The common diabetes drug metformin can diminish the action of citral against Rhabdomyosarcoma cells in vitro

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Rhabdomyosarcoma (RMS) is a rare type of soft tissue sarcoma most commonly found in pediatric patients. Despite progress, new and improved drug regimens are needed to increase survival rates. Citral, a natural product plant oil can induce cell death in cancer cells. Another compound, metformin, isolated originally from French lilac and used by diabetics, has been shown to reduce the incidence of cancer in these patients. Application of citral to RMS cells showed increase in cell death, and RD and RH30 cells showed half maximal inhibitory concentration (IC50) values as low as 36.28 μM and 62.37 μM, respectively. It was also shown that the citral initiated cell apoptosis through an increase in reactive oxygen species (ROS) and free calcium. In comparison, metformin only showed moderate cell death in RMS cell lines at a very high concentration (1,000 μM). Combinatorial experiments, however, indicated that citral and metformin worked antagonistically when used together. In particular, the ability of metformin to quench the ROS induced by citral could lead to the suppression of activity. These results clearly indicate that while clinical use of citral is a promising anti-tumor therapy, caution should be exercised in patients using metformin for diabetes.

KEYWORDS
chemotherapy, citral, glycolysis, metformin, mitochondrial respiration, Rhabdomyosarcoma

INTRODUCTION

To investigate new drugs which may have activity against Rhabdomyosarcoma (RMS) cells, we screened the PECKISH (Plant Extract Collection Kiel in Schleswig-Holstein) open access library (Onur, Stöckmann, Zenthofer, Piker, & Döring, 2013). The library extracts were obtained from health shops, outdoor cultivated plants, marine algae, and herbs from traditional Chinese medicine. Our studies showed cell death activity against RMS cells with extracts from Verbena officinalis (Common verbain), and to a lesser extent Galega officinalis (French lilac). Literature searches showed that the most likely active compounds from these plant extracts were citral and metformin. V. officinalis (and its main component citral) has previously been shown to have pro-apoptotic activity in chronic lymphocytic leukemia (De Martino et al., 2009). Citral (3,7-dimethyl-2,6-octadienal) is also found in other lemon scented plants, such as Melissa officinalis and Lemon grass, and is a mixture of the two monoterpenoid isomers geranial and neral. The structure of two citral isomers is shown as Figure S3. Citral is a small molecule which is hydrophobic and able to diffuse through cell membranes. In addition it can be readily metabolized by mammalian cells since it is a strong electrophile with an α,β-unsaturated carbonyl group (Diliberto et al., 1990). We have previously shown that in addition to causing cell death in RMS, citral causes changes in the mitochondrial morphology (White, Evison, 2004).
At low concentrations of citral, the mitochondria change into toroidal structures, most likely as a protective mechanism which enables the mitochondria to tolerate volume increases. At higher concentrations of citral, the mitochondria swell and eventually reach a threshold after which the inner mitochondrial membrane ruptures. A number of different in vitro studies have shown that in comparison to cisplatin which has an half maximal inhibitory concentration (IC$_{50}$) of between 5 and 15 μM, citral has an IC$_{50}$ of between 20 and 50 μM (Kapur et al., 2016). In vivo mice experiments have also shown efficacy against 4T1 mammary tumors resulting in smaller tumor size and delayed tumorigenicity after reimplantation of the primary tumor cells into normal mice (Nigeh, Nordin, & Rosli, 2019). G. officinalis is a perennial herb which is 3 ft tall with purple, blue, or white flowers, and was used in folk medicine to treat diabetes from the Middle Ages. Guanidine was found to be the active ingredient of French lilac and in the 1950s metformin and phenformin, the two main biguanides, were introduced as a treatment for diabetes. Today metformin is prescribed to almost 120 million people for the treatment of type II diabetes (Ida et al., 2020). Interestingly, retrospective studies have shown that metformin is associated with a decreased cancer risk in diabetic patients (DeCensi et al., 2010). In general, the incidence of tumor development is higher in diabetic patients than healthy controls, and cancer patients with diabetes mellitus are less sensitive to chemotherapy and less likely to survive (Currie et al., 2012). However, studies showed that the incidence of tumorigenesis and mortality of patients with diabetes was significantly reduced in the population of patients taking metformin (Kumar et al., 2013). Further in vivo and in vitro studies have shown that metformin may repress the proliferation of tumor cells, induce apoptosis, autophagy, and cell cycle arrest (Bost, Sahra, Le Marchand-Brustel, & Tanti, 2012). As such, metformin has recently been repurposed, and there are currently several clinical trials under way to test the efficacy of metformin as an adjuvant to conventional chemotherapy as well as in combination with new, targeted agents for breast, prostate cancer, and other solid malignancies (Kourelis & Siegel, 2012). This study therefore aimed to investigate the action of citral and metformin in RMS cells, and in combination, to explore whether there are synergistic effects of the drugs on cancer cells.

2 | METHODS

2.1 | Immortalized cell lines

Two different immortalized human cancer cell lines and one fibroblast control cell line were tested in this study. RH30 (American Type Tissue Culture Collection [ATCC no. CRL-2061]), an alveolar RMS was originally derived from the bone marrow metastasis of 17-year-old male. Alveolar RMS (aRMS) commonly occurs during adolescence in the limbs, chest, genitalia, and abdomen. RD (ATCC no. CRL-7763), an embryonal RMS (eRMS) was derived from the muscle carcinoma of a 7-year-old female. The eRMS is a childhood malignant tumor and more likely to occur in children with a high birth weight. Tumors often arise in the head, neck, and genitourinary tract. The cells are adherent with spindle shape and histologically resemble fetal muscle. RH30 and RD were both obtained from the American Type Culture Collection (ATCC; Manassas; VA). The fibroblast control cell line was a kind gift from Dr Jo Poulton (Nuffield Department of Women’s & Reproductive Health, Oxford University).

2.2 | Cell culture

Cells were plated at a density of $1 \times 10^4$ cells per well (96-well microplate; Nunc, Thermofisher Scientific, Renfrew, UK) in 200 μL of growth media (Dulbecco’s Modified Eagle’s Media [DMEM] with 25 mM glucose, 1 mM pyruvate [Sigma- Aldrich, Poole, UK], supplemented with 10% fetal bovine serum [Sigma- Aldrich, Poole, UK], 2 mM 1-glutamine [Gibco, Life Technologies Ltd, Renfrew, UK], 100 U/mL Penicillin and 0.1 mg/mL Streptomycin [Gibco, Life Technologies Ltd, Renfrew, UK]) in 96-well microplates and incubated at 37°C in 5% CO$_2$ atmosphere.

2.3 | Cell survival assay

Cells were rinsed with pre-warmed phosphate buffered saline (PBS) and fixed with 1% glutaraldehyde solution for 30 min. Fixed cells were stained with crystal violet solution (1% crystal violet [Sigma-Aldrich, Poole, UK] dissolved in a mixture of methanol:glacial acetic acid: H$_2$O, 5:1:4 v/v/v) for 1 hr at room temperature. After staining, the supernant was removed, and cells washed carefully three times with water. The microplate was then drained and dried overnight. Before reading, 100 μL solubilizer (1% sodium dodecyl sulphate (SDS) in 10% glacial acetic acid) was added to each well and re-suspended vigorously. The absorbance was read in a plate reader at 540 nm.

2.4 | Cell metabolic assay (MTT)

The colorimetric MTT \((3-[4,5\text{-dimethylthiazol}-2\text{-yl}]-2,5\text{-diphenyltetrazolium bromide})\) assay was applied for quantifying the cellular proliferation and metabolic level after drug treatment. After 24 hr of drug incubation with cells, the media was removed and replaced with 100 μL of growth media containing 0.5 mg/mL of MTT. Formazan crystals were confirmed after 3 hr incubation at 37°C. All media in the well was carefully aspirated and 100 μL of DMSO (dimethyl sulfoxide) was then used to solubilize the formazan. The microplate was then incubated for 20 min and shaken for 2 min before reading. The absorbance was read in a microplate reader at 575 nm.

2.5 | Lactate measurements

Lactate generation was evaluated with Accutrend BM-lactate strips (Roche, Welwyn Garden City, UK). For each sample, 25 μL cell culture
media from each treatment was added on to the reactive pad of a new lactate strip. The result was read by an Accutrend Lactate Meter (Roche, Welwyn Garden City, UK) after incubation for 1 min.

### 2.6 Western blot

Cells were lysed in tissue extraction buffer (TEB; 50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, plus 1 tablet of protease inhibitor in every 10 mL added to the buffer just before use). Each extract was collected and transferred to a 1.5 mL sterile tube (Eppendorf, Loughborough, UK). Protein concentrations were determined using a bicinchoninic acid assay (Pierce™, Bio-rad, Watford, UK).

10 μg of protein from each sample was resolved by SDS-Polyacrylamide gel electrophoresis. (Thermofisher Scientific, Renfrew, UK) 8–16% Precise Protein Gel; Thermo Scientific BuPH™ Tris-HEPES-SDS running buffer) at 180 V for 1 hr, and transferred to nitrocellulose membranes in cold transfer buffer (200 mM Glycine, 25 mM Tris, 20% Methanol) at 100 V for 1 hr. To visualize and confirm the transfer of the protein bands the membranes were stained in Ponceau S solution (0.1% [w/v] Ponceau S in 5% acetic acid) for 5 min, and de-stained in water. Membranes were blocked in Tris buffered saline- Tween (TBST) buffer (10 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20), and blocked in 5% non-fat milk in TBST solution for 2 hr at room temperature. The primary antibody, γ-tubulin (1/1,000 dilution; Biolegend, London, UK), VDAC-1 (1/1,000 dilution; Biolegend, London, UK), or β-actin (1/200; Santa Cruz, Texas, UK) was diluted in 5% non-fat milk in TBST solution. Membranes were incubated in a primary antibody dilution overnight at 4°C. After the membranes were washed three times in TBST, they were incubated in 1/1,000 secondary anti-rabbit antibody in 5% non-fat milk TBST solution for 1 hr. The membranes were washed three times and incubated in 2 mL working solution of Pierce™ (Bio-rad, Watford, UK) ECL Western Blotting Substrate (Life technologies™) for 2 min. The chemiluminescence was detected using a gel imaging system (G:Box F3; Syngene, Cambridge, UK).

### 2.7 Reactive oxygen species (ROS) assay

Carboxy-H2DCFDA is a non-fluorescent compound oxidized in the presence of ROS to a fluorescent green compound. The carboxy derivative carries additional negative charges that improve its retention inside cells compared to noncarboxylated forms. The assay was performed according to manufacturer’s instructions (Thermofisher Scientific, Renfrew, UK) as described briefly: Growth media was removed from adherent cells, and carboxy-H2DCFDA added in PBS to a final concentration of 10 μM. The cells were then incubated at 37°C, 5% CO2 for 60 min. The buffer was then removed and replaced with pre-warmed cell culture media. The baseline fluorescence was measured (Ex. 465 nm, Em. 510 nm) with a Tecan (Teale, UK) Infinite f200 series plate reader, and then the test compounds (citral [95%, Sigma-Aldrich, Poole, UK] and metformin hydrochloride [Pharmaceutical Secondary Standard, Sigma-Aldrich, Poole, UK]) added. The fluorescence was measured periodically, and returned to the incubator between measurements.

### 2.8 Intracellular calcium assay

Cells were seeded in 96 well plates at 1 x 10^4 cells per well in growth media and left overnight in a cell culture incubator for the cells to adhere. The following day cells were washed in PBS and pre-stained with 2 μM Fluo4 AM (Invitrogen, Life Technologies, Renfrew, UK) in PBS for 1 hr. The acetoxymethyl (AM) ester is conjugated to the Fluo-4 dye which results in an uncharged molecule that can permeate cell membranes. Once inside the cell the lipophilic blocking groups are cleaved by non-specific esterases which significantly reduce leakage of the parent compound from the cell. The change in fluorescence was measured over 4.5 hr, at 30-min intervals, using a Tecan (Teale, UK) Infinite f200 series plate reader equipped with filters for FITC (Ex. 495 nm, Em. 519 nm). Samples were blank corrected and the maximum slope of the linear region of each curve was calculated. Experiments were performed in triplicate on three separate occasions.

### 2.9 Mitochondrial membrane potential measurement

Mitochondrial membrane potential was assessed using tetramethylrhodamine methyl ester (TMRM: Thermofisher Scientific) which is a cell-permeant, cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria. Cells were seeded in 96 well plates and grown as per Section 2.1 overnight. The media was then removed and the cells were washed with PBS. The TMRM (100 nM) was then resuspended in phenol red free Roswell Park Memorial Institute (RPMI) I media and added to the cells, the plate wrapped in foil to protect from light, and returned to the incubator for 30 min. The media was then removed and washed with PBS, and RPMI media (without TMRM) added back to the cells. Citral and metformin were then added to the cell media. The plate was returned to the incubator and the emission measured periodically in a Tecan f200 plate reader (Ex. 544 nm, Em. 612 nm).

### 2.10 Incubation of cells with sodium phenylbutyrate

To investigate whether cell death is mediated via an endoplasmic reticulum stress response, the molecular chaperone sodium phenylbutyrate (4-PBA) was used to alleviate protein misfolding by serving as a molecular chaperone. Cells were seeded at 1 x 10^4 cells/well in 96 well plates. Cells were incubated for 24 hr to allow adherence of the cells to the plate. Citral and 4-PBA were added to the wells and incubated for a further 24 hr. Media was removed from the
plates by aspiration. Cells were then either stained with crystal violet (Section 2.3) or MTT.

2.11 Statistical analysis

All experimental data were analyzed with Microsoft™ Excel platform, and plotted with GraphPad Prism 8.0.2 (GraphPad Prism, La Jolla, CA, USA). All results were presented as mean ± SD. Significant differences were shown when the p values were less than .05. Two-tailed homoscedastic Student’s t test & one-way analysis of variance were applied for statistical analysis when two or more groups were compared.

2.12 Other software

The citral isomers structures were plotted with ChemDraw 19.1 (PerkinElmer, Massachusetts, US). The western blotting images were quantified with ImageJ 1.53 (NIH, US) and normalized with the control groups’ data for further plotting.

2.13 Experimental methodology

Experiments were not randomized, or blinded to the researcher. All samples were assessed against cells incubated with the appropriate vehicle solvent as a negative control. The action of citral was taken to be a positive control.

The authors have also taken into account the guidelines for best practice in natural products pharmacological research (Heinrich et al., 2020; Izzo et al., 2020).

3 RESULTS

3.1 Cell death after incubation with citral and metformin

We have previously shown that citral is an effective drug to increase cell death in RMS cells (White et al., 2017). Herein we have looked at the relationship between cell death with citral and the addition of the diabetic drug metformin, which has been suggested to affect the energy supply to cancerous cells, and subsequently cause cell death. Figure 1 shows that a high concentration of metformin (1,000 μM) significantly reduced RD and RH30 cell numbers by over 30% following a 24 hr incubation. Citral alone shows a statistically significant cell death in RD cells over all concentrations tested (50 μM to 1,000 μM). With RH30 cells and citral treatment there was a trend for a reduction in cell survival at all concentrations tested, but this is only highly statistically significant above 500 μM.

Interestingly when RD cells were co-incubated with citral (50 μM or 100 μM) and metformin (50 μM, 100 μM, 500 μM, or 1,000 μM) the addition of metformin abrogated the effects of citral when used alone (Figure 1c). When high concentrations of citral (500 μM or 1,000 μM) were used, the addition of metformin was not able to significantly enhance cell survival. RH30 cells showed a similar tendency for metformin to reduce the effects of citral but failed to reach significance due to citral treatment alone having less of an impact on this cell line (Figure 1d).

3.2 The order of citral and metformin addition does not affect cell survival

Since metformin was shown to affect the activity of citral (Figure 1a, b), the order of addition of the compounds was examined further. RMS cells were either incubated with citral or metformin for 6 hr prior to the addition of the other compound, or the two compounds were added simultaneously. The results are shown in Figure 1, and described as either C + M, or M + C to denote the order of addition, or C/M to indicate that the compounds were added together. It can be seen that irrespective of whether cells are pre-incubated for 6 hr with either compound or added simultaneously, there is no change in the cell viability in either RD (Figure 1e) or RH30 cells (Figure 1f).

3.3 Cell death after incubation with either citral or metformin at reduced glucose levels

The experimental data shown in Figure 1 were from cells grown under conventional conditions of media containing 25 mM glucose (4,500 mg/L). However, in vivo such supraphysiological levels of glucose would represent an extreme diabetic state (Li, Télémaque, Miller, & Marsh, 2005). We therefore compared the action of the compounds in cells grown in high glucose (25 mM) and low glucose (5 mM). Normal serum glucose is usually maintained between 4 and 6 mM, and a value of 5 mM glucose was chosen for "low glucose" since lower concentrations affect cell growth rates in culture (Bhattacharya et al., 2014).

Incubation of RMS cells with citral showed that cell death was only minimally affected by low glucose compared to high glucose (Figure 2a,c). However, the action of metformin can be affected by the concentration of glucose in the media. When glucose is plentiful (25 mM) metformin does not cause cell death in RH30 cells (Figure 2d), however, a significant increase in cell death occurs when cells are grown under low glucose media with effects observed in the lower μM range consistent with an increase in respiration under low glucose conditions (Potter, Newport, & Morten, 2016). In RD cells, there is a concentration dependent decrease in cell viability with increasing metformin concentrations in RD cells under high glucose concentrations at high μM levels (Figure 2b). This is further enhanced by growth in 5 μM glucose with metformin now becoming active as a killing agent at the low μM levels (Figure 2b). The effect of metformin on the RD cells at the high glucose levels but not on the RH30 cells suggests that the RD cells have a greater requirement for mitochondrial respiration under high glucose conditions. As would be expected, when glucose levels are reduced and mitochondrial respiration becomes more important (Potter et al., 2016) as glucose is removed from the media, both cell lines show increased metformin activity reducing...
cell number. This could explain why in Figure 1 it can be seen that above 100 μM citral (i.e., 500 μM and 1,000 μM) the co-addition of citral and metformin results in greater cell death than citral alone, in contrast to the decrease in cell death seen after addition of metformin when incubated with either 50 μM or 100 μM citral. The addition of citral increases the metabolic rate of the cells which removes glucose from the media, and under low glucose conditions metformin causes cell death.

3.4 | Cell death after co-incubation with both citral and metformin in low glucose

As shown in Figure 2 there was little difference in the amount of cell death caused by incubation with citral in low or high glucose. However, since the degree of cell death after incubation with metformin could be affected by the concentration of glucose in the media, co-incubation with metformin and citral was repeated under low glucose conditions (Figure 3a). Comparison of Figures 1 and 3a shows that there is little difference between the experiments with high and low glucose. However, this could be due to depletion of glucose from the media by the metabolism of the cells.

3.5 | Lactate levels after incubation with citral and metformin

In RH30 cells it can be seen that there is no increase in lactate with the addition of citral (Figure 3b). The co-addition of metformin does not affect the amount of lactate released. Conversely, in RD cells increasing lactate levels are seen with increasing citral concentrations indicating glycolysis. However, the co-addition of metformin in RD cells prevents the increase in lactate levels. This indicates that the co-addition of metformin decreased glycolysis and hence lactate production.

3.6 | Metformin quenches citral-induced ROS

Citral was shown to cause a statistically significant increase in ROS generation in both RD and RH30 cells when compared to cell only controls (Figure 3c). The amount of ROS generated by the addition of either 500 μM or 1,000 μM citral to either RD or RH30 cells appears to increase the amount of ROS by the same amount in the two cell lines. (ROS was below the level of detection for citral concentrations below 500 μM; data not shown.) Metformin was also...
seen to cause a small increase in ROS although the increase does not seem to be concentration dependent (between 50 $\mu$M and 1,000 $\mu$M). Both RD and RH30 cells are similarly affected. However, the co-addition of metformin to cells incubated with either 500 $\mu$M or 1,000 $\mu$M citral shows quenching of ROS generation in RD cells. Quenching is seen in RH30 cells only when incubated with 1,000 $\mu$M citral (except for 500C + 1,000M). RD cells use mitochondrial respiration to a greater extent so metformin could be acting at a mitochondrial site. In RD, metformin quenches citral-induced lactate increase, and citral-induced ROS.

3.7 | Calcium flux after incubation with citral and metformin

The rate of change of calcium flux was measured over 24 hr. Incubation with citral showed an increase in calcium flux at concentrations of 500 $\mu$M and 1,000 $\mu$M in both RD and RH30 cells. All concentrations of metformin showed no significant change in the calcium flux, in either RD or RH30 cells.

Co-incubation of 50 $\mu$M or 100 $\mu$M citral with metformin (50 $\mu$M to 1,000 $\mu$M) did not show any significant differences in the amount of calcium for either RD or RH30 cells (Figure 3d,e). Co-incubation of RD cells with metformin and 500 $\mu$M or 1,000 $\mu$M citral showed a statistically significant decrease in calcium compared to the citral alone. While a similar decrease could be seen in RH30 cells, this was not statistically significant. Calcium signaling is intrinsically linked with ROS signaling. Since ROS was only generated in significant amounts at 500 $\mu$M and 1,000 $\mu$M (Figure 3c), the increased calcium concentrations at 500 $\mu$M and 1,000 $\mu$M citral could be linked to ROS signaling. Metformin can quench ROS and could therefore prevent signaling to generate an increase in calcium.

3.8 | Citral and metformin do not cause cell death in an ER dependent manner

Sodium phenylbutyrate (4-PBA) has been shown to alleviate protein misfolding by serving as a molecular chaperone, and as such can relieve ER stress. Therefore, if cell death is reduced in the presence of 4-PBA it is likely that there is ER involvement. Crystal violet proliferation assays (Figure 4a) were used to look at the effects of 4-PBA on the activity of citral in RMS cell lines. First, 2.5 mM and 5 mM 4-PBA alone was tested, and could be seen to decrease cell survival in both RD and RH30 cells. This was not seen in control fibroblast cells. Equally, 200 $\mu$M and 500 $\mu$M citral did not affect cell survival in fibroblast cells.
but could be seen to affect the RMS cell lines (although this was only statistically significant for RH30). 4-PBA was not seen to attenuate the effects on citral on the RMS cell lines. Cell metabolism assays (MTT) were also undertaken in addition to crystal violet cell proliferation assays and showed very similar results (Figure 4d).

3.9 Citral and metformin modify gamma tubulin and voltage-dependent anion channel (VDAC) levels

Increasing concentrations of citral from 0.1 μM to 200 μM showed a decrease in expression levels of gamma tubulin in RD cells (Figure 4c and S2). Metformin (50 μM) was shown to decrease expression levels of gamma tubulin compared to the control. The co-addition of citral (all concentrations) and metformin decreased gamma tubulin levels to that of metformin alone. VDAC levels increased at 0.1 μM citral, but then decreased to control levels with increasing concentrations of citral up to 100 μM citral (Figure 4d and S2). However, at 200 μM citral, there was an increase in VDAC expression. Metformin alone did not significantly affect VDAC levels.

4 DISCUSSION

4.1 Increasing citral concentration results in increased ROS generation, and cell death

We investigated the actions of citral and metformin alone, and in combination against RMS cells. Citral is a relatively small and hydrophobic molecule which diffuses readily through cell membranes. It is also an ω1-unsaturated aldehyde which can participate in a Michael addition reaction with sulfhydryl groups of glutathione and the cysteine residues of proteins (Kapur et al., 2016). Citral has previously been shown to induce caspase-3 activity, activate p53, and decrease Bcl-2 expression.
It was also shown to result in apoptosis in two endometrial cancer cell lines (ECC-1 and Ishikawa) at IC_{50} of 15–25 μM (Liu et al., 2012). In this study we have demonstrated that citral causes cell death in the RMS cell lines, RD and RH30 with IC_{50} 36.28 μM and 62.37 μM, respectively. Mice in vivo experiments have shown that for 4T1 tumors, concentrations of between 40 and 80 mg/kg of citral showed antitumor effects whereas 100 mg/kg body weight of citral caused abnormalities (Zeng, Kapur, Patankar, & Xiong, 2015). Metformin is a complex drug with multiple sites of action and multiple molecular mechanisms, although it is widely used in relation to glucose metabolism, and is thought to inhibit mitochondrial respiration (Rena, Hardie, & Pearson, 2017). Therapeutic concentrations of metformin have been reported for up to 1,800 mg/L (14 mM) (Kajbaf, De Broe, & Lalau, 2016), although this is far above the usually reported value for metformin intoxication (267 mg/L or 2 mM) (Al-Abri, Hayashi, Thoren, & Olson, 2013). Metformin only shows significant cell death in the RMS lines at concentrations of 1,000 μM (Figure 1). When metformin and citral were added in combination cell death was prevented at low concentrations of citral, but the metformin was unable to prevent cell death at concentrations of citral of 500 μM and above.

### 4.2 Low glucose levels increased glycolysis rates in cells

While “high glucose” media creates an optimal environment for cancer cell proliferation, these glucose levels may complicate the interpretation of drug efficacy studies (Zhuang, Chan, Haugrud, & Miskimins, 2014) since normal serum glucose levels in vivo are usually maintained between 4 and 6 mM. Cell death after incubation with citral alone showed little difference between high and low glucose states, although metformin was more affected (Figures 1 and 2). When the
compounds were used in combination there were no significant differences between cell death in high and low glucose (Figures 1 and 3a). However, it is possible that the glucose is depleted by rapidly metabolizing cells. It has been shown in the literature that in 25 mM glucose-containing media that metformin inhibited oxygen consumption rate (OCR; Zhuang et al., 2014), which was consistent with an effect on electron transport. The metformin also stimulated glycolysis as measured by the extracellular acidification rate. However, in media containing low levels of glucose it is not possible to maintain the high levels of glycolysis which contributes to the depletion of adenosine triphosphate (ATP), and eventually cell death. Glycolytic flux from cells in culture can be quantified by measuring lactate excretion. RD cells showed an increase in lactate concentrations with increasing citral implying an increase in excretion (Figure 3b). These increases were prevented by the addition of metformin. In addition to lactate, cell death was also increased in RD compared to RH30 cells.

4.3 | Citral increases ROS which is quenched by metformin

We have shown that citral significantly increased ROS levels in both RD and RH30 cells (Figure 3c). This has also been shown previously in both HeLa and ME180 cells (Ghosh, 2013). Co-incubation of 1,000 μM citral with metformin showed quenching of ROS in both cell lines for all concentrations of metformin. Metformin has been shown to reduce ROS levels in human leukocytes by either directly scavenging the free radicals or modulating their intracellular production (Bonnefont-Rousselot et al., 2003). Furthermore, Kapur et al., showed apoptosis was reduced in cells treated with citral in the presence of an oxygen scavenger (Kapur et al., 2016). This is in agreement with our data which show that metformin is unable to quench the increased amounts of ROS generated by higher concentrations of citral (Figure 1).

4.4 | Calcium involvement in cell death

It has been previously observed that during apoptosis calcium is released from the ER and floods the cytosol with free calcium. This causes the activation of degradative processes and dysfunction of organelles, in particular the mitochondria. We therefore assessed calcium levels in response to the two drugs under investigation. There was a general trend for increasing calcium with increasing citral for both RD and RH30 cells (Figure 3d,e). Interplay between calcium signaling and ROS production is well described, although the communication between ROS and calcium is highly dependent on cell type (Gordeeva, Zvyagilskaya, & Labas, 2003). The increases that we see in calcium mirror those of ROS seen with increasing citral (Figure 3c). Calcium release may also open the mitochondrial permeability transition pore which can cause mitochondrial depolarization, resulting in the uncoupling of oxidative phosphorylation (OXPHOS) and mitochondrial swelling (Toné et al., 2007). We have previously shown that low concentrations of citral induce toroidal-shaped mitochondria in RMS cells, while higher concentrations show mitochondrial swelling (White et al., 2017).

4.5 | Stress response to citral does not involve the endoplasmic reticulum

4-PBA is a chemical chaperone which stabilizes proteins. It can stop the activation of ER-stress induced cell death by reducing the amount of misfolded protein (Perlmuter, 2002). We therefore investigated whether 4-PBA could prevent cell death in RMS cells in the presence of citral. Our results indicated that citral is not working through an ER-stress response in RMS cells (Figure 4a,b). A previous study has shown that 4-PBA attenuates the effects of citral in SKOV-3 cells, but not in ECC-1 or OVCAR-3 cells (Kapur et al., 2016). The authors concluded that citral works in a p53-dependent manner, unless p53 is absent, in which case it works via an ER stress-dependent mechanism. This implies that in RMS cells citral is working in a p53-dependent manner. In addition to the use of 4-PBA as a molecule to investigate stress responses involving the ER, it appears that it can also act as an inducer of apoptosis. 4-PBA at concentrations of 5 mM showed a significant increase in cell death in the RMS cell lines, although not control fibroblasts (Figure 4a,b). A study looking at glioblastoma cell lines also found that LN-18 cells were insensitive to 4-PBA whereas LN-229 cells showed increased apoptosis (Kusaczuk, Krętowski, Bartoszewicz, & Checowska-Pasko, 2016). Therefore, it appears that the effects on 4-PBA on cell death are not only cell-type-specific but also determined by the particular cell line.

4.6 | Reduction in γ-tubulin levels results in initiation of apoptosis

We have shown in RD cells that there is an inverse relationship between citral levels and the expression of γ-tubulin protein (Figure 4c,d). γ-tubulin is an important regulator of microtubule formation and nucleates αβ-tubulin dimers at the minus end of growing microtubules. Tubulin, the heterodimeric subunit of microtubules has been shown to bind mitochondria via the VDAC (Bernier-Valentin & Rousslet, 1982; Carré et al., 2002). Dimeric tubulin closes VDAC channels reconstituted into planar phospholipid membranes (Rostovtseva et al., 2008). The microtubule destabilizing compounds rotenone, colchicine, and nocodazole were shown to increase free tubulin and decrease the mitochondrial membrane potential (Maldonado, Patnaik, Mullins, & Lemasters, 2010). Thus, free tubulin can decrease VDAC conductance and ultimately lead to outer mitochondrial membrane permeabilization and cell death. This inhibition of VDAC by free tubulin limits mitochondrial respiration, and may explain the Warburg effect in cancer cells, which by nature require excess tubulin to support rapid division (McCommis & Baines, 2012).

Microtubule nucleation is enhanced in tubulin solutions by the addition of gamma-tubulin or various gamma-tubulin complexes. In vivo, microtubule assembly is usually seeded by gamma-tubulin ring
complexes (Job, Valiron, & Oakley, 2003). The decrease seen in gamma tubulin with increasing citral is likely to result in decreased nucleation of microtubules and consequently more free tubulin. As discussed above, free tubulin can decrease the mitochondrial membrane potential.

It has been shown that γ-tubulin is involved in coordinating cell cycle progression (Höög, Zarrizi, Stedingk, Jonsson, & Alvarado-Kristensson, 2011) and in particular the G1 to S phase transition, since γ-tubulin governs E2F transcriptional activities by directly binding to E2Fs on E2F-regulated promoters. It is the interaction of γ-tubulin which represses the activity of E2F and assures a transient expression of genes necessary for G1 to S phase entry. It has also been demonstrated that reduced γ-tubulin protein levels results in an E2F mediated upregulation of RB1 protein levels, and consequently G1 phase arrest (Ehlén et al., 2012). In this way γ-Tubulin and RB1 moderate the expression of genes leading to apoptosis (Biswas & Johnson, 2012). This is consistent with the results shown in Figure 4c which show that incubation with increasing concentrations of citral, which result in increased apoptosis, decreases levels of γ-tubulin.

VDAC is a major mitochondrial outer-membrane transporter and as such has an important role in energy production by controlling metabolite traffic, and is also recognized as a key protein in mitochondrial-mediated apoptosis. VDAC also plays an important role as a controlled passage for adenine nucleotides, Ca ++, and other metabolites between the cytosol and mitochondria. In addition, VDAC functions as a docking site for cellular kinases, such as hexokinase, providing the enzyme with preferential access to ATP derived from oxidative phosphorylation (Abu-Hamad, Sivan, & Shoshan-Barmatz, 2006). Therefore, VDAC expression levels may serve as a crucial factor in the process of mitochondria-mediated apoptosis. Due to the fact that VDAC controls the transport of ATP, adenosine diphosphate, and other metabolites between the cytosol and mitochondria, down-regulation of VDAC expression should lead to disrupted energy production. We found that between 0.1 μM and 100 μM citral VDAC expression decreased (Figure 4c,d), although at 200 μM expression was dramatically increased. It can be seen in Figure 1 that cell death increased up to 100 μM citral, but was not subsequently increased with increasing concentrations of citral. VDAC1 has also been shown to be overexpressed in some cancer cells, and this may be related to its multifunctional activities as required by high-energy demanding cells (Shoshan-Barmatz & Golan, 2012).

5 | CONCLUSIONS

Citril appears to be an effective chemotherapy agent against RMS cells in culture. The compound induces an increase in ROS and free calcium. Low concentrations of citral show mitochondrial accumulation around the nuclei, and display toroidal structures (White et al., 2017). As the concentration of citral increases, the mitochondria swell and upon reaching a threshold rupture, and the nuclei condense, and OXPHOS uncouples. Proceeding toward apoptosis the cells become glycolytic. When metformin was applied to the cells with citral, it could be seen that at higher concentrations of citral, when the cells were undergoing glycolysis, that cell death was not diminished. Since metformin is a drug commonly used by diabetics, this study indicates that caution should be exercised when used alongside other drug treatments.

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