6-26-2017

Investigating the Synergistic Effects of Cisplatin and Two Curcuminoid Compounds on Cancer

Denis Hodzic

INVESTIGATING THE SYNERGISTIC EFFECTS OF CISPLATIN AND TWO CURCUMINOID COMPOUNDS ON CANCER

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

Denis Hodzic

May 2017

*****

CE/T Committee:

Dr. Michael Smith, Chair

Dr. Dave Monroe

Dr. Kevin Williams

I dedicate this thesis to my mother, who is my rock, my father, who provided the inspiration for my research, my step-dad for his advice and support, and my fellow Bosnian refugees. “Ući školu sine.”

ACKNOWLEDGEMENTS

For their consistent and steadfast guidance, I would like to extend a very special thank you to Dr. Michael Smith and Dr. Dave Monroe; the mentorship both of you have provided the last four years has been invaluable. For their input and contribution to methodology, I thank Dr. Kevin Williams and Dr. Sigrid Jacobshagen. Thank you to John Paul Abah for caring for the zebrafish. Lastly, thank you to my family for providing stability and security in my personal life, and for understanding if I could not make it to family dinner. This project was supported by a National Institutes of Health R15 grant (1R15CA188890-01A1), a Kentucky Biomedical Research Infrastructure Network grant (8P20GM103436-14), a Western Kentucky University Research and Creative Activities Grant to M.E.S., and Western Kentucky University Faculty-Undergraduate Student Engagement and Honors Development grants.

ABSTRACT

Cisplatin is an anti-cancer drug effective against several cancers which can produce the serious side-effect of hearing loss. Curcumin, a natural plant compound, can increase the activity of cisplatin against cancer and counteract cisplatin's effect against hearing. Because curcumin exhibits poor bioavailability, there is considerable interest in developing synthetic curcumin analogs (curcuminoids) that are more soluble and which retain anti-cancer activity and otoprotective function. This study investigated whether two curcuminoids, EF-24 and CLEFMA, increase the cytotoxic and ototoxic effects of cisplatin against the lung cancer cell line, A549, and the colorectal cancer cell line, Caco2. Cytotoxicity was measured by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ototoxicity was quantified by measuring hearing thresholds acquired by the auditory evoked potential technique in a zebrafish (*Danio rerio*) model. The results of this study indicate that a combination of cisplatin with either CLEFMA or EF-24, produces a dose-dependent effect against the cancer cell-lines which is not synergistic or additive. The hearing tests showed that both curcuminoids could prevent hearing loss caused by cisplatin treatment. However, the curcuminoid vehicle, DMSO, could also play a role in the effect on hearing. These results suggest that curcuminoid treatment may increase the effect of cisplatin against these cancers and might also reduce hearing damage produced by cisplatin treatment. Future research is needed to investigate the signaling pathways that regulate the function of cisplatin, CLEFMA, EF-24 and DMSO in cancer and auditory physiology.

VITA

EDUCATION

Western Kentucky University, Bowling Green, KY May 2017
B.A. in Science – Mahurin Honors College Graduate
Honors Capstone: *Investigating the Synergistic Effects of Cisplatin and Two Curcuminoid Compounds on Cancer*

Bowling Green High School, Bowling Green, KY May 2013

PROFESSIONAL EXPERIENCE

Laboratory Assistant, Smith Neurobiology Lab January 2017-present

Honors Undergraduate Researcher, Smith Neurobiology Lab January 2014-present

AWARDS & HONORS

Summa Cum Laude, WKU, May 2017
Undergraduate Research Oral Presentation Award, 1st place, Student Research Conference, Western Kentucky University (2017)
Undergraduate Research Oral Presentation Award, 3rd place, Physiology & Biochemistry, Kentucky Academy of Sciences (2016)
Undergraduate Research Poster Presentation Award, 1st place, Student Research Conference, Western Kentucky University (2015)

PRESENTATIONS

Hodzic, D., Monroe, J.D., Smith, M.E. 2017. Investigating the Synergistic Effects of Cisplatin and Two Curcuminoid Compounds on Cancer. 47th Annual WKU Student Research Conference, Western Kentucky University, Bowling Green, KY. Oral Presentation
Hodzic, D., Monroe, J.D., Smith, M.E. 2016. Investigating the Synergistic Effects of Cisplatin and Two Curcuminoid Compounds on Cancer. 102nd Annual Kentucky Academy of Sciences Meeting, University of Louisville, Louisville, KY. Oral Presentation
Hodzic, D., Smith, M.E. 2015. The Role of Melanin in Auditory Function of Zebrafish (*Danio rerio*). 45th Annual WKU Student Research Conference, Bowling Green, KY. Poster Presentation

CONTENTS

LIST OF FIGURES

Figures Pages

INTRODUCTION

Cancer is the second leading cause of death in the world, making the development of new chemotherapeutics an urgent priority (Ferlay et al. 2012; Cheung-Ong et al. 2013). One drug design approach, utilized in the platinum-based compound, cisplatin, is to induce DNA damage, which activates apoptotic pathways and initiates mitochondrial-dependent reactive oxygen species (ROS) release (Cepeda et al. 2007; Marullo et al. 2013). Another approach is to target a broad set of pathways, and is exemplified by the phytochemical, curcumin, which is able to act against various cancers by modulating the cell cycle, apoptotic mechanisms, microRNAs, the proteasome, Wnt/β-catenin and NF-κB signaling, as well as several protein kinases (Tuorkey 2014). The diverse mechanistic targeting of these two drugs suggests that combining them could produce an additive or even synergistic response beyond their individual effects. However, there are serious limitations present in combining cisplatin with curcumin. Cisplatin can cause multiple side-effects, including permanent hearing-loss by releasing ROS (Sergent et al. 2002; Cepeda et al. 2007; Benard et al. 2014; Salehi et al. 2014; Chen et al. 2015; Horibe et al. 2015). Elevated hearing threshold have been reported in 75–100% of patients treated with cisplatin (Mckeage 1995). Although curcumin can act as an otoprotectant and counteract cisplatin resistance mechanisms (Salehi et al. 2014; Chen et al. 2015; Shanmugam et al. 2015), curcumin exhibits very poor bioavailability, which restricts its efficacy (Teiten et al. 2014; Fridlender et al. 2015). To address the deficiencies found with curcumin solubility, researchers have developed synthetic curcumin alternatives (curcuminoids) that retain anti-cancer activity, but also possess improved bioavailability.

However, little is known about whether these curcuminoids enhance anti-cancer effects when combined with cisplatin or if they can counteract its side-effects.

Two new curcuminoids, (3E,5E)-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), demonstrate significant anti-cancer effects and improved solubility. EF-24 causes cell cycle arrest followed by alteration of mitochondrial function leading to apoptosis, increased ROS production and decreased cell proliferation in cancer cell-lines (Adams et al. 2005). Another study has shown that EF-24 induces apoptosis and suppresses cancer cell viability more effectively than curcumin in cisplatin-sensitive and -resistant cell-lines (Tan et al. 2010). Interestingly, this study also demonstrated that EF-24 acts as an anti-oxidant (Tan et al. 2010), which suggests that this curcuminoid might be able to counteract cisplatin modulated ROS release and damage to auditory hair cells. Unlike EF-24, CLEFMA has been shown to reduce proliferation in a lung cancer cell-line through an autophagic, and not apoptotic, mechanism (Lagisetty et al. 2011). CLEFMA has also been shown to upregulate ROS release from the mitochondria in lung cancer cells by deregulating redox pathways (Sahoo et al. 2012). Surprisingly, this study also found that ROS release was not increased, nor was cellular viability decreased in a normal lung fibroblast cell-line, suggesting that CLEFMA may not target non-cancerous cells (Sahoo et al. 2012). Therefore, these studies indicate that EF-24 and CLEFMA may function through distinct pathways and could potentially act against cancer without producing ROS that cause auditory side-effects.

My project examined if EF-24 and CLEFMA could enhance cisplatin's activity against cancer cell viability and reduce hearing damage caused by cisplatin treatment. The first phase utilized the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine if these curcuminoids altered cisplatin's effect on cellular viability in the small-cell lung cancer line, A549, and the colorectal cancer line, Caco2. Then, the second phase applied the auditory evoked potential (AEP) technique in a zebrafish (*Danio rerio*) inner ear model to determine if the curcuminoids reduced auditory side-effects produced by cisplatin treatment. Thus, this project investigated if these two new curcuminoid compounds act synergistically with cisplatin against cancer and also counteract cisplatin-mediated hearing loss.

METHODOLOGY

Cell viability assay

The colorimetric MTT assay was used to determine if the two curcuminoids, EF-24 and CLEFMA, enhanced the effect of cisplatin against cancer cell viability. The small-cell lung cancer cell line, A549 (ATCC), was seeded in 96-well plates in F12K media (with 10% fetal bovine serum and 1% penicillin/streptomycin supplementation) at a density of 5,000 cells per well and incubated for 24 hours at 37°C in 5% CO_2. Then, the 96-well plates were treated with a dilution series (500, 50, 5, 0.5, 0.05 or 0.005 µM) of either EF-24 or CLEFMA (solubilized in dimethyl sulfoxide, DMSO) for 72 hours at 37°C in 5% CO_2 in replicates of six. In addition, a negative control (cells with media only), positive control (Triton X-100) and media only blanks were run in replicates of six. After 72 hours elapsed, the MTT assay was run for 2 hours. The media was then ejected from all the wells, and solubilization solution was added. The plates were vortexed for 15 minutes, and then their absorbance was determined using a spectrophotometer at 570 and 690 nm. IC_{50} values of cisplatin and both curcuminoids were calculated in GraphPad Prism (version 6) using a sigmoidal, four parameter logistic equation. Then, another set of plates were prepared using the same procedure as above except that after 24 hours, the experimental wells were treated with the IC_{50} value of cisplatin (10 µM of cisplatin in A549 and 12 µM of cisplatin in Caco2 solubilized in media) for 24 hours followed by treatment with a curcuminoid for 48 hours using the same dilution series and then the MTT assay as before. A separate plate with a DMSO only treatment was

prepared using the same dilution series and protocol above to determine whether it affected cellular viability.

Auditory evoked potentials

The auditory evoked potential (AEP) technique was used in a zebrafish model to assess whether the curcuminoids counteract cisplatin-induced hearing side-effects. AEP recording is a commonly employed electrophysiology technique used to assess hearing in fishes (Smith et al. 2006; Smith et al. 2011; Uribe et al. 2013). Zebrafish were obtained from commercial suppliers and maintained individually in 170 liter tanks. Mean (± S.E.) standard length and mass of the zebrafish (N=45) was 31.0 (± 0.66) millimeters and 0.47 (± 0.03) grams, respectively. Zebrafish were microinjected with cisplatin, a curcuminoid, or cisplatin with a curcuminoid. The cisplatin vehicle was 0.9% sodium chloride, while the curcuminoid vehicle was DMSO. Separate sodium chloride and DMSO vehicle controls were also performed. For cisplatin, the injection was based on the ratio of 25 mg/kg (cisplatin mg/kg body weight). For both curcuminoids, we injected 5 mg/kg. Hearing tests were performed on cisplatin, EF-24, CLEFMA and the two vehicle group treatment fish 48 hours after injection. However, for treatments using a combination of cisplatin and either EF-24 or CLEFMA, the cisplatin was injected first and then after 24 hours, a curcuminoid was injected, and the fish were placed back into an aquarium for an additional 24 hours before AEP testing.

For each treatment category, a minimum of six zebrafish (range: 6-8) were injected. After the treatment interval, the fish were subjected to the auditory evoked potential technique. Briefly, the fish were lightly anaesthetized with tricaine

methanesulfonate (MS-222) and then placed into a mesh harness suspended 6 cm from the water surface and 22 cm above a University Sound UW-30 underwater speaker (Electro-Voice, Burnsville, MN) in a 19-L tank containing 27-28°C water. Electrical interference was minimized by keeping the tank within a Faraday cage. This cage was located within a sound-attenuation room to reduce background noise (Whisper Room, Inc., Knoxville, TN). Three stainless steel subdermal electrodes (27 gauge; Rochester Electro-Medical, Inc., Tampa, FL) were attached 1-2 mm sub dermally to the fish- a recording electrode over the brainstem, a reference electrode between the nares, and a ground electrode in the tail musculature. Sound stimuli were presented and AEP waveforms collected using BioSig software running on a TDT physiology system (Tucker Davis Technologies, Inc., Alachua, FL). Pure tone pip stimuli at eight different frequencies (100, 250, 400, 600, 800, 1000, 1500 and 3000 Hz) were presented to the fish. Each frequency was tested by decreasing decibel levels in 5 dB steps until an AEP trace was no longer visible.

The sound pressure levels of each frequency were confirmed using a calibrated hydrophone (calibration sensitivity of -195 dB re 1 V/µPa: ± 3 dB, 0.02-10 kHz omnidirectional, GRAS Type 10CT, Denmark), placed in the same location where fish were held during AEP recording. The last sound pressure level at which an AEP trace was visible was noted as the threshold for each frequency. The collective thresholds for these eight frequencies were used to produce audiograms. All procedures were conducted under the approval of the Western Kentucky University Institutional Animal Care and Use Committee (Animal Welfare Assurance # A3558-01).

Statistical analysis

For the MTT assays, a two-way ANOVA using a Sidak's multiple comparisons test, with curcuminoid concentration and cisplatin treatment as factors, was performed. For the AEPs, a two-way ANOVA, with frequency and injection treatment as factors, was used. When the overall ANOVA exhibited significant treatment effects, a Tukey's multiple comparisons *post hoc* test was performed. A p-value of <0.05 was considered to be significant. Mean and standard error of the mean (SEM) was calculated for each treatment group and used to plot the data. All statistical analysis was processed in the statistical modeling program Prism (GraphPad Prism version 6, La Jolla, California, USA).

RESULTS

Cellular Viability Assay

We determined the cellular viability of the selected cancer lines using the MTT assay for each treatment condition. The IC_{50} value for cisplatin was determined to be 10 µM in the A549 cancer cell line; in the Caco2 cancer cell line, the IC_{50} value for cisplatin was determined to be 12 µM. Furthermore, the IC_{50} values for EF-24 and CLEFMA in both cell lines were determined to be 2 µM and 15 µM respectively.

In the A549 cell line, we found a dose-dependent decrease in the absorbance values, indicating a decrease of cellular viability when cells were treated with either CLEFMA or cisplatin and CLEFMA. CLEFMA-treated cells had higher cell viability than CLEFMA and cisplatin-treated cells, although this effect was only significant at 0.5 µM CLEFMA (Figure 1). There was no significant difference in cell viability between EF-24 and EF-24 plus cisplatin-treated cells, although a concentration-dependent effect was present (Figure 1).

For the Caco2 cell line, there was also a dose-dependent decrease in cellular viability with increased concentration of either the curcuminoid, or either curcuminoid plus cisplatin. The MTT assay showed lower absorbance values (i.e., lower cell viability) for the CLEFMA and cisplatin treatment when compared to CLEFMA alone, although they were significantly different only at 500 µM CLEFMA (Figure 2). For EF-24 and cisplatin, lower absorbance values were shown compared to EF-24 controls, although they were significantly different only at 0.5 µM EF-24 (Figure 2). In both A549 and

Caco2 cell lines, the DMSO control showed no effect on the viability of the cancer cells, as cell viability was similar across all DMSO concentrations (Figure 3).

Auditory Evoked Potentials

Treatment with 0.9% sodium chloride (NaCl) and DMSO resulted in a typical zebrafish audiogram (Uribe et al. 2013). Cisplatin treatment caused an approximately 10 dB upward threshold shift in the audiograms at 800, 1000, and 1500 Hz relative to NaCl controls (Fig. 4A). Treatment with the curcuminoids showed a slight increase of approximately 5 dB in hearing thresholds at three frequencies (800, 1000, and 3000Hz) when compared to the vehicle for the curcuminoids, DMSO (Figure 4B). Across most frequencies in the audiogram, cisplatin-injected zebrafish exhibited significantly higher hearing thresholds than those of zebrafish injected with cisplatin plus either EF-24, CLEFMA, or DMSO, showing that these compounds can mitigate cisplatin-induced hearing loss in zebrafish (Figure 5). This protective effect was more prominent at intermediate frequencies at which cisplatin-induced threshold shifts were the greatest.

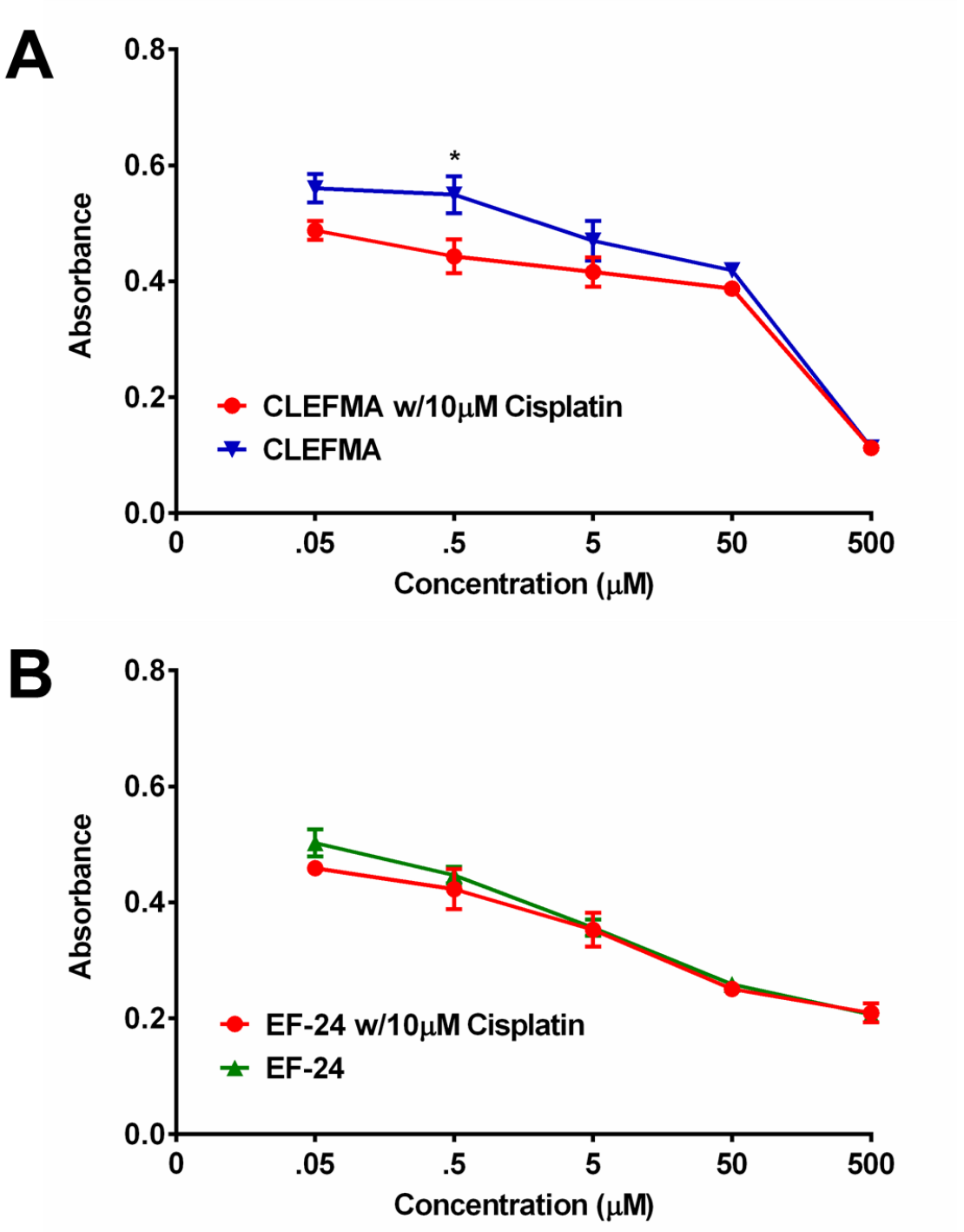

Figure 1. Absorbance at 570 nm of formazan dye in the A549 cancer cell line as a function of concentration of (A) CLEFMA with and without cisplatin and (B) EF-24 with and without cisplatin. *$P \leq 0.05$.

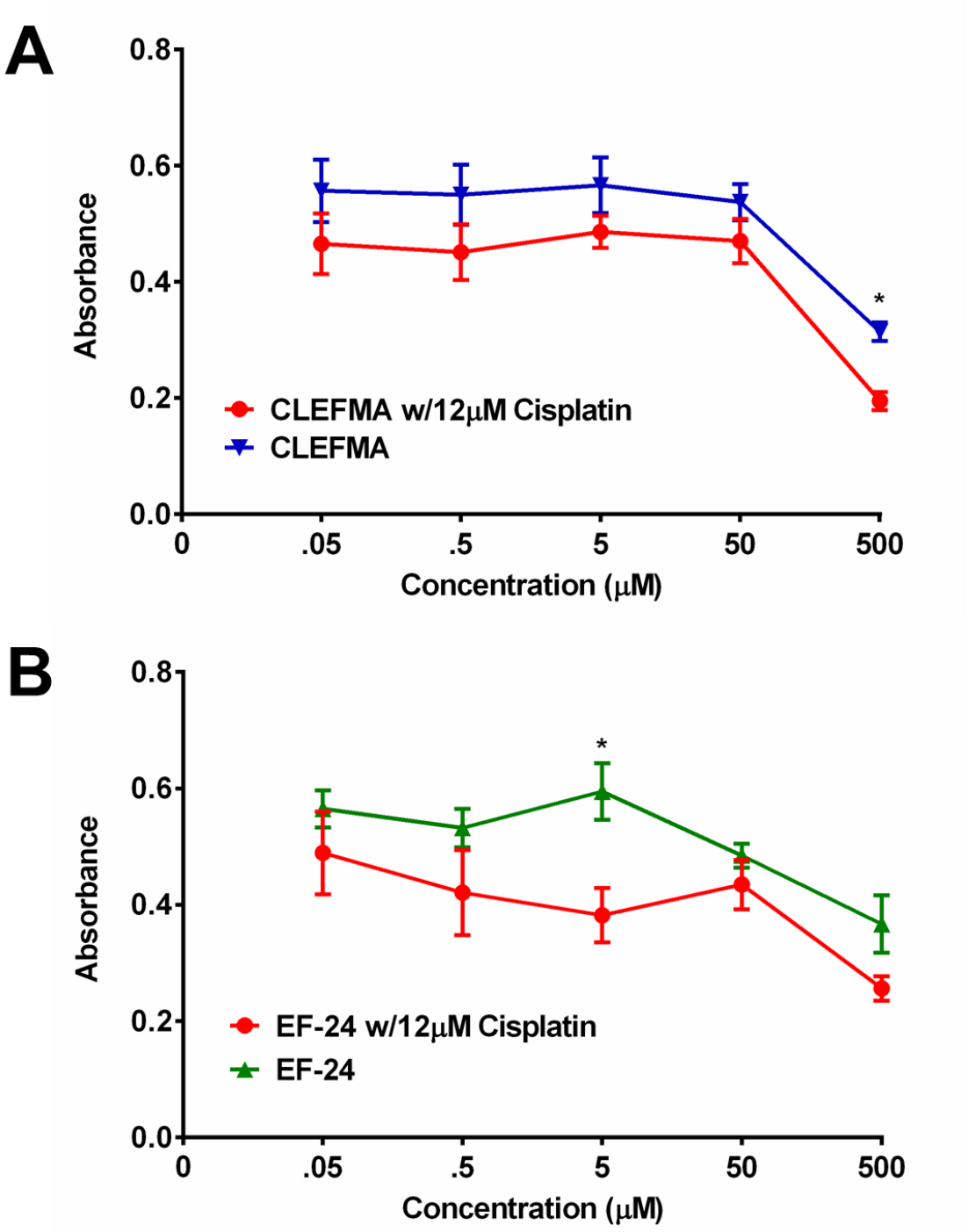

Figure 2. Absorbance at 570 nm of formazan dye in the Caco2 cancer cell line as a function of concentration of (A) CLEFMA with and without cisplatin and (B) EF-24 with and without cisplatin. *$P \leq 0.05$.

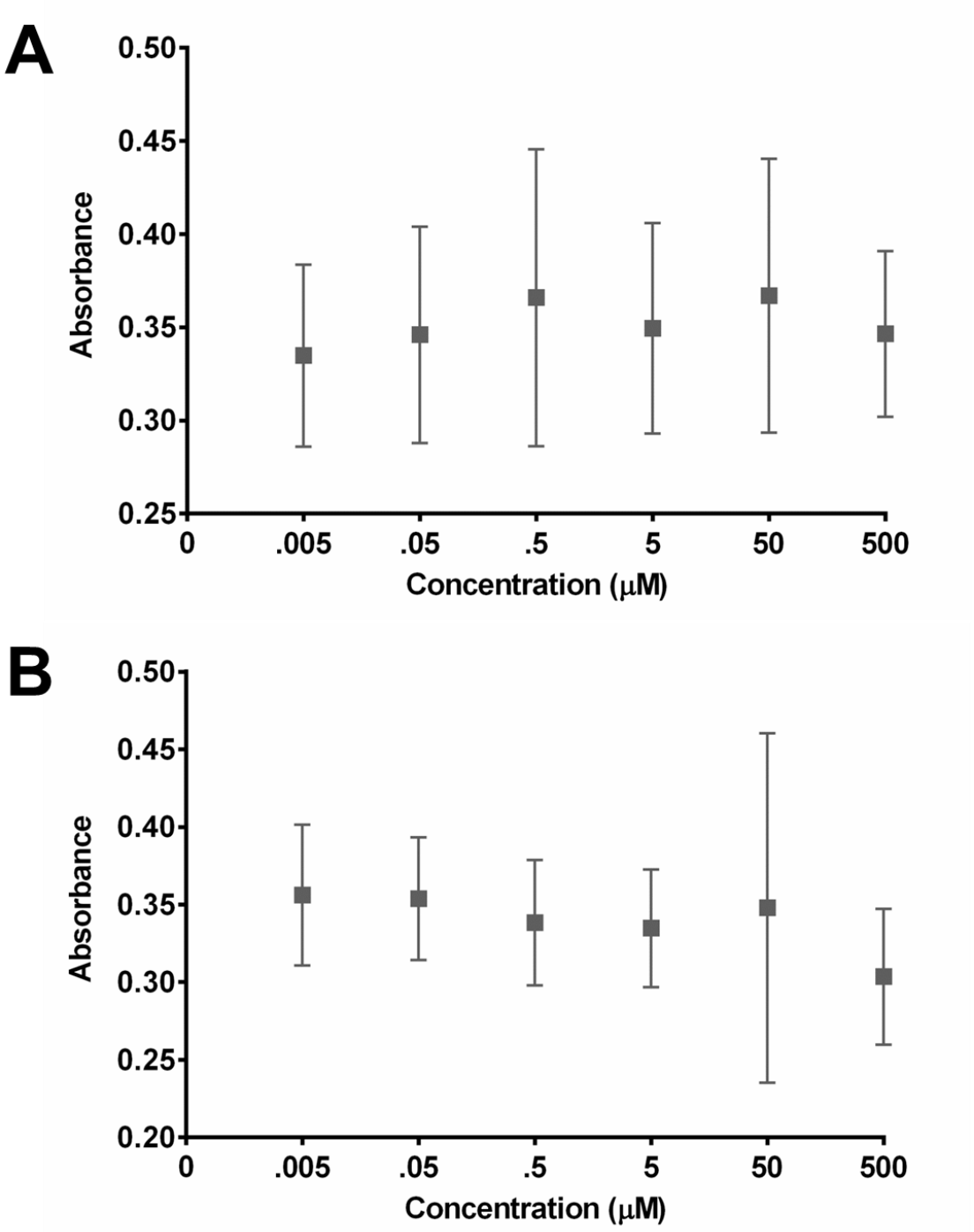

Figure 3. Absorbance at 570 nm of formazan dye in the (A) A549 and (B) Caco2 cancer cell lines as a function of concentration of DMSO.

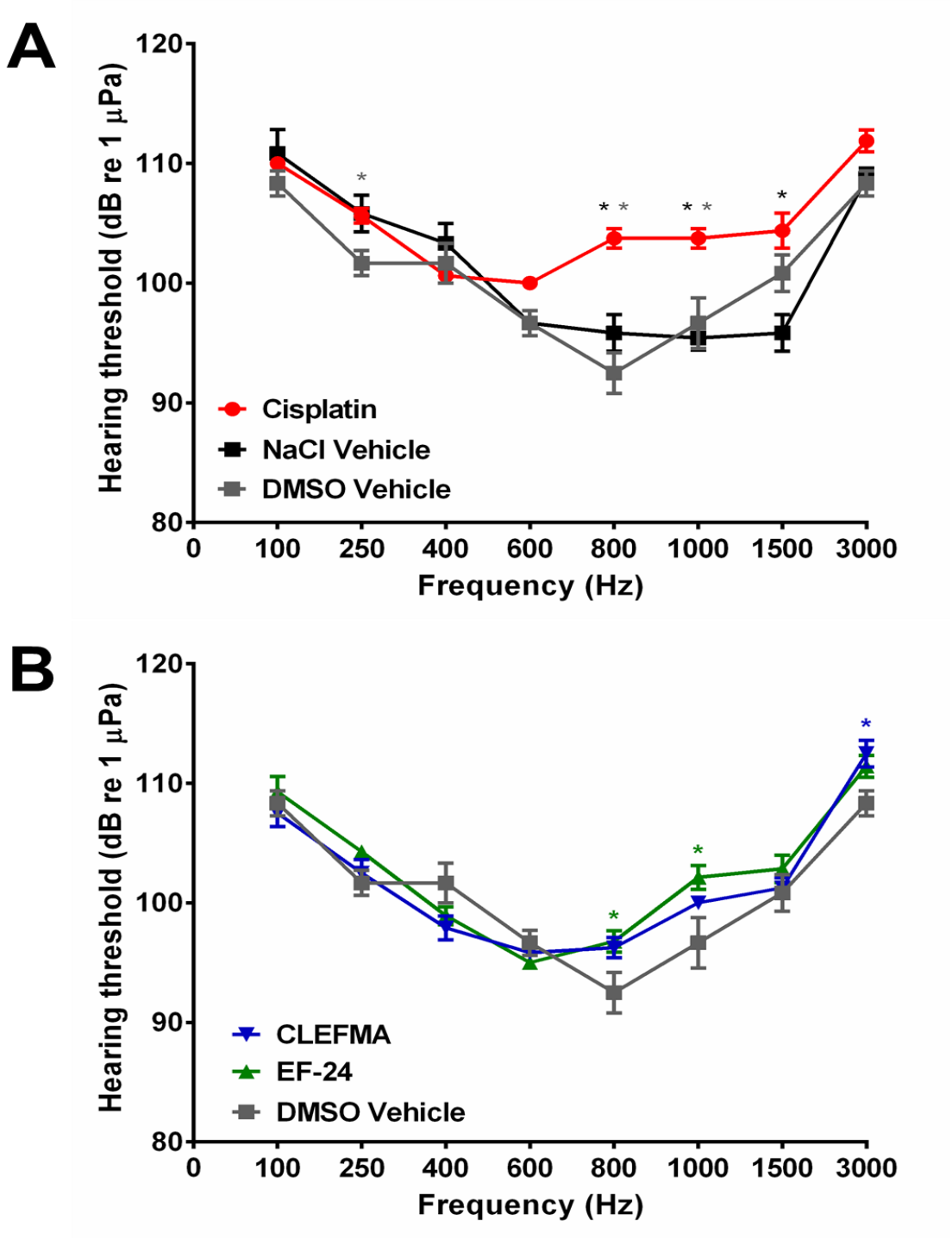

Figure 4. Hearing thresholds as a function of frequency of tone pip stimuli in (A) cisplatin, sodium chloride, and DMSO-microinjected zebrafish and (B) CLEFMA, EF-24, and DMSO-injected zebrafish. *$P \leq 0.05$, compared with cisplatin (A) or vehicle (B).

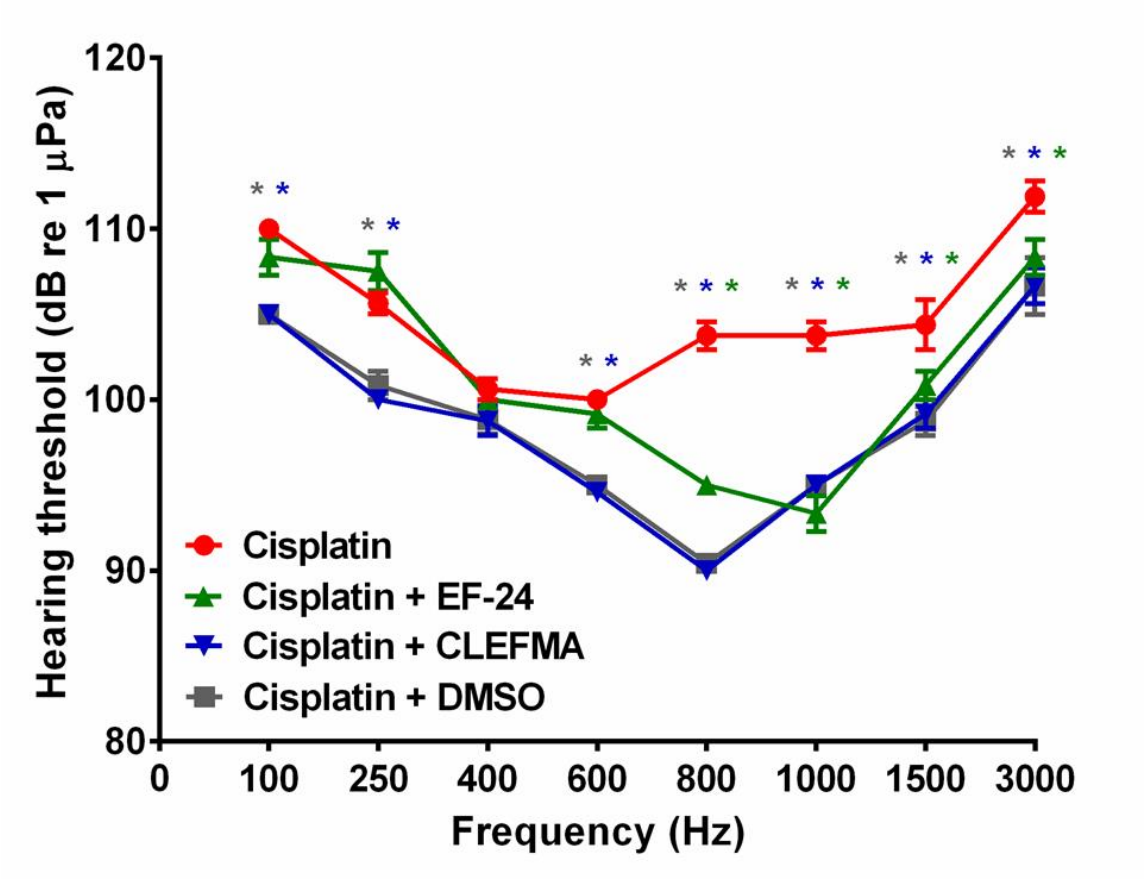

Figure 5. Hearing thresholds as a function of frequency of tone pip stimuli in cisplatin, cisplatin and EF-24, cisplatin and CLEFMA, and cisplatin and DMSO-microinjected zebrafish. At 100, 250, and 600 Hz, cisplatin is significantly different from the cisplatin + CLEFMA, cisplatin + DMSO; cisplatin + EF-24 is statistically different from cisplatin + CLEFMA and cisplatin + DMSO. At 800 Hz, cisplatin is statistically different from cisplatin + EF-24, cisplatin + DMSO, and cisplatin + CLEFMA; cisplatin + EF-24 is statistically different from cisplatin + DMSO and cisplatin + CLEFMA. At 1000, 1500, and 3000 Hz, cisplatin is significantly different from cisplatin + EF-24, cisplatin + DMSO, and cisplatin + CLEFMA. *$P \leq 0.05$ for all treatment conditions.

DISCUSSION

Chemotherapy often combines therapeutic agents to enhance the effect of individual drugs while simultaneously reducing the side-effects produced by one of these components alone. This study indicates that a combination of cisplatin with either CLEFMA or EF-24 produces a dose-dependent effect against two cancer cell-lines and may also prevent hearing loss from cisplatin treatment in a zebrafish auditory model. Increasing CLEFMA or EF-24 concentration decreased cancer cell viability and the cisplatin co-treatment produced an equivalent effect or further reduced viability. Treatment with either CLEFMA or EF-24 can counteract hearing loss caused by cisplatin, but there could be an effect from the DMSO vehicle. These results suggest that cisplatin and both curcuminoids have a compatible but not synergistic or additive effect against these two cancer cell-lines. Further, these curcuminoids may prevent cisplatin-modulated hearing loss but the timing of curcuminoid treatment either before or after cisplatin exposure and the curcuminoid vehicle used may be important factors to consider.

This study attempted to identify synergisms between cisplatin and the two curcuminoids, CLEFMA and EF-24, by measuring their effects on cancer cell viability. Co-administration of curcumin with cisplatin can sensitize cancer cells that normally are not responsive to cisplatin treatment (Mimeault and Batra, 2011; Huq et al. 2014). Curcumin can inhibit the FA/BRCA pathway in cisplatin resistant A549 cells leading to enhanced apoptosis and cell death (Chen et al. 2015). Similarly, curcumin can improve the efficacy of cisplatin in the A549 cell line by targeting p21 and cyclin D1 leading to

increased apoptosis (Baharuddin et al. 2016). Cisplatin induces mechanisms associated with apoptosis and mitochondrial-mediated ROS release that cause cancer cell death (Knox et al. 1986; Jamieson et al. 1999; Cepeda et al. 2007; Wang et al. 2010; Marullo et al. 2013; Choi et al. 2015; Zou et al. 2015). Curcumin has been shown to reduce ROS release in cancer cells via a mitochondrial dependent pathway (Jung et al. 2016) and could potentially negate cisplatin's effect to promote mitochondrial ROS release and kill cancer cells. However, studies of CLEFMA and EF-24 suggest that these two curcuminoids suppress cancer by acting through distinct pathways. CLEFMA prevents cancer proliferation by activating a non-apoptotic autophagic pathway and promotes increased ROS production from the mitochondria (Lagisetty et al. 2011; Sahoo et al. 2012). EF-24 reduces cancer proliferation by signaling through an apoptotic pathway and, like CLEFMA, increases mitochondrial ROS release (Adams et al. 2005; Selvendiran et al. 2007; Subramaniam et al. 2008; Tan et al. 2010; Yadav et al. 2013). These results suggest that either curcuminoid could act to enhance cisplatin's effect by amplifying the same pathway, e.g., mitochondrial ROS release and apoptosis, or by acting alongside cisplatin via a distinct pathway, e.g., CLEFMA's promotion of autophagy.

Synergistic anti-cancer activity between cisplatin and other drugs can be identified using the MTT cellular viability assay (Onen et al. 2015; Tian et al. 2017). My MTT results in the A549 cell line (Figure 1) show that treating cells with 10 μM cisplatin in a concentration series (500 μM to .05 μM) of either CLEFMA or EF-24 does not produce a significant difference in cellular viability from either curcuminoid alone in the same concentration series in all but one concentration (0.5 μM CLEFMA). The MTT

assay results for the Caco2 cell line using the same concentration series for either curcuminoid with or without treatment with 12 µM cisplatin also produced only two data points that were significantly different (500 µM CLEFMA and 0.5 µM EF-24: Figure 2). Results for both the A549 or Caco2 do not suggest either an additive or synergistic effect is produced by combining either curcuminoid with cisplatin. This result implies a lack of targeting, by either CLEFMA or EF-24, of a pathway that enhances cisplatin activity. However, an alternative interpretation is that either curcuminoid might modulate a pathway that nullifies cisplatin's effect but that the curcuminoid can still signal through other cancer targeting pathways causing a decrease in cancer cell viability. This interpretation is supported by the data which shows that there is a general decline in cellular viability as the concentration of the curcuminoid is increased (Figure 1 and 2).

The curcuminoid treatments used the solvent, DMSO, which can neutralize cisplatin (Fischer et al. 2008; Hall et al. 2014). Although this could explain the absence of an enhanced effect on viability from combining cisplatin with a curcuminoid, cisplatin treatment occurred 24 hours prior to curcuminoid/DMSO treatment. It has been shown that significant cisplatin uptake to the nucleus occurs within 3 hours (Park et al. 2012), so it is unlikely that introducing DMSO 24 hours after cisplatin treatment would cause DMSO to directly neutralize cisplatin. My MTT results testing the effect of DMSO treatment in both the A549 and Caco2 cell lines indicates that this solvent itself does not influence cellular viability (Figure 3). However, DMSO might function as an antioxidant to neutralize ROS produced during a later stage of the cisplatin-induced cell death pathway. Although DMSO has been shown to act as an antioxidant that counteracts the effect of pro-oxidant compounds in brain tissue homogenates (Sanmartin-Suarez et al.,

2011), no study has examined the effect of this solvent on cisplatin-modulated ROS production. Thus, the cellular viability assay does not identify a synergistic or additive effect from combining either CLEFMA or EF-24 with cisplatin but does support a concentration dependent effect against both cancer cell-lines from these curcuminoids.

Examination of whether CLEFMA and EF-24 compounds could counteract hearing side-effects produced by cisplatin treatment was also conducted. Cisplatin is an ototoxin which damages auditory hair cells resulting in reduced hearing thresholds (Karasawa et al. 2015; Waissbluth et al. 2015). In the cochlea, cisplatin initially damages DNA leading to an increase in ROS generation that alters the activity of many enzymes leading ultimately to apoptotic hair cell damage and death (Deavall et al. 2012; Schacht et al. 2012; Paken et al. 2016). Curcumin treatment can induce hemeoxygenase, an antioxidant enzyme, and counteract auditory threshold shifts caused by cisplatin (Fetoni et al. 2014). A subsequent study in the same laboratory implicated STAT3 and Nrf2 signaling in curcumin's otoprotective effect against cisplatin (Fetoni et al. 2015). No studies have yet characterized the effect of either CLEFMA or EF-24 on hearing. It is known that EF-24 can increase ROS release in cancer cells, but can also act as an anti-oxidant (Adams et al. 2005; Tan et al. 2010). This suggests that if EF-24 acted as an anti-oxidant in auditory hair cells, then it could counteract ROS release caused by cisplatin treatment and possibly prevent auditory hair cell apoptosis. CLEFMA has been shown to increase ROS release in cancer cells, but studies show that it does not increase ROS levels in normal cells (Lagisetty et al. 2011; Sahoo et al. 2012). This could mean that CLEFMA may not act as an ROS scavenger in auditory hair cells, but it is not known

whether this curcuminoid's activity against ROS or associated mechanisms would be altered in hair cells that have become physiologically aberrant due to cisplatin exposure.

The zebrafish auditory electrophysiology model can detect threshold shifts caused by ototoxins (Uribe et al. 2013). As cisplatin causes damage to the hair cells of the zebrafish inner ear (Giari et al. 2012), the AEP technique should detect auditory threshold shifts produced from cisplatin treatment and identify otoprotective effects from curcuminoids. My AEP results showed that cisplatin caused significant hearing threshold shifts of 10 dB or more above its NaCl vehicle and the curcuminoid vehicle, DMSO at three frequencies (Figure 4A). This suggests that cisplatin creates an ototoxic effect relative to both vehicle treatments. A similar experiment compared the two curcuminoids with their DMSO vehicle and found that a significant threshold shift occurred for CLEFMA at only one frequency, and at two frequencies for EF-24 with less than a 5 dB effect at these three endpoints (Figure 4B). These results suggest that both curcuminoids generally do not induce an ototoxic effect.

The AEP experiments performed on zebrafish treated initially with cisplatin and either a curcuminoid or DMSO suggest that DMSO could act as an otoprotectant. Both curcuminoids significantly reduced threshold shifts at multiple frequencies (Figure 5). However, the CLEFMA data suggests that this curcuminoid might be a better otoprotectant than EF-24, as the hearing thresholds in zebrafish treated with CLEFMA + cisplatin were significantly lower than EF-24 + cisplatin. Furthermore, treating cisplatin-injected fish subsequently with just DMSO, caused a reduction in threshold shifts at the same frequencies that CLEFMA did and produced an almost identical audiogram. These results suggest that DMSO, and not the curcuminoid, could be responsible for the

otoprotective effect. As DMSO can neutralize cisplatin (Fischer et al. 2008; Hall et al. 2014), DMSO was injected with or without a curcuminoid 24 hours after the initial cisplatin injection. This protocol was premised on results from biochemical assays where significant platinum uptake into the nucleus occurred within 3 hours of cisplatin treatment (Park et al. 2012). Although, this study was performed in dissociated cancer cells and intact auditory tissue may exhibit different and slower platinum uptake physiology.

DMSO can, depending on the molecular target, act as both an antioxidant and pro-oxidant (Sanmartin-Suarez et al., 2011) and in the former capacity it could target and neutralize ROS generated downstream from cisplatin treatment. This could mean that the temporal separation in these experiments between cisplatin and DMSO injection may be insufficient to prevent DMSO from counteracting the downstream effects from cisplatin and was responsible for the reduced threshold shifts. However, one study of DMSO and cisplatin in rats showed that intraperitoneally injecting cisplatin immediately after an intratympanic injection of DMSO caused significant hearing threshold shifts (Roldan-Fidalgo et al. 2014). Thus, temporally proximate treatments of cisplatin and DMSO do not necessarily negate the ototoxic effect of this platinum-based drug. Evidently, additional research is needed to more precisely determine the mechanistic interplay between cisplatin, CLEFMA, EF-24 and DMSO in cancer cell and auditory physiology.

REFERENCES

Adams, B.K., Cai, J., Armstrong, J., Herold, M., Lu, Y.J., Sun, A., Snyder, J.P., Liotta, D.C., Jones, D.P., Shoji, M. (2005). EF24, a novel synthetic curcumin analog, induces apoptosis in cancer cells via a redox-dependent mechanism. *Anti-Cancer Drugs*., 16: 263–75.

Baharuddin P., Satar N., Fakiruddin K.S., Zakaria N., Lim M.N., Yusoff N.M., Zakaria Z., Yahaya B.H. (2016). Curcumin improves the efficacy of cisplatin by targeting cancer stem-like cells through p21 and cyclin D1-mediated tumour cell inhibition in non-small cell lung cancer cell lines. *Oncol. Rep*., 35:13-25. doi: 10.3892/or.2015.4371.

Benard, A., Janssen, C.M., van den Elsen, P.J., van Eggermond, M.C., Hoon, D.S., van de Velde, C.J., Kuppen, P.J. (2014). Chromatin status of apoptosis genes correlates with sensitivity to chemo-, immune- and radiation therapy in colorectal cancer cell lines. *Apoptosis*., 19:1769-78. doi: 10.1007/s10495-014-1042-8. PMCID: PMC4358803.

Cepeda, V., Fuertes, M.A., Castilla, J., Alonso, C., Quevedo, C., Pérez, J.M. (2007). Biochemical mechanisms of cisplatin cytotoxicity. *Anticancer Agents Med. Chem*., 7:3-18. PMID: 17266502.

Chen, P., Li, J., Jiang, H.G., Lan, T., Chen, Y.C. (2015). Curcumin reverses cisplatin resistance in cisplatin-resistant lung cancer cells by inhibiting FA/BRCA pathway. *Tumour Biol*. 36:3591-9. doi: 10.1007/s13277-014-2996-4. PMID: 25542235.

Chen, Y., Hao, L., Hung, C., Chen, J., Leu, S., Huang, B. (2013). Apoptotic effect of cisplatin and cordycepin on OC3 human oral cancer cells. *Chin. J. Integr. Med.*, *20*:624-32. doi:10.1007/s11655-013-1453-3.

Cheung-Ong, K., Giaever, G., Nislow, C. (2013). DNA-damaging agents in cancer chemotherapy: serendipity and chemical biology. *Chem. Biol., 20*:648-59. doi:10.1016/j.chembiol.2013.04.007.

Choi, Y., Kim, H., Shim, W., Anwar, M.A., Kwon, J., Kwon, H., Choi, S. (2015). Mechanism of cisplatin-induced cytotoxicity is correlated to impaired metabolism due to mitochondrial ROS generation. *Plos One.*, 10:e0135083 doi:10.1371/journal.pone.0135083.

Deavall D.G., Martin E.A., Horner J.M., Roberts R. (2012). Drug-induced oxidative stress and toxicity. *J. Toxicol*., 2012:645460. doi: 10.1155/2012/645460.

Ferlay J., Soerjomataram I., Ervik M., Dikshit R., Eser S., Mathers C. (2013). GLOBOCAN 2012 v1.0: Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. Lyon, France: International Agency for Research on Cancer; 2013.

Fetoni A.R., Eramo S.L., Paciello F., Rolesi R., Podda M.V., Troiani D., Paludetti G. (2014). *Curcuma longa* (curcumin) decreases in vivo cisplatin-induced ototoxicity through heme oxygenase-1 induction. *Otol. Neurotol*., 35:e169-77. doi: 10.1097/MAO.0000000000000302.

Fetoni A.R., Paciello F., Mezzogori D., Rolesi R., Eramo S.L., Paludetti G., Troiani D.

(2015). Molecular targets for anticancer redox chemotherapy and cisplatin-Induced ototoxicity: the role of curcumin on pSTAT3 and Nrf-2 signaling. *Br. J. Cancer*., 113:1434-44. doi: 10.1038/bjc.2015.359.

Fischer S.J., Benson L.M., Fauq A., Naylor S., Windebank A.J. (2008). Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity. *Neurotoxicology*, 29:444-52. doi: 10.1016/ j.neuro.2008.02.010.

Fridlender, M., Kapulnik, Y., Koltai, H. (2015). Plant derived substances with anti-cancer activity: from folklore to practice. *Front. Plant. Sci*., 6:799. doi: 10.3389/fpls.2015.00799. PMID: 26483815.

Giari L., Dezfuli B.S., Astolfi L., Martini A. (2012). Ultrastructural effects of cisplatin on the inner ear and lateral line system of zebrafish (*Danio rerio*) larvae. *J. Appl. Toxicol*., 32:293–99.

Hall M.D., Telma K.A., Chang K.E., Lee T.D., Madigan J.P., Lloyd J.R., Goldlust I.S., Hoeschele J.D., Gottesman M.M. (2014). Say no to DMSO: dimethylsulfoxide inactivates cisplatin, carboplatin, and other platinum complexes. *Cancer Res*., 74:3913-22. doi: 10.1158/0008-5472.CAN-14-0247.

Horibe, S., Matsuda, A., Tanahashi, T., Inoue, J., Kawauchi, S., Mizuno, S., Ueno, M., Takahashi, K., Maeda, Y., Maegouchi, T., Murakami, Y., Yumoto, R., Nagai, J., Takano, M. (2015). Cisplatin resistance in human lung cancer cells is linked with dysregulation of cell cycle associated proteins. *Life Sci*., 124:31-40. doi: 10.1016/j.lfs.2015.01.011. PMID: 25625243.

Huq F., Yu J.Q., Beale P., Chan C., Arzuman L., Nessa M.U., Mazumder M.E. (2014)

Combinations of platinums and selected phytochemicals as a means of overcoming resistance in ovarian cancer. *Anticancer Res.,* 34:541-5.

Jamieson, E.R., & Lippard, S.J. (1999). Structure, Recognition, and Processing of Cisplatin−DNA Adducts. *Chem. Rev., 99*:2467-98. doi:10.1021/ cr980421n.

Jung K.H., Lee J.H., Park J.W., Moon S.H., Cho Y.S., Choe Y.S., Lee K.H. (2016). Effects of curcumin on cancer cell mitochondrial function and potential monitoring with ^{18}F-FDG uptake. *Oncol. Rep*., 35:861-8. doi: 10.3892/ or.2015.4460.

Karasawa, T., & Steyger, P.S. (2015). An integrated view of cisplatin-induced nephrotoxicity and ototoxicity. *Toxicology Lett., 237*:219-27. doi:10.1016/j.toxlet.2015.06.012.

Knox, R.J., Friedlos, F., Lydall, D.A., & Roberts, J.J. (1986). Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1-cyclobutanedicarboxylato) platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res., 46*:1972-79.

Lagisetty, P., Vilekar, P., Sahoo, K., Anant, S., & Awasthi, V. (2010). CLEFMA—An anti-proliferative curcuminoid from structure–activity relationship studies on 3,5-bis(benzylidene)-4-piperidones. *Bioorgan. Med.Chem., 18*:6109-20. doi:10.1016/j.bmc.2010.06.055.

Marullo, R., Werner, E., Degtyareva, N., Moore, B., Altavilla, G., Ramalingam, S.S.,

Doetsch, P.W. (2013). Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. *PLoS One,* 8:e81162. doi:10.1371/journal.pone.0081162.

Mckeage, M. J. (1995). Comparative adverse effect profiles of platinum drugs. *Drug Safety*, 13:228-244. doi:10.2165/00002018-199513040-00003.

Mimeault M, Batra S.K. (2011). Potential applications of curcumin and its novel synthetic analogs and nanotechnology-based formulations in cancer prevention and therapy. *Chin. Med*., 6:31. doi: 10.1186/1749-8546-6-31.

Onen H.I., Yilmaz A., Alp E., Celik A., Demiroz S.M., Konac E., Kurul I.C., Menevse E.S. EF24 and RAD001 potentiates the anticancer effect of platinum-based agents in human malignant pleural mesothelioma (MSTO-211H) cells and protects nonmalignant mesothelial (MET-5A) cells. *Hum. Exp. Toxicol*., 34:117-26. doi: 10.1177/0960327114542965.

Paken J, Govender C.D., Pillay M., Sewram V. (2016). Cisplatin-associated ototoxicity: a review for the health professional. *J. Toxicol*., 2016:1809394. doi: 10.1155/2016/1809394.

Park G.Y., Wilson J.J., Song Y., Lippard S.J. (2012). Phenanthriplatin, a monofunctional DNA-binding platinum anticancer drug candidate with unusual potency and cellular activity profile. *Proc. Natl. Acad. Sci. U.S.A.*, 109:11987-92. doi: 10.1073/pnas.1207670109.

Roldán-Fidalgo A., Trinidad A., Rodríguez-Valiente A., García-Berrocal J.R., Millán I.,

Coronado M.J., Ramírez-Camacho R. (2014). Effect of intratympanic dimethyl sulphoxide (DMSO) in an in vivo model of cisplatin-related ototoxicity. *Eur. Arch. Otorhinolaryngol*., 271:3121-6. doi: 10.1007/s00405-014-2957-y.

Sahoo, K., Dozmorov, M.G., Anant, S., Awasthi, V. (2010). The curcuminoid CLEFMA selectively induces cell death in H441 lung adenocarcinoma cells via oxidative stress. *Invest. New Drug*., 30:558-67. doi: 10.1007/s10637-010-9610-4. PMID: 21181232.

Salehi, P., Akinpelu, O.V., Waissbluth, S., Peleva, E., Meehan, B., Rak, J., Daniel, S.J. (2014). Attenuation of cisplatin ototoxicity by otoprotective effects of nanoencapsulated curcumin and dexamethasone in a guinea pig model. *Otol. Neurotol*., 35:1131-9. doi: 10.1097/MAO.0000000000000403. PMID: 24841915.

Sanmartín-Suárez C., Soto-Otero R., Sánchez-Sellero I., Méndez-Álvarez E. (2011). Antioxidant properties of dimethyl sulfoxide and its viability as a solvent in the evaluation of neuroprotective antioxidants. *J. Pharmacol. Toxicol. Methods*., 63:209-15. doi: 10.1016/j.vascn.2010.10.004.

Schacht J, Talaska A.E., Rybak L.P. (2012). Cisplatin and aminoglycoside antibiotics: hearing loss and its prevention. *Anat Rec (Hoboken).*, 295:1837-50. doi: 10.1002/ar.22578.

Selvendiran, K., Tong, L., Vishwanath, S., Bratasz, A., Trigg, N.J., Kutala, V.K., Hideg, K., Kuppusamy, P. (2007). EF24 induces G2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by increasing PTEN expression. *J. Biol. Chem*., 282:28609–18.

Sergent, C., Franco, N., Chapusot, C., Lizard-Nacol, S., Isambert, N., Correia, M.,

Chauffert, B. (2002). Human colon cancer cells surviving high doses of cisplatin or oxaliplatin in vitro are not defective in DNA mismatch repair proteins. *Cancer Chemother Pharmacol*., 49:445-52. PMID: 12107548.

Shanmugam, M.K., Rane, G., Kanchi, M.M., Arfuso, F., Chinnathambi, A., Zayed, M.E., Alharbi, S.A., Tan, B.K., Kumar, A.P., Sethi, G. (2015). The multifaceted role of curcumin in cancer prevention and treatment. *Molecules*, 20:2728-69. doi: 10.3390/molecules20022728. PMID: 25665066.

Smith M.E., Coffin A.B., Miller D.L., Popper A.N. (2006). Anatomical and functional recovery of the goldfish (*Carassius auratus*) ear following noise exposure. *J. Exp. Biol.*, 209:4193–4202.

Smith, M.E., Schuck, J.B., Gilley, R.R., and Rogers, B.D. (2011). Structural and functional effects of acoustic exposure in goldfish: evidence for tonotopy in the teleost saccule. *BMC Neurosci.*, 12:19, Doi:10.1186/1471-2202-12-19.

Subramaniam, D., May, R., Sureban, S.M., Lee, K.B., George, R., Kuppusamy, P., Ramanujam, R.P., Hideg, K., Dieckgraefe, B.K., Houchen, C.W., Anant, S. (2008). Diphenyl difluoroketone: a curcumin derivative with potent in vivo anticancer activity. *Cancer Res*., 68:1962–69.

Tan, X., Sidell, N., Mancini, A., Huang, R.P., Wang, S., Horowitz, I.R., Liotta, D.C., Taylor, R.N., Wieser, F. (2010). Multiple anticancer activities of EF24, a novel curcumin analog, on human ovarian carcinoma cells. *Reprod Sci*., 17:931-40. doi: 10.1177/1933719110374239. PMID: 20693500.

Teiten, M.H., Dicato, M., Diederich, M. (2014). Hybrid curcumin compounds: a new

strategy for cancer treatment. *Molecules*, 19:20839-63. doi: 10.3390/molecules191220839. PMID: 25514225.

Tian J, Liu R, Qu Q. (2017). Role of endoplasmic reticulum stress on cisplatin resistance in ovarian carcinoma. *Oncol. Lett.*, 13:1437-43. doi: 10.3892/ol.2017.5580.

Tuorkey, M. (2014). Curcumin a potent cancer preventive agent: Mechanisms of cancer cell killing. *Interv. Med. Applied Sci.,* 6:139-146. doi:10.1556/imas.6.2014.4.1.

Uribe P. M., Sun H., Wang K., Asuncion J. D., Wang Q., Chen C. W., et al. . (2013). Aminoglycoside-induced hair cell death of inner ear organs causes functional deficits in adult zebrafish (*Danio rerio*). *PLoS One.*, 8:e58755. 10.1371/journal.pone.0058755.

Uribe P. M., Mueller M. A., Gleichman J. S., Kramer M. D., Wang Q., Sibrian-Vazquez M., et al. (2013). Dimethyl sulfoxide (DMSO) exacerbates cisplatin-induced sensory hair cell death in zebrafish (*Danio rerio*). *PLoS One.,* 8:e55359. 10.1371/journal.pone.0055359.

Waissbluth, S., & Daniel, S. J. (2013). Cisplatin-induced ototoxicity: Transporters playing a role in cisplatin toxicity. *Hear. Res.,* 299: 37-45. doi:10.1016/ j.heares.2013.02.002.

Wang, Q., Zheng, X., Yang, L., Shi, F., Gao, L., Zhong, Y., Wang, X. (2010). Reactive oxygen species-mediated apoptosis contributes to chemosensitization effect of saikosaponins on cisplatin-induced cytotoxicity in cancer cells. *J. Exp. Clin. Canc. Res.,* 29:159. doi:10.1186/1756-9966-29-159.

Yadav, V.R., Sahoo, K., Roberts, P.R., Awasthi, V. (2013). Pharmacologic suppression

of inflammation by a diphenyldifluoroketone, EF24, in a rat model of fixed-volume hemorrhage improves survival. *Pharmacol. Exp. Ther*., 347:346-56. doi: 10.1124/jpet.113.208009. PMID: 23995597.

Zou, W., Ma, X., Hua, W., Chen, B., & Cai, G. (2015). Caveolin-1 mediates chemoresistance in cisplatin-resistant ovarian cancer cells by targeting apoptosis through the Notch-1/Akt/NF-κB pathway. *Oncol. Rep.,* 34:3256-63. doi:10.3892/or.2015.432.