic cell killing in human breast and lung epithelial cancer cells as compared to normal non-transformed human breast and lung epithelial cells. Intracellular labile iron pool is significantly higher in lung cancer cells compared to normal non-immortalized lung epithelial cells

(A) Normal lung epithelial cells (HBEpC) and lung cancer cells (H1299) were treated 100 μM DPEN + 15 μM CuSO$_4$ for 3 hours then subjected clonogenic assay. (B) Normal breast epithelial cells (HMEC) and breast cancer cells (MB231) similarly treated with 100 μM DPEN + 15 μM CuSO$_4$ followed by clonogenic assay. (C) HMEC and MB231 cells were treated for 3 hours with 50 μM H$_2$O$_2$ followed by the clonogenic assay. (D) Relative intracellular labile iron pools measured using Calcein AM dye followed by flow cytometry comparing HBEpC and H292 cells. Cells were pre-treated with 250 μM Fe sucrose or 250 μM DFO as a positive and negative control. *Significantly different. $p<0.05$; n=3. Errors represent ± 1 SEM.
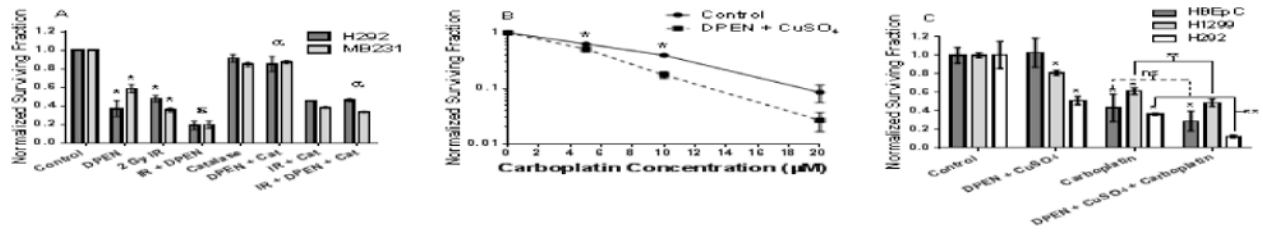
Figure 5. DPEN + Cu enhances cancer cell clonogenic cell killing when combined with ionizing radiation (IR) or Carboplatin

(A) H292 and MB231 cancer cells were treated with 100 μM DPEN + 15 μM CuSO$_4$ for a total of 3 hours, with exposure to 2 Gy IR after one hour of drug treatment. Catalase protected the cancer cells from the enhanced cell killing of the combination of IR + DPEN. *-Significantly different than IR alone control; p< 0.05; n= 3. ɛ Significantly different than DPEN alone. α Significantly different than IR + DPEN. (B) H292 cells were treated with DPEN + CuSO$_4$ and the indicated concentrations of carboplatin for 24 hours followed by clonogenic cell survival analysis. Survival data were normalized to the toxicity of DPEN + CuSO$_4$ alone. *Significantly different than untreated control. (C) HBEpC (n = 2 experiments plated into at least 6 cloning dishes per treatment group), H292 (n = 3 experiments plated into at least 9 cloning dishes per treatment group), and H1299 (n = 2 experiments plated into at least 6 cloning dishes per treatment group) were treated with 10 μM carboplatin for 24 hours with DPEN added for the last 3 hours, followed by clonogenic assay. CuSO$_4$ was present in all dishes at the beginning of treatment. ** Significantly different than either drug alone. p<0.05, Errors represent ± 1 SEM.