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Disulfiram/Copper-Disulfiram Damages Multiple Protein Degradation and Turnover Pathways and Cytotoxicity is Enhanced by Metformin in Oesophageal Squamous Cell Carcinoma Cell Lines

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ABSTRACT

Disulfiram (DSF), used since the 1950s in the treatment of alcoholism, is reductively activated to diethyldithiocarbamate and both compounds are thiol-reactive and readily complex copper. More recently DSF and copper-DSF (Cu-DSF) have been found to exhibit potent anticancer activity. We have previously shown that the anti-diabetic drug metformin is anti-proliferative and induces an intracellular reducing environment in oesophageal squamous cell carcinoma (OSCC) cell lines. Based on these observations, we investigated the effects of Cu-DSF and DSF, with and without metformin, in this present study. We found that Cu-DSF and DSF caused considerable cytotoxicity across a panel of OSCC cells, and metformin significantly enhanced the effects of DSF. Elevated copper transport contributes to DSF and metformin-DSF-induced cytotoxicity since the cell-impermeable copper chelator, bathocuproinedisulfonic acid, partially reversed the cytotoxic effects of these drugs, and interestingly, metformin-treated OSCC cells contained higher intracellular copper levels. Furthermore, DSF may target cancer cells preferentially due to their high dependence on protein degradation/turnover pathways, and we found that metformin further enhances the role of DSF as a proteasome inhibitor. We hypothesized that the lysosome could be an additional, novel, target of DSF. Indeed, this acid-labile compound decreased lysosomal acidification, and DSF-metformin co-treatment interfered with the progression of autophagy in these cells. In summary, this is the first such report identifying the lysosome as a target of DSF and based on the considerable cytotoxic effects of DSF either

Abbreviation: AO, Acridine orange; AVO, Acidic vesicular organelles; BCS, Bathocuproinedisulfonic acid; Cu-8HQ, Copper-8-hydroxyquinoline; Cu-DSF, Copper-disulfiram; DMSO, Dimethyl sulfoxide; DPTD, Dipyrrolidine thiuram disulphide; DSF, Disulfiram; Suc-LLVY-AMC, N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; MeDTC-SO, Methyl diethylthiocarbamoyl sulfoxide; OSCC, Oesophageal squamous cell carcinoma; TTD, Tetrapropyl thiuram disulphide.

Rupal Jivan and Leonard Howard Damelin are first authors.

Disclosure of Potential Conflicts of Interest: LHD and DMD are co-applicants on a patent for the treatment of OSCC using disulfiram and copper-disulfiram with and without metformin.

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alone or in the presence of metformin, in vitro, and we propose these as novel potential chemotherapeutic approaches for OSCC. J. Cell. Biochem. 116: 2334–2343, 2015.

KEY WORDS: AUTOPHAGY; CANCER; COPPER; DISULFIRAM; LYSOSOME; PROTEASOME

Disulfiram (DSF) (tetraethylthiuram disulfide) has been used since the 1950's to treat alcohol abuse [Johansson 1992]. In vivo, DSF is rapidly converted to its monomer, diethyldithiocarbamate (DDC), which is then methylated and subsequently oxidised to methyl diethylthiocarbamoyl sulfoxide (MeDTC-SO) [Ververka et al., 1997]. Both DSF and MeDTC-SO can irreversibly inhibit aldehyde dehydrogenase, which leads to an accumulation of acetaldehyde in the blood upon alcohol intake, causing, amongst other effects, severe nausea, vomiting and tachycardia [Johansson 1992; Ververka et al., 1997].

DSF was shown to have anticancer activity in the 1970's [Wattenberg 1974] and in recent years, there has been renewed interest in DSF for cancer therapy primarily because DSF is an attractive candidate for drug repurposing given its well characterized toxicity profile and good safety track record [Cvek 2011; Johansson 1992]. DSF and DDC are thiol-reactive and can react with free thiol groups on proteins and glutathione; they also have high affinity for, and form stable complexes with, heavy metals such as copper and zinc [Johansson 1992]. Much of the anticancer activity of DSF has been attributed to its copper complex, which has been found to exhibit highly efficacious and specific toxicity both in vitro and in vivo through a variety of mechanisms, including acting as an inhibitor of activating transcription factor/cyclic AMP-responsive element binding protein (ATF/CREB), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), P-glycoprotein, DNA topoisomerases, DNA methyltransferases, as a potent inhibitor of the proteasome and of invasion and angiogenesis [Yakisich et al., 2001; Wang et al., 2003; Brar et al., 2004; Cen et al., 2004; Loo et al., 2004; Lovberg et al., 2006; Chen et al., 2006; Lin et al., 2011].

In a previous study, in which we investigated the effects of metformin on cisplatin cytotoxicity in oesophageal squamous cell carcinoma (OSCC) cell lines, we established that metformin induces a reducing intracellular environment, which whilst protective against cisplatin toxicity, allows cells to be targeted by compounds that are activated under reducing conditions. As a case in point, we showed that two copper-bis(thiosemicarbazone) compounds, copper diacetyl-bis(4-methylthiosemicarbazonato) copper(II) (Cu-ATSM) and copper glyoxal-bis(4-methylthiosemicarbazonato) copper(II) (Cu-GTSM), which are understood to be reductively activated, are highly cytotoxic to OSCC cells in the presence of metformin [Damelin et al., 2014]. Given these promising results, we decided to investigate the effect of the reductively activated DSF and Cu-DSF on OSCC cells in combination with metformin; we and others have shown that metformin exhibits excellent antiproliferative effects on carcinoma cell lines [Quinn et al., 2013; Damelin et al., 2014].

In this study, we show that Cu-DSF and interestingly, DSF alone, display considerable cytotoxic activity towards OSCC cell lines, and that this cytotoxicity is enhanced by metformin co-treatment. In addition, we show that DSF toxicity is only partially copper-dependent and, for the first time, we identify that a major contributing mechanism of DSF toxicity (in OSCC cells) is perturbed lysosomal acidification and autophagic activity, which in combination with proteasomal inhibition, confer damage to multiple protein degradation/turnover pathways in these cells. This study therefore identifies a novel mechanism of action for DSF and highlights the potential use of DSF, with or without metformin, as a potential chemotherapy strategy for the treatment of OSCC.

MATERIALS AND METHODS

REAGENTS

All reagents were purchased from Sigma Aldrich unless otherwise specified.

SYNTHESIS OF CU-DSF AND DSF ANALOGUES

Cu-DSF was synthesized by adding equimolar amounts of DSF and cupric chloride in DMSO. Analogues of DSF were synthesized, as previously described [Cramer 1935].

Dipyrrolidine thiuram disulphide (DPTD). ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.86 (dt, J = 61.6, 7.0 Hz, 1H), 2.03 (dt, J = 73.0, 6.9 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 187.71, 57.43, 51.40, 26.63, 24.21.

Tetrapropyl thiuram disulphide (TTD). ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.89 (dt, J = 10.1, 4.9 Hz, 1H), 1.78 (dq, J = 87.4, 7.6 Hz, 1H), 0.92 (dt, J = 57.1, 7.4 Hz, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 192.10, 58.88, 54.89, 21.73, 19.49, 11.42.

CELL CULTURE

The human oesophageal squamous carcinoma cell (OSCC) lines, WHCO1, WHCO5, [Veale and Thornley, 1989] and SNO [Bey et al., 1976] were cultured in DMEM/F12 (3:1) with 10% foetal bovine serum (Lonza) and 100 U/ml penicillin/0.1 mg/ml of streptomycin, in a humidified atmosphere at 37°C with 5% CO_2. OSCC cell lines were isolated from moderately differentiated tumours from South African OSCC patients. The SNO cell line harbours an p53-R175H mutation [Fanucchi and Veale, 2009].

CYTOTOXICITY ASSAYS

Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, 7,500 cells per well were seeded into 96-well plates and after 24 h, exposed to cytotoxic agents DSF, Cu-DSF or the DSF analogues DPTD or TTD for a further 48 h. To determine the effect of metformin on cytotoxicity, cells were pre-treated with 10 mM metformin for 24 h then co-treated with metformin and DSF, Cu-DSF or the DSF analogues DPTD or TTD for a further 48 h. To investigate the role

of copper in DSF mediated cytotoxicity, cells were treated as described but with the addition of the cell impermeable copper chelator, bathocuproinedisulfonic acid (BCS) (200 μM) [Furuta et al., 2002]. After treatments, the medium was replaced with 100 μl of MTT solution (0.5 mg/ml MTT in cell culture medium) and incubated at 37°C for 1.5 h. MTT solution was then removed, and MTT formazan dissolved in 100 μl dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a Thermo Scientific Multiskan GO microplate reader ($n = 3 \pm SD$). Cells were also visualized microscopically and images captured on an Olympus BX63 microscope at 40x magnification and images processed using CellSense Dimension software.

INDUCTIVELY COUPLED PLASMA MASS SPECTOMETRY FOR COPPER QUANTIFICATION

Inductively Coupled Plasma-Mass Spectometry (ICP-MS) was used to quantify total intracellular copper levels as previously described [Price et al., 2011]. Cells were seeded in 100 mm dishes and treated with metformin, DSF or metformin and DSF, (with or without BCS) as previously indicated. Cells were washed with cold 1x Phosphate Buffered Saline (PBS) three times, scraped and collected by centrifugation for 10 min at 8,000 rpm at 4°C. A relative portion of cells was removed for protein quantification by the Bradford assay [Bradford 1976]. Cell pellets were then incubated with 65% nitric acid (Suprapure, Merck) at 65°C for 2 h. Samples were then diluted to a final concentration of 5% nitric acid and analysed for copper on an Agilent 7,700 ICP-MS with He collision gas ($n = 3 \pm SD$).

WESTERN BLOTTING FOR AUTOPHAGY AND PROTEIN UBIQUITINATION

Autophagy and protein ubiquitination were assessed by western blotting by LC3B and total ubiquitinated protein levels, respectively. Cells were treated as described above (the positive control was 50 μM chloroquine (CQ) for 24 h) and harvested in triple detergent cell lysis buffer. Protein was quantified (Bradford assay), 40 μg electrophoresed on 5–10% polyacrylamide gels at 25 mA for 45 min and protein was subsequently electroblotted for 2 h at 200 mA onto nitrocellulose [Bradford 1976]. Membranes were blocked in 5% fat-free milk powder in 1xTris Buffered Saline with 0.1% Tween (TBS-T) then incubated with the primary antibodies, rabbit anti-LC3B or rabbit anti-ubiquitin (Cell Signalling Technology) in 5% bovine serum albumin in 1xTBS-T overnight, following which, membranes were washed in 1xTBS-T and then incubated with a goat anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnologies). Chemiluminescent signal was obtained with SuperSignal® West Pico Chemiluminescent substrate (Thermo Scientific) and detected using x-ray film. Densitometry was performed using QuantityOne software.

PROTEASOME FUNCTION

Proteasome function was assessed in treated cells using the fluorogenic proteasome substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Suc-LLVY-AMC), which indicates levels of chymotrypsin-like activity, as previously described [Daniel et al., 2004]. Cells seeded at equal density in 100 mm dishes were left to settle for 24 h then treated with LD_{30} concentrations of DSF, Cu-DSF or the DSF analogues DPTD and TTD, as for the MTT assays, or with the positive control copper-8-hydroxyquinoline (5 μM for 24 h) (Cu-8HQ), which is a previously established inhibitor of proteasomal function [Daniel et al., 2004]. Cells were then washed in 1xPBS, collected by scraping and centrifugation, lysed in lysis buffer (25 mM Tris-HCl, pH 8.0; 75 mM NaCl; 0.05% SDS; 0.5% Triton X-100; 0.25% Sodium deoxycholate), and lysates cleared by centrifugation. Protein was quantified by Bradford assay and 40 μg added to assay buffer (50 mM Tris–HCl pH 7.5) to a final volume of 400 μl. Suc-LLVY-AMC in DMSO was added to a final concentration of 40 μM and the reaction mixture incubated at 37°C for 1 h, followed by the addition of trichloroacetic acid to a final concentration of 4% to stop the reaction. Fluorescence was measured at $360\,nm_{excitation}/460\,nm_{emission}$ using an Ascent multi-well plate fluorimeter (Thermo Scientific) ($n = 3 \pm SD$).

FLUORESCENCE MICROSCOPY FOR ACIDIC VESICULAR ORGANELLE ACIDIFICATION

Acidic vesicular organelles (AVO), including lysosomes, were visualized using the pH sensitive dye acridine orange (AO), which displays a clear shift from red to yellow to green fluorescence, with increasing pH, unlike other commercially available dyes [Paglin et al., 2001; Pierzynska-Mach et al., 2014]. OSCC cells were grown on glass coverslips until 50% confluent then treated with LD_{30} concentrations of DSF, Cu-DSF, DPTD or TTD, for 24 h, with or without 10 mM metformin. Chloroquine was used as the positive control (1 mM for 1 h). Cells were then incubated with 3 μM AO added to culture medium for 1 h at 37°C. Cells were visualized using the Olympus BX41 fluorescence microscope (100 W equipped with a camera and lamp using a 490 nm band-pass blue excitation filter and a 515 nm long-pass barrier filter) and images captured and processed with CellSense Dimensions software.

ELECTRON MICROSCOPY FOR AUTOPHAGY

Electron microscopy (EM) was used to look for autophagosome formation [Yla-Antilla et al., 2009]. Cells were cultured as previously described in 100 mm dishes and when 60% confluent were incubated with 10 mM metformin for 24 h, or LD_{30} dose of DSF, metformin plus DSF, Cu-DSF, or Cu-DSF plus metformin. Cells were then rinsed with cold 1x PBS three times and then fixed with 2.5% glutaraldehyde in 0.1 M HEPES buffer pH 7.2 for 1 h. Cells were then scraped into a microfuge tube and collected by centrifugation at 1,000 xg for 10 min. Pelleted cells were routinely prepared for transmission electron microscopy as previously described [Hayat 2000]. Briefly, pelleted cells were fixed overnight in 2.5% glutaraldehyde in 0.1 M HEPES buffer (pH 7.2), rinsed several times in HEPES buffer followed by post-fixation in 1% buffered osmium tetroxide for 1 h, repeatedly rinsed, dehydrated in a graded ethanol series (30 min intervals, absolute ethanol thrice), infiltrated with a low viscosity epoxy resin (Agar Scientific®) and polymerised in BEEM® capsules at 70°C overnight. 70nm sections were cut on a Leica EM UC6, picked up on 0.25% formvar-coated copper slot grids, double stained with uranyl acetate and lead citrate, and viewed at 80 kV on an FEI BioTwin Spirit transmission electron microscope fitted with an Olympus Quemesa CCD camera.

STATISTICAL ANALYSIS

All experiments were performed a minimum of three times as indicated. Comparisons were made by Student's t-tests and $P < 0.05$ was considered statistically significant. LD_{50} and LD_{30} values were calculated using GraphPad Prism version 6.

RESULTS

OSCC CELLS ARE HIGHLY SUSCEPTIBLE TO CU-DSF AND DSF ALONE AND METFORMIN ENHANCES DSF CYTOTOXICTY

MTT cytotoxicity assays indicated that Cu-DSF was highly toxic to all OSCC cell lines (LD_{50} values: WHCO1 $3.6 \pm 0.3\,\mu M$, WHCO5 $2.8 \pm 0.2\,\mu M$ and SNO $2 \pm 0.08\,\mu M$) (Fig. 1A and 1B). Interestingly, DSF alone was also highly cytotoxic to OSCC cells with LD_{50} values of $6 \pm 0.4\,\mu M$ for WHCO1, $6.8 \pm 0.3\,\mu M$ for WHCO5 and $11.9 \pm 0.6\,\mu M$ for SNO cells (Fig. 1A and 1B). The addition of metformin significantly enhanced the cytotoxicity of DSF alone (by 25–40% across the three cell lines), but not of Cu-DSF (Fig. 1A and 1B). Cell morphology following treatments reflected the findings of the MTT assays (Fig. 1C).

PERTURBED COPPER HOMEOSTASIS IS PARTIALLY RESPOSNSIBLE FOR DSF AND METFORMIN-ENHANCED DSF CYTOTOXICTY

The treatment of OSCC cell lines with DSF and doubling cupric chloride concentrations, with or without metformin, indicated that DSF-induced cytotoxicity increased with increasing cupric chloride concentrations (Fig. 2A), but interestingly, metformin-enhanced cytotoxicity was only observed at low and not at high levels of cupric chloride concentrations (not shown). This suggested that metformin-enhanced DSF-cytotoxicity was due to increased copper transport (leading to increased intracellular Cu-DSF and/or Cu-dithiocarbamate formation), and that this effect is saturating at high copper concentrations. To confirm this, intracellular copper levels for DSF treated cells, with or without metformin co-treatment, were quantified using ICP-MS analysis. Predictably, DSF-treated cells exhibited a significant increase in intracellular copper levels vs. untreated controls; DSF facilitates increased copper import via the formation of stable, neutral lipophilic copper complexes (Fig. 2B). Metformin treated cells exhibited higher intracellular copper levels overall vs. untreated controls while DSF-metformin co-treated cells exhibited an additive increase in intracellular copper levels (Fig. 2B), confirming that metformin treatment did indeed facilitate increased copper transport and this was most likely responsible for the observed increase in cytotoxicity for DSF/metformin co-treatment vs. DSF alone. These findings agree with previous investigations, which have established that metformin can indeed bind copper and alter intracellular copper levels [Zhu et al., 2002; Logie et al., 2012].

Whilst copper-enhanced DSF toxicity in all OSCC cell lines was significant, we also determined the contribution of copper–independent mechanisms of DSF cytotoxicity using the cell impermeable copper chelator, BCS ($200\,\mu M$), which was in

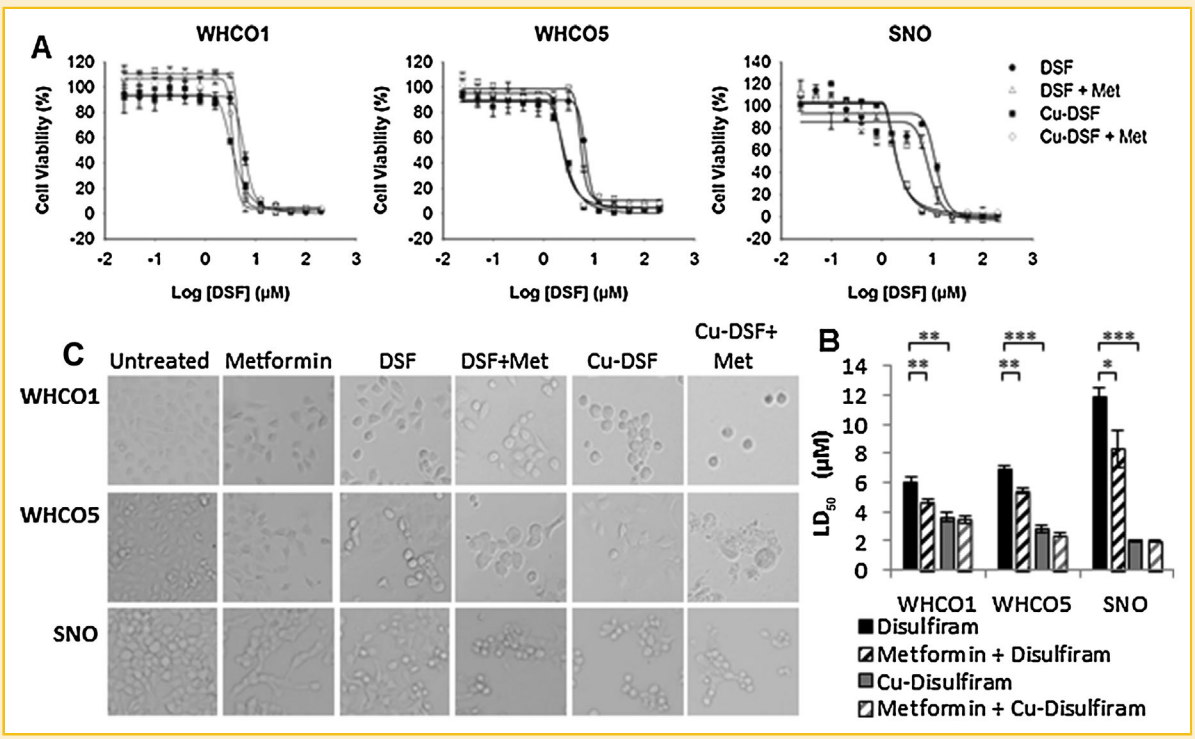

Fig. 1. Cu-DSF and DSF are highly cytotoxic to OSCC cells and DSF cytotoxicity is enhanced by metformin. (A) Cells exposed to Cu-DSF and DSF for 48 h show considerable cytotoxicity across all three OSCC cell lines with a significant leftward shift in toxicity curves for Cu-DSF ($n = 3$, mean ± SD). (B) The addition of 10 mM metformin further enhanced DSF cytotoxicity and whilst Cu-DSF was considerably more toxic than DSF alone, the addition of metformin to Cu-DSF did not significantly change cytotoxicity ($n = 3$, mean ± SD). C: Cell morphology mirrored the observations;1; of the MTT assays.

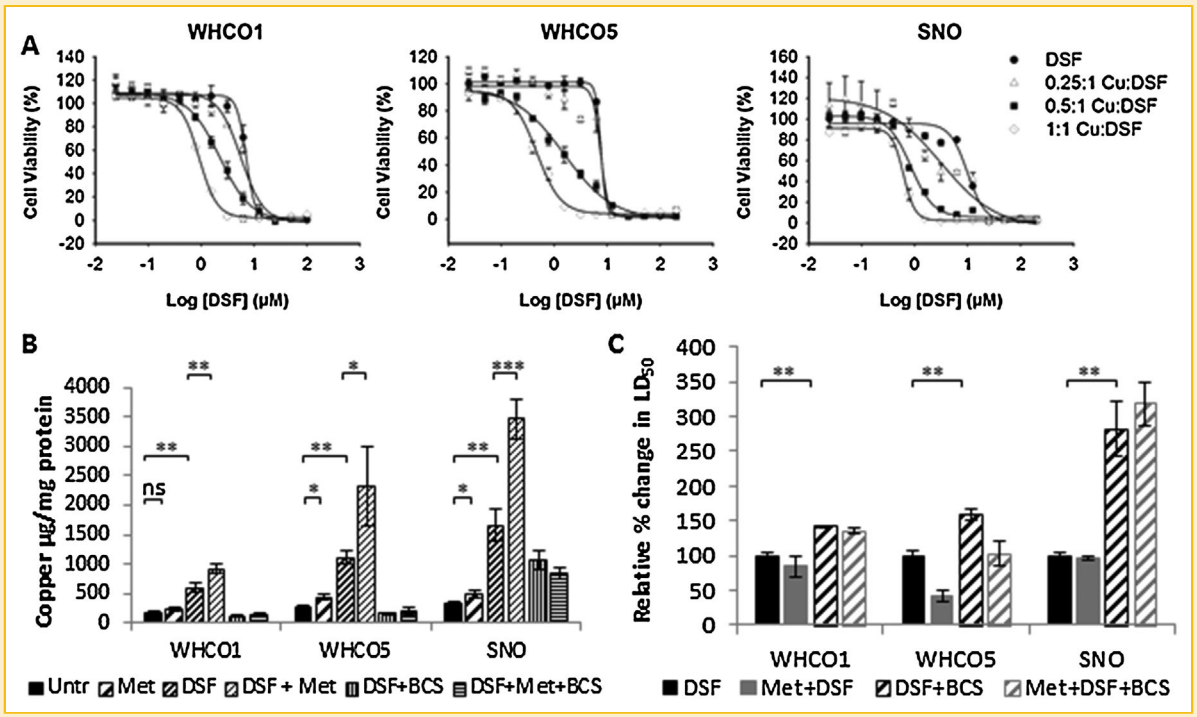

Fig. 2. Copper is partially responsible for DSF toxicity and metformin causes increased copper uptake by OSCC cells. (A) The role of copper in DSF toxicity was observed by increasing copper concentrations to equimolar molar levels of copper and DSF, which had an increasing effect on cytotoxicity as seen by the significant leftward shift in toxicity curves with increasing copper concentration (n = 3, mean ± SD). 2B. Measurement of intracellular copper by ICP-MS showed that as expected DSF treated cells had significantly higher copper levels than untreated cells. Interestingly OSCC cells treated with 10 mM metformin had, overall, higher intracellular levels of copper in comparison to untreated controls. The effect of metformin compounded that of DSF, with DSF-metformin co-treated cells displaying higher copper levels than DSF alone (values expressed per mg of protein), (n = 3, mean ± SD. 1D). Copper content of cells was also assessed in the presence of a cell impermeable copper chelator bathocuproinedisulfonic acid (BCS) to confirm the reduction in intracellular copper, as anticipated. (C) The contribution of copper to cell cytotoxicity was further assessed by the addition of BCS (200 μm), which partially reversed the cytotoxic effects of DSF. All values are expressed as % change in LD_{50} relative to DSF alone (n = 3, mean ± SD).

considerable excess to culture medium copper levels (range 1.4–4 μM). ICP-MS on cells treated with BCS confirmed the reduction in intracellular copper levels (Fig. 2B). Interestingly, a significant but only partial (approximately 50% for WHCO1 and WHCO5 cells) increase in the LD_{50} value was observed for DSF-treated cells in the presence of BCS vs. DSF alone (Fig. 2C), indicating that while copper significantly contributed to DSF-cytotoxicity, copper-independent mechanisms were also involved in DSF-mediated cytotoxicity in OSCC cell lines.

DSF PERTURBS MULTIPLE PROTEIN DEGRADATION AND TURNOVER PATHWAYS

DSF inhibits proteasome function. The above observation that DSF alone was cytotoxic to OSCC cells was interesting as previous investigations have indicated that cytotoxicity occurs largely through proteasome inhibition by Cu-DSF, and is the primary contributor to DSF-cytotoxicity [Cvek and Dvorak, 2008]. To confirm that DSF treatment inhibited proteasome function in OSCC cell lines, we quantified levels of ubiquitinated proteins as a gross indicator of protein turnover and observed an increase in total ubiquitinated proteins for DSF and Cu-DSF treated cells, (WHCO1 cells shown in Fig. 3A, WHCO5 and SNO cells in supplementary data). Next, proteasome chymotrypsin-like activity was fluorescently quantified for treated cells using the fluorogenic substrate Suc-LLVY-AMC. This assay indicated that DSF and Cu-DSF markedly reduced proteasome function, with metformin co-treatment exacerbating this effect. Cu-8HQ served as an effective positive control for proteasome inhibition (WHCO1 cells shown in Fig. 3B, WHCO5 and SNO cells in supplementary data). In order to gain further insight into the contribution of proteasome inhibition to DSF-induced cytotoxicity in OSCC cell lines, two DSF analogues, dipyrrolidine thiuram disulphide (DPTD) and tetrapropyl thiuram disulphide (TTD) were synthesized and their effect on proteasome activity in OSCC cells tested. The monomers of DPTD and TTD, namely pyrrolidine dithiocarbamate and dipropyl dithiocarbamate, respectively, have significantly smaller decomposition rates than the diethyl dithiocarbamate monomer of DSF: second order decomposition rate constants for pyrrolidine dithiocarbamate, dipropyl dithiocarbamate and diethyl dithiocarbamate are 0.1, 500 and 2.5×104 L mol-1 min-1, respectively [Topping and Jones, 1998], but their copper complexes are still expected to exhibit proteasome inhibition, as previously established for Cu-(DPTD). We found that, DPTD and TTD were significantly better at inhibiting proteasome function in OSCC cells in comparison to DSF (40% and 20% more effective, respectively) (WHCO1 cells shown in Fig. 3C, WHCO5 and SNO cells in supplementary data), but importantly, MTT assays revealed DPTD and TTD to be far less toxic than DSF for all OSCC cell lines (approximately a log fold and half a log fold less toxic,

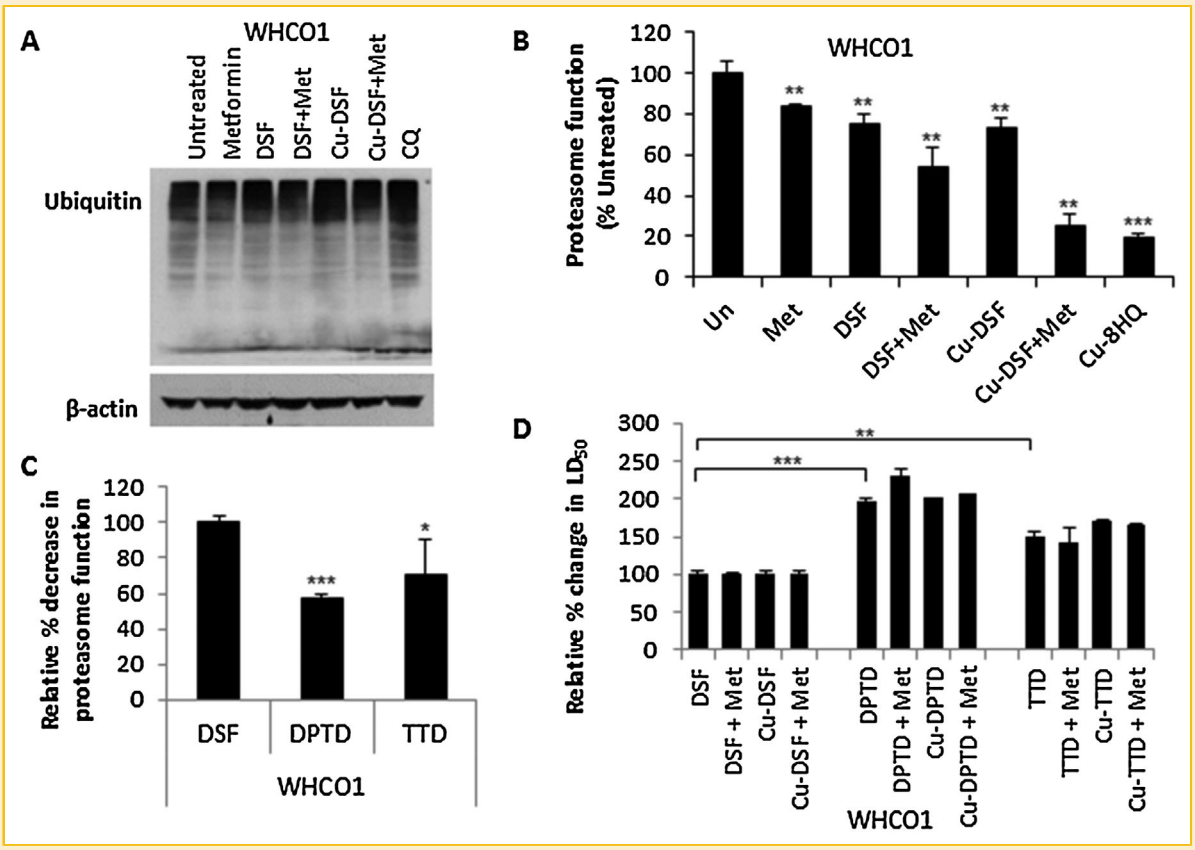

Fig. 3. DSF inhibits proteasomal function. (A) OSCC cells treated with LD_{30} concentrations of DSF for 48 h had higher amounts of total ubiquitinated proteins in comparison to untreated controls. Metformin (Met) treated cells (10 mM for 24 h) had slightly lower levels, as did cells treated with both DSF and metformin; as expected, chloroquine treated cells showed high levels of total ubiquitinated proteins. (B) Proteasomal function for chymotrypsin-like activity using the fluorogenic peptide LLVY-AMC showed that, as expected, DSF decreased proteasome function. Interestingly, metformin also had an inhibitory effect with slightly decreased fluorescence exhibited, and when combined with DSF or copper-DSF, proteasome activity was even further diminished. The positive control, Cu-8HQ, caused a considerable reduction in fluorescence as expected, ($n = 3$, mean $\pm$ SD). (C) DPTD and TTD, two analogues of DSF that have lower rates of decomposition, inhibited proteasome function more effectively in comparison to disulfiram. (D) DPTD and TTD displayed lower levels of OSCC cell toxicity in comparison to their respective controls ($n = 3$, mean $\pm$ SD) (all data shown for WHCO1 cells).

respectively) (WHCO1 cells shown in Fig. 3D, WHCO5, and SNO cells in supplementary data).

Collectively, these results suggested that: (1) proteasome inhibition by Cu-DSF was only a partial contributor to DSF-induced cytotoxicity in OSCC cell lines and (2) that the intracellular decomposition of the DSF monomer, DDC, may be important for DSF-induced cytotoxicity.

DSF alters lysosomal acidification. Dithiocarbamates are acid labile and rapidly break down at low pH to their parent amine and carbon disulfide. We therefore hypothesized that since the decomposition rates of the monomers (dithiocarbamates) for DSF, TTD and DPTD were found to be proportional to their cytotoxicities, a potential novel mechanism of toxicity for DSF could be via its diffusion into lysosomes, its conversion to acid labile DDC and subsequent decomposition of DDC within the low pH (~4) of the lysosomal environment to diethylamine and carbon disulfide, resulting in amine accumulation within lysosomes, amine-dependent lysosomal alkalinisation, and ultimately, reduced lysosomal function. Therefore to investigate the effect of DSF and DSF analogues on lysosomal pH, cells were treated with LD_{30} levels of DSF, DPTD, and TTD with or without cupric chloride and then stained with AO and examined by fluorescent microscopy. AO is a weekly basic, lysomsomotropic fluorescent dye, which upon accumulation in AVOs, including lysosomes, exhibits a red shift in fluorescence. Therefore acidic lysosomes stained with AO display orange/red fluorescence while lysosomes with reduced acidity display a progressive shift to yellow then green fluorescence with increasing alkalinisation. DSF treated cells displayed a marked increase in AVO (lysosomal) pH and this effect was exacerbated by the presence of copper, where overall fluorescence was both shifted towards green (DSF alone) and also reduced in intensity (Cu-DSF) in comparison to untreated controls, as seen across all OSCC cell lines (Fig. 4). DPTD had a minimal effect on lysosomal pH, with and without copper, while TTD treatment alone had a minimal effect on lysosomal pH while some reduction in overall fluorescence was observed in the presence of copper (Fig. 4). Chloroquine, a standard lysosomal alkaliniser, which served as a positive control, completely dissipated lysosomal pH as expected, as evidenced by bright green lysosomes (Fig. 4). These results strongly indicated that DSF did indeed perturb lysosomal pH/function and supported the above hypothesis that

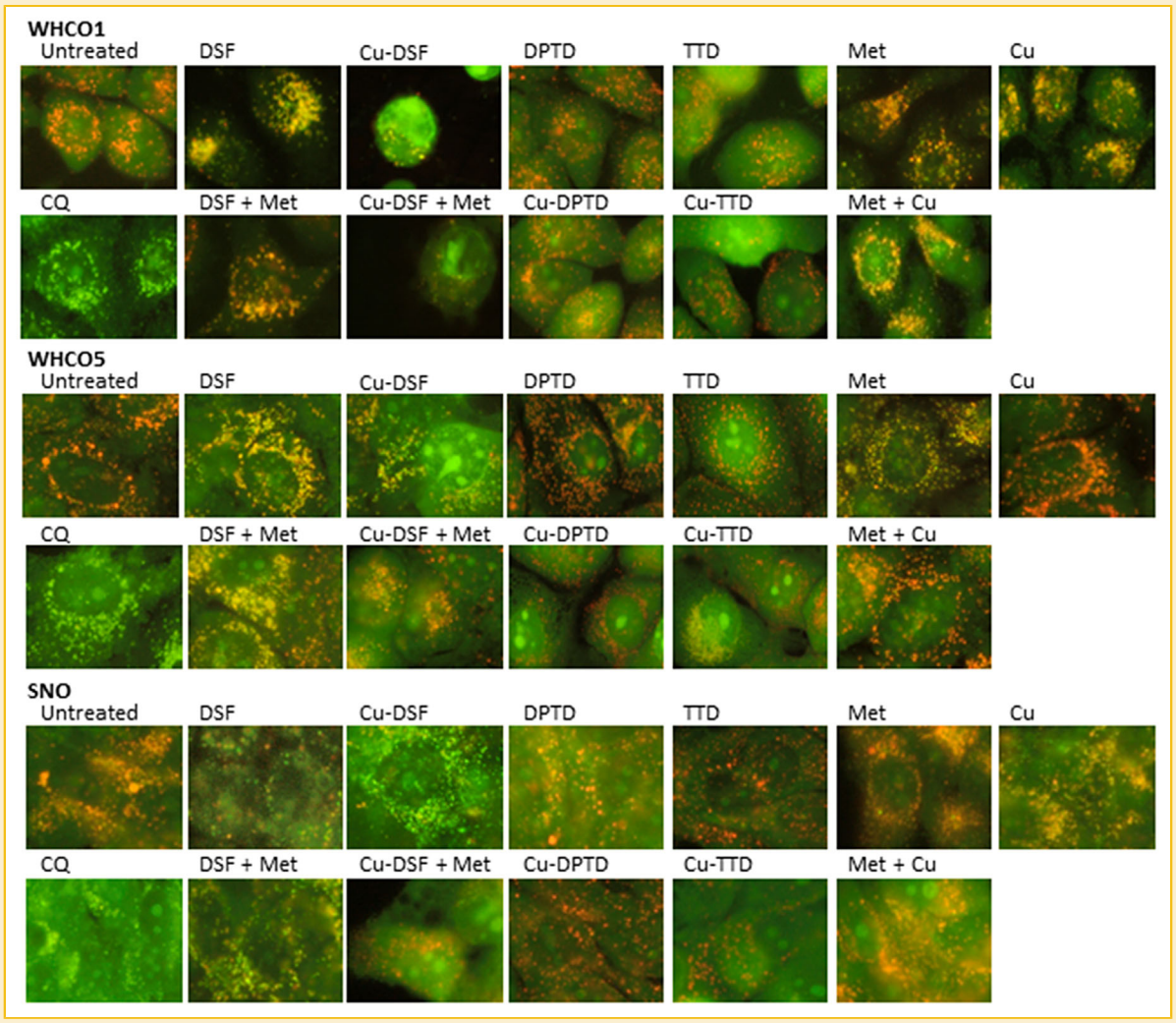

Fig. 4. DSF decreases AVO (lysosomal) acidification. Lysosomal acidification as visualized by AO staining shows a shift from red (low pH) to yellow to green (higher pH). DSF treated and metformin-DSF (DSF + Met) co-treated cells clearly had yellow lysosomes, in comparison the red lysosomes in untreated cells, clearly indicating an increase in pH of these organelles upon DSF treatment. Cu-DSF and metformin-Cu-DSF (Cu-DSF + Met) treatment was highly cytotoxic to cells. Cells treated with the known lysosomal inhibitor chloroquine (CQ) (1 mM for 1 h) as the positive control, had only green lysosomes, as expected. The DSF analogues DPTD and TTD did not show the same extent of perturbation of lysosomal acidification as DSF.

diethyl dithiocarbamate decomposition within lysosomes and subsequent diethylamine-dependent lysosomal alkalinisation may be responsible for this effect. DPTD and TTD, that have monomers with significantly smaller decomposition rates than the monomer of DSF (diethyl dithiocarbamate), exhibited a minimal effect on lysosomal pH, as would be expected for this model to be valid.

DSF inhibits autophagy. Damaged lysosomal function leads to perturbed autophagy [Yang et al., 2011]. We observed, by electron microscopy, that a representative OSCC cell line (WHCO1) exhibited numerous autophagic vesicles, which increased in number and size after metformin treatment (Fig. 5A), indicating a highly autophagic phenotype for OSCC cells and potentially, as with many other cancer cell lines, a high susceptibility to autophagic perturbation. We therefore investigated the effect of DSF on autophagic activity by assessing LC3B levels in OSCC cell lines. Cells treated with DSF exhibited a significant increase in LC3B-II relative to β-actin in response to DSF treatment and this effect was highly exacerbated by metformin, copper and copper/metformin co-treatment (Fig. 5B), suggesting that both perturbed lysosomal function and Cu-DSF mediated oxidative effects acted in concert to severely damage autophagic function in Cu-DSF treated cells, and this is not surprising given the sensitivity of the autophagic cascade to altered redox state [Filomeni et al., 2010]. Electron microscopy further supported the above findings in that DSF-treated cells contained large irregular autophagosomes while DSF-metformin treated cells exhibited a considerable accumulation of autophagosomes with poorly cleared autolysosomes. This suggested an inhibition in the maturation of autophagosomes to autolysosomes, which occurs upon fusion of the autophagosome with the lysosome, and is a potential consequence of perturbed lysosomal function (Fig. 5A).

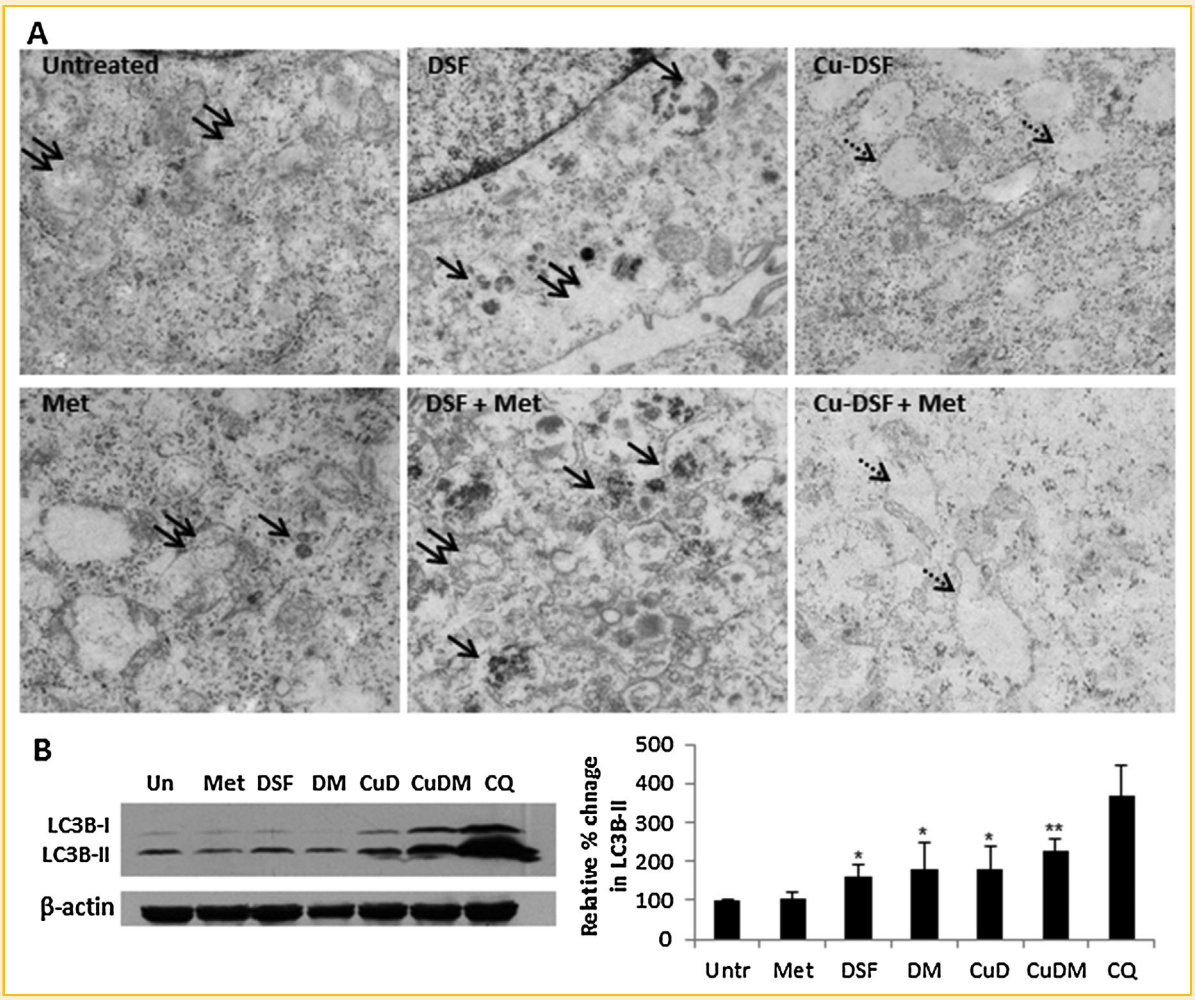

Fig. 5. DSF inhibits autophagy. (A) Electron microscopy showed a small increase in the numbers of autophagosomes and autolysosomes in metformin (Met) only treated cells. DSF only treated cells also exhibited increased numbers of autophagosomes, in comparison to untreated control. Interestingly, metformin-DSF (DSF + Met) co-treated cells, showed a considerable accumulation of autophagosomes (single arrow) with protein aggregates and far fewer cleared autolysosomes (double arrow). Cu-DSF and metformin-CU-DSF (Cu-DSF+Met) treated cells show considerable vacuolization (dashed arrow). (B) Autophagy by LC3B western blot showed that metformin (Met) alone did not significantly alter autophagy but that DSF alone treated cells had slightly higher LC3B-II levels relative to β-actin in comparison to untreated controls (Un), but that for metformin-DSF (DM), Cu-DSF (CuD) and metformin-CuDSF (CuDM) treated cells this increase in LC3B-II was significant. The positive control chloroquine (CQ) displayed a large proportion of the LC3B-II form as expected.

Cu-DSF and Cu-DSF-metformin treated cells exhibited vacuolization (Fig. 5A) along with a significant increase in LC3B-II (Fig. 5B), which is likely to be indicative of cell death with autophagy [Kroemer et al 2009].

DISCUSSION

Cancer cells have a higher dependence on protein degradation and turnover pathways than normal cells, in order to accommodate for their elevated metabolic rates [Mancias and Kimmelman, 2011; Molineaux 2012]. These pathways comprise the ubiquitin-proteasome system, where proteins are targeted by polyubiquitination for degradation by the proteasome; and lysosomal proteolysis, which involves the degradation of proteins by proteases in this acidic organelle. This latter pathway also includes autophagy since autophagosomes, containing cellular material for degradation, fuse with lysosomes to form autolysosomes, in which organelles and proteins are degraded [Yang et al., 2011]. Cancer cells are therefore very sensitive to inhibition of these pathways and this has led to the identification and development of new classes of inhibitors that have a greater effect on cancer cells over normal cells; proteasome inhibitors and modifiers of autophagy such as bortezomib and hydroxychloroquine, respectively, are under extensive clinical trial evaluation [Molineaux 2012].

In recent years, DSF (and Cu-DSF), has been shown to exhibit anticancer properties both in vitro and in vivo [Cvek, 2011]. We have found in this study that both Cu-DSF and DSF are highly cytotoxic to OSCC cells lines. OSCC is a particularly aggressive carcinoma and there are very few treatment options, especially in the resource poor

countries in which OSCC is prevalent [Ilson 2008; Ferlay et al., 2010], and as such, drug "repurposing" is an attractive prospect. DSF displays a high specificity in its toxicity towards cancer cells. We show that in addition to proteasome inhibition, which is evidenced in this study and has been previously documented [Cvek and Dvorak 2008], DSF also perturbs AVO (lysosomal) acidification and subsequently autophagy (an effect greatly enhanced by copper) in OSCC cells. This simultaneous inhibition of multiple protein degradation/turnover pathways in cells that are so highly dependent on these processes, may additionally explain the cancer-cell specific toxicity of DSF over normal cells.

In this study we also show that metformin, which is highly anti-proliferative in OSCC cells [Damelin et al., 2014], significantly increases DSF cytotoxicity. We have shown that a major reason for this is a metformin-dependent increase in copper transport but as metformin also induces a reductive phenotype in OSCC cells [Damelin et al., 2014], metformin treatment may increase the rate of reduction of DSF to its monomer, diethyl dithiocarbamate, the decomposition of which is likely responsible for lysosomal alkalinisation. Electron microscopy in this study, and in previous studies, also indicate that metformin may increase autophagy [Tomic et al., 2011], therefore also potentially increasing cell susceptibility to DSF-dependent autophagic perturbation.

Our findings therefore highlight the potential use of DSF in OSCC therapy and the use of metformin/DSF as a highly efficacious and economical drug therapy combination in the treatment of this highly aggressive malignancy. Such repurposing of existing drugs that are already very well characterized could be particularly beneficial in resource poor settings where OSCC is prevalent.

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AUTHOR'S CONTRIBUTIONS

RJ carried out toxicity assays, western blots, fluorescence microscopy, proteasome assays, statistical analysis and assisted in drafting the manuscript; LHD conceived the study, participated in its design, synthesized the disulfiram analogues, performed fluorescence microscopy, conducted data analysis and interpretation, and assisted in drafting the manuscript; MB prepared electron microscopy cells and images; ALR performed NMR spectroscopic analysis; RBV assisted in manuscript preparation; DMD conceived the study, participated in its design, conducted data analysis and interpretation, drafted and approved the final manuscript.

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