Spring 4-25-2018

Investigating the Synergistic Effects of Two Curcuminoids and Cisplatin on Cancer Cell Reactive Oxygen Species

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INVESTIGATING THE SYNERGISTIC EFFECTS OF TWO CURCUMINOIDS AND
CISPLATIN ON CANCER CELL REACTIVE OXYGEN SPECIES

A Capstone Project Presented in Partial Fulfillment

of the Requirements for the Degree Bachelor of Science

with Honors College Graduate Distinction at

Western Kentucky University

By

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May 2018

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I dedicate this thesis to all those who have suffered from cancer and for families that have

lost a loved one to the disease. I also dedicate this thesis to Dr. Jerry D. Monroe and all

others who labor in research for those who suffer from cancer.

ACKNOWLEDGEMENTS

ABSTRACT

Cisplatin is an anticancer drug which can cause the production of reactive oxygen species (ROS) that kill cancer cells. Curcumin is a naturally occurring plant compound that can increase ROS levels in cancer cells and enhance the activity of cisplatin against cancer, but it exhibits poor bioavailability. We investigated whether two synthetic curcumin analogs (curcuminoids), EF-24 and CLEFMA, with anti-cancer activity and improved bioavailability, increased cisplatin's effect against cancer. A spectrophotometric fluorescent ROS assay was used to determine if cisplatin, the curcuminoids or combinations of cisplatin with a curcuminoid affected the level of ROS in the A549 non-small cell lung cancer cell line. Cisplatin treatment significantly increased cancer cell ROS levels, while both curcuminoids caused the level of ROS to significantly decline. When we combined cisplatin with either curcuminoid, there was a significant and even greater reduction in ROS than in the curcuminoid only treatments. Our results suggest that cisplatin and these curcuminoids signal through different pathways or pathway components to regulate the level of ROS in A549 cells.

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Annual Kentucky Academy of Sciences Meeting, Murray State University, Murray, KY.
Poster Presentation.

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INTRODUCTION

It is estimated 1.7 million people were diagnosed with cancer in 2017 and that 600,000 people will die from this disease in the United States (Siegel, 2017). Limitations that arise from the use of anti-cancer drugs include harmful side effects and resistance. This study investigated cisplatin, which has been shown to cause, nephrotoxicity, neurotoxicity, and ototoxicity (Karasawa & Steyger, 2015). Moreover, resistance can develop to cisplatin treatment (Shen et al., 2012; Sarin et al., 2017). One strategy for addressing these limitations is to combine drugs to produce synergistic effects greater than those found with usage of the individual compounds alone. This project assesses the combination of two synthetic curcumin analogs and the anti-cancer drug, cisplatin, to determine if together these drugs act synergistically.

Cisplatin is an FDA-approved platinum-based chemotherapy drug that is particularly effective against cancers of the urogenital tract, including testicular, ovarian, and bladder cancer (The National Cancer Institute, 2007). Cisplatin is also effective against melanomas, non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), lymphomas, and myelomas (Wheate et al., 2010). Cisplatin's mode of action includes being taken up into the cancer cells and then traveling into the nucleus where it typically binds primarily to guanine residues located on both strands of DNA (Skowron, 2018). Bound cisplatin causes DNA to bend and recruit proteins that activate apoptotic pathways (Wang & Lippard, 2005; Wheate et al., 2010), including pathways that signal through reactive oxygen species (ROS) to cause cell death (Karasawa & Steyger, 2015). Moreover, the cytotoxic effect of cisplatin has been connected to an upregulation of

mitochondrial ROS which in turn activates cell death signaling in a variety of pathways (Choi et al., 2015).

Curcumin is a naturally occurring plant compound that can improve the effect of cisplatin and increases the level of reactive oxygen species in cancer cells but also acts as a ROS scavenger to prevent auditory and renal tissue damage (Trujillo et al., 2013; Fetoni et al., 2015; Baharuddin et al., 2016; Huang et al., 2017; Liao et al., 2017; Larasati et al., 2018), but curcumin exhibits low bioavailability and solubility (Trujillo et al., 2013; Ali, 2017). Mechanistically, curcumin functions by inhibiting key transcription factors associated with cancer cell viability; more specifically, NF-κB and STAT-3 are inhibited and both are associated with anti-apoptotic pathways in cancer cells (Vallianou et al., 2015). Synthetic analogs of curcumin, known as curcuminoids, have been developed that have anti-cancer activity and improved solubility, and may affect cisplatin-mediated ROS production. This project used two curcuminoids, 3,5-bis[(2-fluorophenyl)methylene]-4-piperidinone (EF-24) and 4-[3,5-Bis[(2-chlorophenyl)methylene]-4-oxo-1-piperidinyl]-4-oxo-2-butenoic acid (CLEFMA). Although the interaction of CLEFMA and cisplatin has not yet been characterized, EF-24 and cisplatin can signal through pathways that regulate ROS and incorporate glutathione (GSH). GSH binds to cisplatin and hinders its ability to induce cancer cell apoptosis (Kasherman et al., 2009; Cadoni et al., 2017). EF-24 can form adducts with glutathione leading to ROS production, and a decrease of GSH, which is associated with reduced cell viability (Skoupa et al., 2017). These studies suggest that combining a curcuminoid with cisplatin could increase their individual effect against cancer cell viability by regulating a ROS pathway. Further, it has been shown

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that cancer cell ROS production can underlie synergistic mechanisms that cause cell death in combination-style treatments (Chen et al., 2016).

I used a fluorescent spectrophotometric assay to determine if EF-24 and CLEFMA increased cisplatin-mediated ROS production in a NSCLC cell line, A549. My project showed that cisplatin treatment and the positive control, H_2O_2, caused increased ROS production in cancer cells. However, curcuminoid treatment caused ROS yields to decrease. Interestingly, the combination of cisplatin with a curcuminoid caused a decrease in the level of ROS in cancer cells. These results suggest that cisplatin and these two curcuminoids might function in different pathways or target different components of the same pathway.

METHODOLOGY

Reactive Oxygen Species (ROS) Assay

A ROS assay was used to determine if the curcuminoids effect the level of ROS in cisplatin-treated cancer cells. Seven 10 centimeter (cm) dishes were prepared each with 1 x 10^6 A549 cells in 10 mL of F12K media with 10% fetal bovine serum and 1% penicillin/streptomycin supplementation. Dishes were incubated for 24 hours at 37 °C and 5% CO_2. Media was then aspirated out of the dishes and replaced with 10 mL of the following treatments (all in F12K media): media only for 48 hours (negative control), 100 µM 30% hydrogen peroxide (H_2O_2) for 48 hours (positive control), 10 µM cisplatin for 48 hours, 2 µM EF-24 for 48 hours, 15 µM CLEFMA for 48 hours, 10 µM cisplatin for 24 hours followed by 10 mL of 2 µM EF-24 for another 24 hours, and 10 µM cisplatin for 24 hours followed by 10 mL of 15 µM CLEFMA for another 24 hours. After 48 hours, incubation was considered complete and the media was aspirated out. Each dish was then washed 3X with 2 mL of phosphate-buffered saline (PBS). The cells were then detached by adding 1 mL of 1X accutase to each dish. Then, the cells were transferred to individual microcentrifuge tubes and 0.5 mL of PBS was added to each tube. Each tube was spun for five minutes at 1000 revolutions per minute (rpm). The supernatant was then carefully discarded and the pellet was resuspended with 500 µL of 10 µM ROS indicator dye in PBS and each tube was incubated for 45 minutes at 37° C in 5% CO_2 (Ng et al., 2014). Next, the centrifuge tubes were centrifuged for five minutes at 1000 rpm. Then, the supernatant was removed and the cells were washed three times with one mL of PBS. The cells were then resuspended in one mL of PBS. Then, 100 µL of cell

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suspension containing approximately 100,000 cells was placed into each of nine wells of a black 96-well plate. Also, 100 μL of PBS was placed into six wells of the plate (blank treatment). Then, the plate was placed into a spectrophotometer and read at 495 nm (excitation) and 527 nm (emission) wavelengths.

Statistical Analysis

The results were saved to an Excel spreadsheet and data analysis performed in PRISM (Graph Pad, version 6, La Jolla, CA) using a 2-way ANOVA and Tukey's post-hoc t-test ($p < 0.05$). The levels of statistical significance that apply to figures later used include: $P > 0.05$ (no significance), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$.

RESULTS

ROS Assay

To measure how cisplatin, EF-24 and CLEFMA alone and in combination affected ROS production we used a fluorescent spectrophotometric ROS assay. We first measured the effect of cisplatin and the positive control hydrogen peroxide (H_2O_2) and found that they significantly increased ROS compared to the negative control treatment (Figure 1; $p \leq 0.0001$). Cisplatin treatment increased reactive oxygen species to 119% and H_2O_2 to 117% of control. When we measured the level of ROS in cells treated with the curcuminoids individually and in combination with cisplatin, we found that all of these treatment categories caused a significant decrease in reactive oxygen species (Figure 2; $p \leq 0.0001$). For this data set, the level of ROS for EF-24 was 85%, CLEFMA 81%, cisplatin-EF-24 75% and cisplatin-CLEFMA 76% of control treatment. We then compared the ROS yields of cisplatin with the curcuminoids alone and in combination with cisplatin and found that each of these comparisons was statistically significant (Figure 3; $p \leq 0.0001$). In order to better understand the interaction between the curcuminoids and cisplatin-curcuminoid combinations, we compared their ROS yields and found that the combination of cisplatin with either curcuminoid was statistically different than EF-24 treatment, but not CLEFMA (Figure 4; $p \leq 0.01$). Cisplatin with EF-24 treatment was 10% and cisplatin-CLEFMA 9% below the ROS yield of EF-24 alone. Summary data for all ROS experiments with complete statistical analysis are presented in Figure 5.
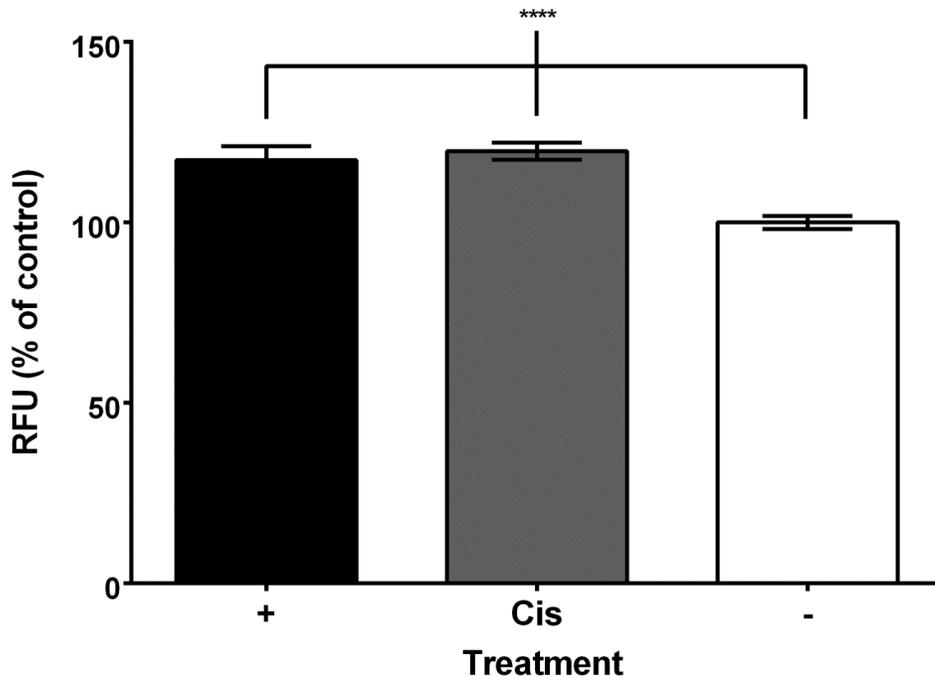
Figure 1: Cisplatin and positive control treatment increases the level of ROS in A549 cells. Code scheme: + = positive control (H_2O_2), Cis = cisplatin, - = negative control. RFU = relative fluorescence units. N = 6; **** = $p \leq 0.0001$.
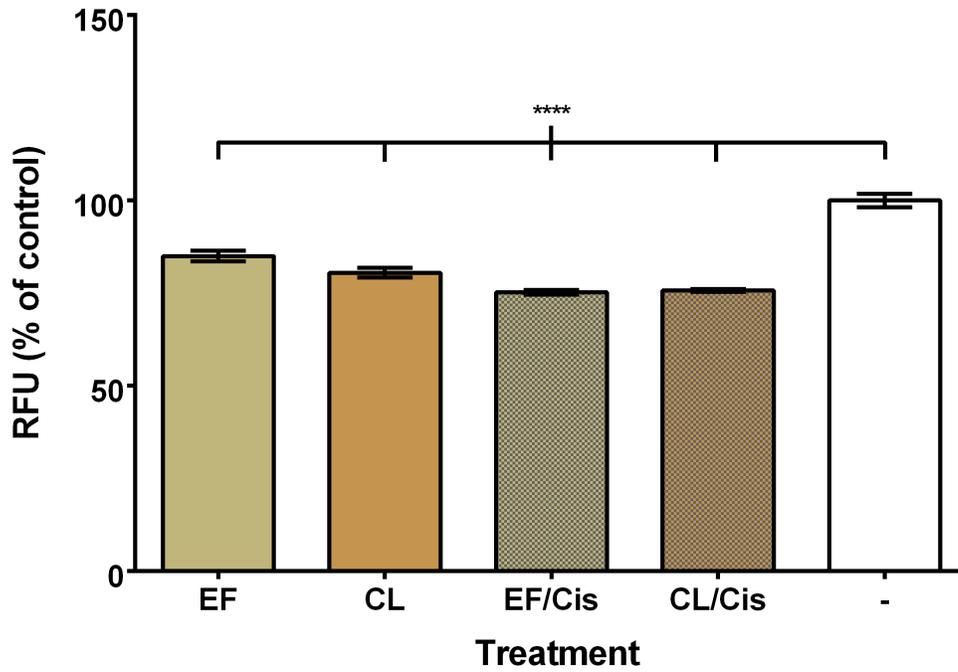
Figure 2: Curcuminoid and cisplatin-curcuminoid combination treatment reduced the level of ROS in A549 cells. Code scheme: EF = EF-24, CL = CLEFMA, EF/Cis = EF-24 with cisplatin, CL/Cis = CLEFMA with cisplatin and - = negative control. RFU = relative fluorescence units. N = 6; $**** = p \leq 0.0001$.
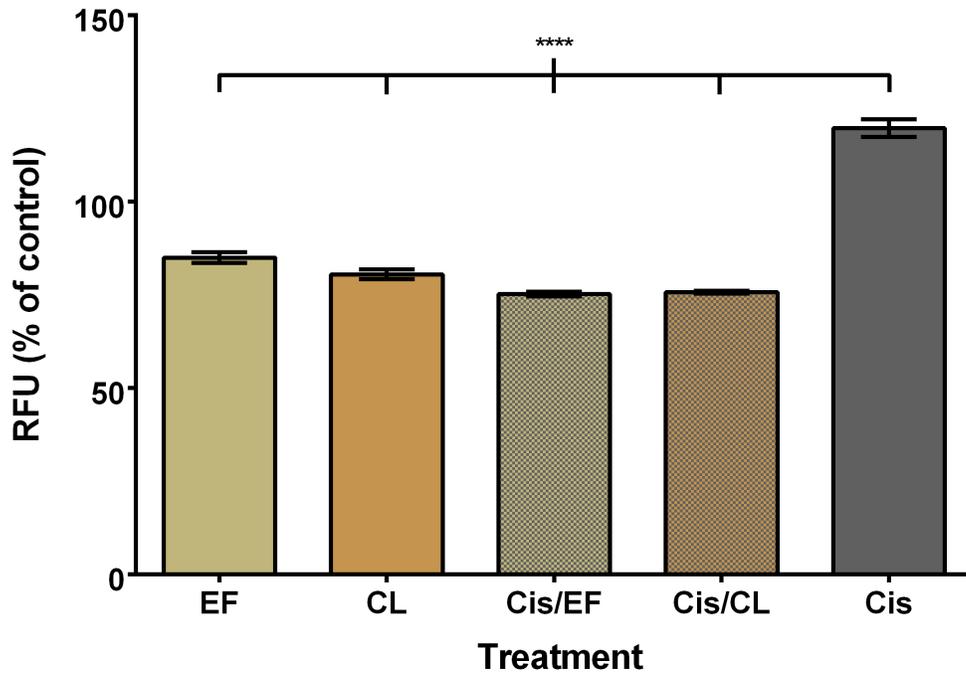
Figure 3: Curcuminoid and cisplatin-curcuminoid combination treatment decreased the level of ROS in A549 cells compared to cisplatin. Code scheme: EF = EF-24, CL = CLEFMA, EF/Cis = EF-24 with cisplatin, CL/Cis = CLEFMA with cisplatin, and Cis = cisplatin. RFU = relative fluorescence units. N = 6; **** = $p \leq 0.0001$.
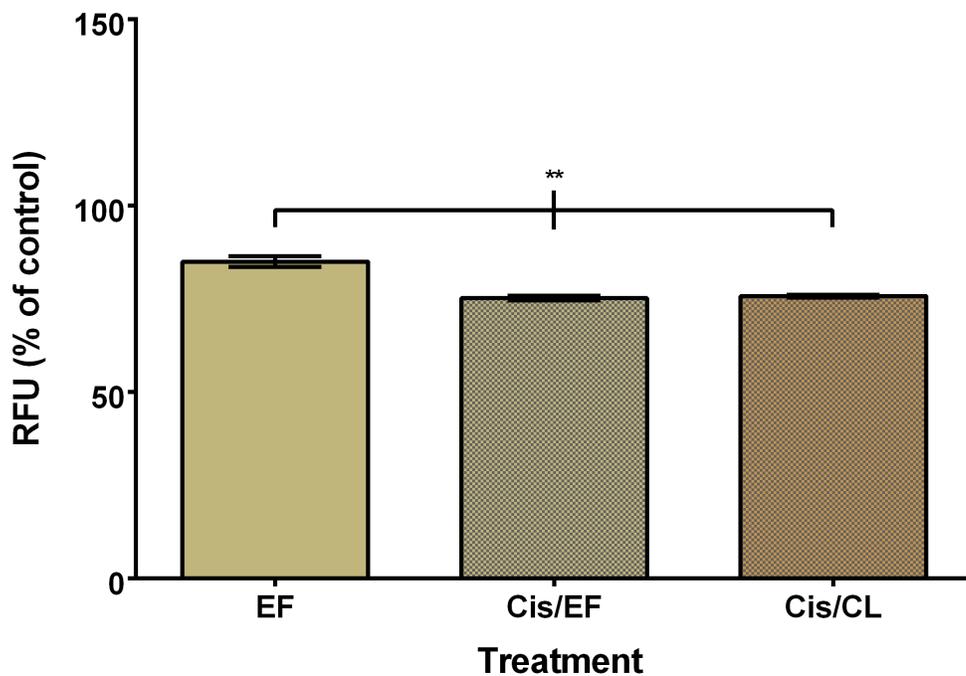
Figure 4: EF-24 and CLEFMA treatment with cisplatin reduced the level of ROS in A549 cells. Code scheme: EF = EF-24, Cis/EF = cisplatin with EF-24, and Cis/CL = cisplatin with CLEFMA. RFU = relative fluorescence units. N = 6; ** = $p \leq 0.01$.
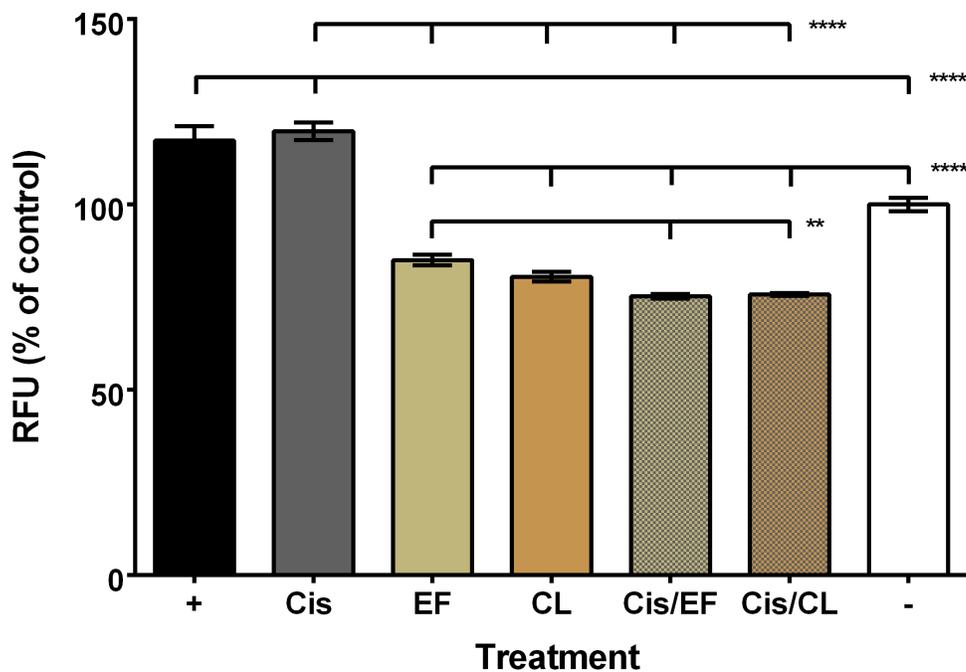
Figure 5: Summary project data showing how cisplatin, controls and curcuminoid alone or in combination with cisplatin affect the level of ROS in A549 cells. Code scheme: + = positive control (H_2O_2), Cis = cisplatin, EF = EF-24, CL = CLEFMA, Cis/EF = cisplatin with EF-24, Cis/CL = cisplatin with CLEFMA, and - = negative control. RFU = relative fluorescence units. N = 6 for each treatment category; ** = $p \le 0.01$, **** = $p \le 0.0001$.

DISCUSSION

Cisplatin and the synthetic curcuminoids both had significant effects on the level of ROS in A549 cells. Cisplatin and curcumin analogs could work in the same or different pathways to modulate the production of reactive oxygen species that can kill cancer cells. I initially investigated whether the A549 cell line would be responsive to cisplatin and H_2O_2 treatments as both of these compounds are reported to induce ROS production in cancer cells (Marullo et al., 2013; Chonghua et al., 2016; Selvi et al., 2017; Park, 2018). I found that both cisplatin and H_2O_2 significantly increased ROS production to 119 and 117% of control (Figure 1; $p \leq 0.0001$). Thus, my results corroborate what others have found with these compounds and validated the usage of the A549 cell line in these experiments.

In contrast, synthetic curcumin analogs decreased ROS production, however, other researchers have found that curcumin and other curcuminoids have increased ROS production in cancer cells. A549 cells treated with curcumin have exhibited increased ROS production which is associated with cellular toxicity (Chen et al., 2010; Luo et al., 2017). However, curcumin has also been found to decrease ROS production in A549 cells and yet promote cellular toxicity in this cell line through other mechanisms (Fan et al., 2015). EF-24 can cause cell death in A549 cells (Thomas et al., 2010) and structurally similar analogs can kill A549 cells through increased ROS production (Wu et al., 2017; Jin et al., 2018). CLEFMA also increased cell death in the lung cancer cell line, H-411, by increasing reactive oxygen species production (Sahoo et al., 2012) and can decrease the viability of A549 cancer cells (Yadav et al., 2013). The curcumin

analog, H-4073, can enhance cisplatin's effect against head and neck cancer migration beyond that associated with cisplatin-only treatments (Kumar et al., 2014). These results suggest that a combination of two drug types, the platinum-based compound cisplatin, and a curcuminoid, like EF-24 or CLEFMA, might cause greater, and even synergistic cytotoxicity, in the A549 cell line through enhancing ROS production.

Surprisingly, we found that EF-24 and CLEFMA significantly reduced reactive oxygen species yields compared to control by 15% and 19% below negative control respectively (Figure 2; $p \leq 0.0001$). Further, when either of the curcuminoids was combined with cisplatin, we found that the level of ROS decreased even more from individual curcuminoid treatment alone (cisplatin-EF-24 was 25% and cisplatin-CLEFMA treatment was 24% below the negative control value (Figure 2; $p \leq 0.0001$). Comparing our results for the EF-24, CLEFMA, cisplatin-EF-24 and cisplatin-CLEFMA treatments with cisplatin showed that the curcuminoids and cisplatin-curcuminoid treatments significantly reduced reactive oxygen species relative to the platinum compound (Figure 3; $p \leq 0.0001$). Further, intercomparison of the curcuminoid with cisplatin-curcuminoid treatments revealed that both cisplatin-EF-24 and cisplatin-CLEFMA treatments caused significantly lower ROS production than EF-24 alone but not CLEFMA alone (Figure 4; $p \leq 0.01$). Thus, our results suggest that both curcuminoids might either neutralize ROS, act in different pathways than cisplatin, or target different components of the same pathway than cisplatin.

These curcuminoids could reduce reactive oxygen species production by acting as ROS scavengers. Curcumin, which is the structural basis for these compounds, can scavenge ROS (Barzegar & Moosavi-Movahedi, 2011; Park et al., 2013). If EF-24 and

CLEFMA have the structural ability to directly scavenge ROS, this could explain our results for these curcuminoids when applied individually. This hypothesis could also explain why the combined cisplatin-EF-24 and cisplatin-CLEFMA caused even lower ROS production than EF-24 and CLEFMA alone. When cisplatin and curcumin treatment are combined, cisplatin might cause an increase in ROS production but curcumin scavenging might counteract this and cause a compensatory effect to reduce the overall level of reactive oxygen species (Liou & Storz, 2010; Trujillo et al., 2013; Fetoni et al., 2015). Thus, EF-24 and CLEFMA might both be able to scavenge ROS produced by cisplatin treatment in a similar manner and promote a cellular compensatory response that reduces overall reactive oxygen species yield.

The time course of curcuminoid and curcuminoid with cisplatin treatments could also be a significant factor. In these experiments, we used results from an MTT assay to determine the curcuminoid and cisplatin concentrations at which these compounds reduced cancer cell viability to 50% of control treatment (IC_{50} value). The IC_{50} value was determined at a 48-hour time point; however, the IC_{50} time point may not correspond to the time course of ROS production generated by these compounds. In other words, it could be that the curcuminoids could generate high, and apoptosis-inducing amounts of ROS, earlier than the 48-hour time point that we measured, and then the ROS could be reduced to lower levels, potentially due to a cellular compensatory mechanism that counteracts the presence of an excessive level of ROS. This interpretation could particularly explain the combined curcuminoid with cisplatin results if high ROS production during the earlier phase of cisplatin treatment was conjoined with a similar high production from either curcuminoid just after its introduction during the second 24-

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hour phase when it was introduced. Such an additional ROS production response could potentiate a strong anti-ROS compensatory effect during the second 24-hour portion of the 48-hour time course. This would account for the reduced ROS level during combined cisplatin-curcuminoid treatments. Studies have shown that curcumin treatment in cancer cells can activate cellular mechanisms that counteract reactive oxygen species production and this causes a rapid decrease in the level of ROS (Lee et al., 2011; Gersey et al., 2017).

Cisplatin and the two curcuminoids may modulate the level of ROS via different cellular pathways. For example, studies of cisplatin and EF-24 have shown that they might be able to target different signaling molecules. Cisplatin can reduce mRNA expression of the anti-apoptosis gene, Bcl2, whereas EF-24 does not have this effect (Onen et al., 2015). In ovarian carcinoma cells, cisplatin does not induce the apoptotic protein, caspase-3, but addition of EF-24 promotes caspase expression (Tan et al., 2010). Another molecule that EF-24 affects is the antioxidant glutathione, which has shown efficacy as a manager of oxidative stress (Kerksick & Willoughby, 2005). In leukemia cancer cells, EF-24 was shown to form an adduct with the antioxidant glutathione, and this binding could be responsible for increased ROS expression (Skoupa et al., 2017). However, cisplatin does not directly target glutathione, but instead can act to reduce the activity of glutathione S-transferase, an enzyme that catalyzes the binding of glutathione to reactive oxygen species (Mukherjea & Rybak, 2011). Therefore, cisplatin treatment might activate a pathway or a specific pathway component to increase ROS production, but this may sensitize the pathway or component to subsequent curcuminoid treatment through a different pathway or component. Thus, later treatment with the curcuminoid

may cause a strong compensatory action resulting in greatly reduced reactive oxygen species production.

Our reactive oxygen species assay shows that the curcuminoids and cisplatin cause significant modulation of the level of ROS in the A549 non-small cell cancer line (Figure 5). Additional work is needed in order to determine the mechanistic interplay of these compounds. We propose to study the effect of these compounds on apoptotic pathways using western blots and caspase activity assays to identify which cellular signaling pathways are activated by these compounds either alone or in combination. Further, we intend to perform the ROS assay at an earlier time point to identify temporal relationships between these compounds on reactive oxygen species production. Another investigation that we propose is to use different concentrations of the curcuminoids to determine if ROS production is conditioned by distinct concentration profiles of EF-24 or CLEFMA. These proposed studies in addition to the work that I have already performed could lead to the development of new chemotherapeutic strategies in clinical settings.

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