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## Combination of esomeprazole with chemotherapeutics results in more pronounced cytotoxic effect via apoptosis on A549 nonsmall-cell lung cancer cell line

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**Abstract:** The vacuolar (H<sup>+</sup>)-ATPases that pump H<sup>+</sup> from the cytoplasm to extracellular compartments can alter the pH of the tumor microenvironment. Esomeprazole can effectively inhibit vacuolar (H<sup>+</sup>)-ATPases and may increase the effectiveness of chemotherapeutics. Therefore, we used esomeprazole in combination with cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, and vinorelbine on the A549 nonsmall-cell lung cancer cell line. Cisplatin and carboplatin combinations with esomeprazole exhibited superior cytotoxicity compared to the other selected chemotherapeutics. Low-dose combinations of esomeprazole with either cisplatin or carboplatin resulted in synergistic interaction. We examined cytotoxic activity of these combinations with the xCELLigence real-time cytotoxicity assay and detected that esomeprazole combinations with both 100% test drug concentrations of cisplatin and carboplatin shifted the antiproliferative effects of these agents towards a cytotoxic effect in a dose-dependent manner. Cell death mode was investigated by M30 assay, Annexin-V-FITC fluorescence imaging, and determination of PARP cleavage in western blotting. The cells treated with the cisplatin and esomeprazole combination displayed characteristic features of apoptosis such as elevated M30 levels, Annexin-V staining, and PARP cleavage. In conclusion, these novel combinations resulted in higher sensitivity of tumors to chemotherapeutics, thereby warranting further *in vivo* experiments for proof of the concept.

**Key words:** Lung carcinoma, cisplatin, carboplatin, esomeprazole, apoptosis, synergism

### 1. Introduction

Lung cancer ranks first in deaths among malignancies and approximately 1.59 million people (19.4% of all cancer deaths excluding nonmelanoma skin cancer) die worldwide from lung cancer every year (Ferlay et al., 2013). The 5-year survival rate for nonsmall-cell lung cancer is estimated to be 10.5% for females and 9.4% for males (Caldarella et al., 2007). No symptoms are detected in the early stage of most lung cancer cases, which results in late diagnosis (Carter-Harris et al., 2014; Shim et al., 2014). Despite the recently developed diagnostic methods, the localization of detected tumors is not limited to the lungs. The most important difficulty in lung cancer therapy is metastasis to different organs or different sites in the lung, which makes it almost impossible to fully eradicate the tumor by surgery, radiotherapy, and chemotherapy. Hence, resistance to chemotherapeutics becomes crucial in patients with no chance of curative surgery.

The acidity of the tumor microenvironment is one of the important factors in chemotherapy resistance. The vacuolar (H<sup>+</sup>)-ATPases (V-ATPases) that pump H<sup>+</sup> from the cytoplasm to extracellular compartments have a critical role in acidity (Perez-Sayans et al., 2009). These multisubunit V-ATPase complexes are basically expressed in all eukaryotic cells, although there are variations in subunits (Wagner et al., 2004). However, they are preferentially expressed in various cancer types including pancreatic cancer, oral squamous cell cancer, and nonsmall-cell lung carcinoma compared to nonmalignant tissues, and higher expression levels of V-ATPases are linked to malignant phenotypes (Sennoune et al., 2004; Chung et al., 2011; Garcia-Garcia et al., 2012; Qiang et al., 2013). Consequently, therapeutic approaches to inhibit V-ATPases are expected to increase sensitivity towards antineoplastic agents or overcome chemoresistance.

Tumor tissues, and especially solid tumors, possess different microenvironmental features (Vaupel, 2004). The

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tumor vasculature system is irregular and bears anomalies with the effect of growth factors, and there are no lymphatics present in the tumor tissue (Leu et al., 2000; Padera et al., 2002). The most important factor contributing to the acidic tumor microenvironment is adaptation to the glycolytic phenotype resulting from the induction of hypoxia-inducible factor 1 $\alpha$  that leads to lactate production during anaerobic glucose metabolism (Gatenby and Gillies, 2004). The pH of solid tumors was observed to be more acidic than that of normal tissues when measured directly with electrodes (Van Den Berg et al., 1982). Hypoxic and acidic tumor microenvironments enable the selection of cells in this unfavorable environment that triggers the transformation from a benign to a malignant phenotype. It has been shown that acidity takes part in chemotherapy resistance, proliferation, and metastatic transformation (Morita et al., 1992; Martinez-Zaguilan et al., 1996; Raghunand et al., 2001).

The acidic tumor microenvironment can have a critical role in chemotherapy resistance (Simon et al., 1994; Mahoney et al., 2003). Some of the mechanisms of chemotherapy resistance resulting from an acidic microenvironment include decreased drug intake, neutralization of weakly basic drugs, and sequestration of drugs into lysosomal vesicles (Simon et al., 1994; Martinez-Zaguilan et al., 1999; Raghunand et al., 1999b, 2003).

Proton pump inhibitors (PPIs) inhibit H<sup>+</sup>/K<sup>+</sup> ATPase function, the last step of acid secretion in parietal cells. The most potent gastric acid secretion-inhibiting PPIs in clinical use are omeprazole, lansoprazole, pantoprazole, rabeprazole, and esomeprazole (Shi and Klotz, 2008). Esomeprazole, an S-enantiomer of omeprazole used in our study, decreases gastric acid secretion with a distinctive mechanism of action (Robinson, 2001). It was shown that PPIs effectively inhibit V-ATPases in vitro, increase chemotherapy efficacy, and are well tolerated in studies with rat and mouse models (Luciani et al., 2004; De Milito et al., 2007, 2010). There is also a phase I clinical study that involves concomitant use of pantoprazole and doxorubicin in advanced solid tumors (Brana et al., 2014).

In this study, it was aimed to investigate whether esomeprazole alters the chemosensitivity of A549, a nonsmall-cell lung carcinoma line, to traditional chemotherapeutic agents used in lung cancer therapy. For this purpose, combinations of esomeprazole with cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, or vinorelbine were used and their cytotoxic effects were investigated with several methods.

## 2. Materials and methods

### 2.1. Cell culture

The A549 human lung cancer cell line was cultured using Ham's F12 without L-glutamine (Pan Biotech,

Aidenbach, Germany) in the presence of 10% newborn calf serum (HyClone, USA), 1% penicillin G (100 U/mL)/streptomycin (100 µg/mL) (HyClone), and 1% L-glutamine (292.3 mg/L) (EuroClone, Italy) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### 2.2. Drugs and experimental groups

All drugs were used at 6.25%, 12.5%, 25%, 50%, 100%, and 200% test drug concentrations (% TDCs) and diluted in cell culture media. A level of 100% TDC is the approximate level of plasma peak concentrations of the chemotherapeutics (Andreotti et al., 1995). Esomeprazole sodium salt (Nexium, AstraZeneca, Turkey) was resuspended in 5 mL of 0.9% NaCl before use and used in 62.5 µM, 125 µM, and 250 µM final concentrations. The anticancer drugs used in the experiments were as follows: cisplatin (100% TDC = 7.6 µg/mL, Cisplatin, Koçak Farma, Turkey), carboplatin (100% TDC = 15.8 µg/mL, Carboplatin, Eczacıbaşı, Turkey), paclitaxel (100% TDC = 13.6 µg/mL, Taxol, Bristol-Myers Squibb, Turkey), docetaxel (100% TDC = 11.3 µg/mL, Taxotere, Sanofi-Aventis, Turkey), gemcitabine (100% TDC = 25 µg/mL, Gemzar, Lilly, Turkey), and vinorelbine (100% TDC = 1.86 µg/mL, Navelbine, Pierre Fabre, Turkey).

### 2.3. The MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed as previously described (Ulukaya et al., 2008). A549 cells were seeded at a density of 5 × 10<sup>3</sup> cells per well of a 96-well plate in 200 µL of culture medium. Two independent experiments, with each run in triplicate, were performed. The cells were treated for 72 h with different concentrations of esomeprazole, and for the combination treatments, the cells were incubated for 72 h with the drugs and esomeprazole. In brief, at the end of the treatment period, 25 µL of MTT solution (5 mg/mL PBS, pH 7.2) was added to each well. Following incubation for 4 h at 37 °C, 100 µL of the solubilizing buffer (10% SDS dissolved in 0.01 N HCl) was added. Viability of treated cells was calculated in reference to the untreated control cells using the following formula: viability (%) = (100 × (sample Abs)/(control Abs)), where Abs is the absorbance value at 570 nm.

### 2.4. Real-time cytotoxicity assay

The xCELLigence system (Roche, Germany) was operated according to the instructions in the user manual. Following the background impedance measurements, A549 cells (2.5 × 10<sup>3</sup> cells in 100 µL) were seeded in each well of an E-Plate 96 (Roche) to increase the final volume to 200 µL. After 60 min of incubation at 37 °C in the cell culture incubator, the E-Plate 96 was placed into the system. Adhesion, growth, and proliferation of the cells was monitored every 60 min for up to 96 h via the incorporated sensor electrode arrays of the E-Plate 96. Twenty-four hours after seeding, 100 µL of medium was removed from the E-Plate 96 wells without

damaging the cells. The cells were then exposed to 100  $\mu\text{L}$  of medium containing different doses of cisplatin or carboplatin alone (6.25%–100% TDC) or combined with esomeprazole (62.5–250  $\mu\text{M}$ ). Only 100  $\mu\text{L}$  of medium was added to wells containing control cells. All experiments were run for 96 h and a time-dependent cell index graph was produced by the device using the real-time cytotoxicity assay software of the manufacturer.

### 2.5. Detection of caspase-cleaved cytokeratin 18 (M30)

Apoptosis was assayed by measuring the level of caspase-cleaved keratin 18 (ccK18, M30) with a commercially available immunoassay kit (M30-Apoptosense ELISA kit, Peviva AB, Sweden) according to the manufacturer's instructions. In the M30 ELISA assay, samples react with the solid-phase capture antibody M5 and the HRP-conjugated M30 antibody, which are directed against K18 and the K18Asp396 neoepitope, respectively. The neoepitope was exposed after caspase cleavage of K18 after aspartic acid residue 396 (Leers et al., 1999). Cleavage at this position occurs early during apoptosis by caspase 9 and during the execution phase by caspase-3 and -7 (Schutte et al., 2004). Cells were seeded at a rate of  $1 \times 10^4$  per well of a 96-well plate in 200  $\mu\text{L}$  of culture medium in triplicates. Cells were treated for 48 h with esomeprazole (250  $\mu\text{M}$ ), carboplatin (100% TDC), and cisplatin (100% TDC) alone and carboplatin or cisplatin in combination with esomeprazole. Two independent experiments were carried out in triplicates. At the end of the treatment period, the cells were lysed with 10% NP-40 for 10 min on a shaker. The content of identical wells was pooled and centrifuged at 2000 rpm for 10 s to remove the debris. All samples were placed into wells coated with a mouse monoclonal antibody as a catcher. After washing, a horseradish peroxidase-conjugated antibody (M30) was used for detection. The absorbance was determined with an ELISA reader at 450 nm (FLASH Scan S12, Analytik Jena, Germany) (Leers et al., 1999; Ueno et al., 2003).

### 2.6. Fluorescence microscopy

When apoptosis occurs, phosphatidylserine molecules translocate to the outside of the cell membrane, which is an early event in apoptotic cells. Annexin-V-FITC can bind to phosphatidylserine, allowing the apoptotic cells to be visible. A549 cells were seeded in a 96-well plate at the density of  $1 \times 10^4$  cells per well. The cells were treated with 100% TDC cisplatin and carboplatin alone, 250  $\mu\text{M}$  esomeprazole alone, and its combination with 100% TDC cisplatin or carboplatin for 12 h. After treatment, the cells were stained with Annexin-V-FITC and propidium iodide (PI) using the Annexin-V-FLUOS staining kit (Roche). Annexin-V-FITC and PI were diluted (1:50) from stock solution with incubation buffer to yield a working solution. Hoechst dye 33342 (5  $\mu\text{g}/\text{mL}$  final concentration) was added to this solution as well to observe all alive and dead

cells. The cells were then incubated for 30 min at room temperature after aspirating the medium and the addition of 50  $\mu\text{L}$  of working solution to each well. Apoptotic cells were visualized under a fluorescence microscope.

### 2.7. SDS-PAGE and western blotting

A549 cells were seeded in 6-well plates ( $7.5 \times 10^5$  cells/well) and incubated with different doses of esomeprazole (62.5  $\mu\text{M}$ , 125  $\mu\text{M}$ , 250  $\mu\text{M}$ ) and cisplatin (100% TDC) or carboplatin (100% TDC) combinations for 24 h. Cells were scraped at the end of the treatment and washed with ice-cold PBS. The cells were then lysed in RIPA lysis buffer (Santa Cruz Biotechnology Inc., USA) containing protease inhibitors. The proteins were extracted at 4 °C for 30 min and centrifuged at 4 °C for 10 min at 10,000  $\times g$ . Equal amounts of protein (30  $\mu\text{g}$  protein/lane) were subjected to 4%–12% gradient gel SDS-PAGE and then transferred to a nitrocellulose membrane. Western blotting was performed using rabbit anti-PARP monoclonal antibody (1:1000 dilution; Cell Signaling, USA) and rabbit anti- $\beta$ -actin monoclonal antibody (1:1000 dilution; Cell Signaling). HRP-linked antirabbit IgG antibodies (1:2000 dilution; Cell Signaling) and LumiGLO reagent and peroxide (Cell Signaling) were used to detect primary antibodies according to the manufacturer's instructions. The membrane was stripped for subsequent detection. Bound antibodies were visualized on a Fusion FX-7 imaging device (Vilber Lourmat, France). The bands were quantified by using ImageJ 1.49v software.

### 2.8. Statistical evaluation

SPSS 22.0 was used for the statistical analysis. Comparison of groups for each dose was examined by one-way analysis of variance. Dunnett's multiple comparison test was used for testing the presence of statistical significance. Differences were considered to be statistically significant when  $P \leq 0.05$ .

Combination index (CI) values were calculated using CalcuSyn Version 2.1 according to the Chou-Talalay method for drug combinations (Chou, 2010). The CI is a parameter that gives information about the effectiveness of drug combinations. Combination effects are defined as very strong synergism ( $CI < 0.1$ ), strong synergism ( $0.1 < CI < 0.3$ ), synergism ( $0.3 < CI < 0.7$ ), moderate synergism ( $0.70 < CI < 0.85$ ), slight synergism ( $0.85 < CI < 0.90$ ), nearly additive ( $0.9 < CI < 1.1$ ), and antagonistic ( $CI > 1.1$ ) (Chou, 2006).

## 3. Results

### 3.1. Cytotoxic activities of esomeprazole and chemotherapeutics by the MTT assay

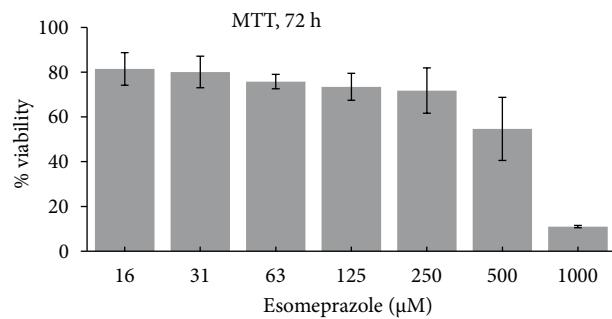
The cytotoxic effect of esomeprazole was investigated by employing the MTT assay on A549 cells. We found that esomeprazole treatment exhibited antigrowth effects in

a dose-dependent manner at most of the doses (Figure 1). The dose of esomeprazole that inhibited 50% of the cell proliferation ( $IC_{50}$ ) corresponded approximately to 500  $\mu$ M. A sublethal dose, 250  $\mu$ M, was selected for combination treatments since 500  $\mu$ M might be considered a suprapharmacological concentration. Drugs that are frequently used in the treatment of lung cancer (cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, vinorelbine) were combined with esomeprazole. It was demonstrated that only carboplatin and cisplatin combinations with esomeprazole led to apparent and significant increases in cytotoxicity. On the other hand, esomeprazole combinations with paclitaxel, docetaxel, gemcitabine, and vinorelbine did not produce any cytotoxic activity. Hence, only carboplatin, cisplatin, and their combinations with esomeprazole were used for further experiments.

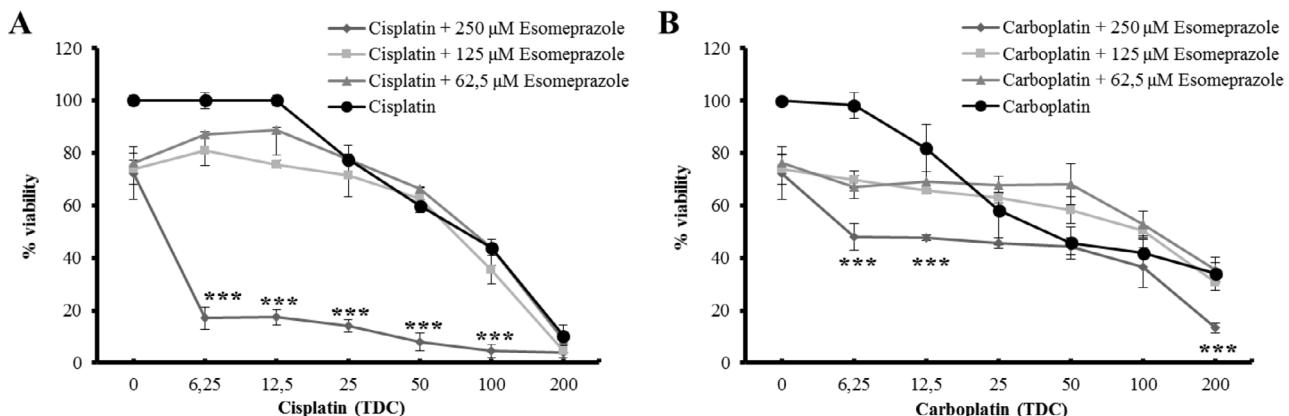
Cell viability was measured with the MTT assay. It was found that both carboplatin and cisplatin combinations with esomeprazole decreased cell viability in a dose-dependent manner (Figure 2). The decrease in % viability

was statistically significant at low doses of carboplatin and cisplatin when combined with 250  $\mu$ M esomeprazole ( $P < 0.001$ ). For the lowest dose of cisplatin, 6.25 TDC, combination with 250  $\mu$ M esomeprazole decreased % viability dramatically whereas cisplatin alone and its combinations with 62.5 and 125  $\mu$ M esomeprazole were ineffective (Figure 2). The growth inhibitory effects of 250  $\mu$ M esomeprazole + carboplatin were significant at 6.25, 12.5, and 100 TDC. However, carboplatin combinations with 62.5 and 125  $\mu$ M esomeprazole did not result in a higher efficacy (Figure 2).

We further examined if there were any synergistic, additive, or antagonistic interactions between esomeprazole and cisplatin or carboplatin. As we expected, the resulting interaction in 250  $\mu$ M esomeprazole combination with cisplatin and carboplatin was synergism at all doses except the 250  $\mu$ M esomeprazole + 100% TDC carboplatin combination, which was an additive interaction (Table). In contrast, there was no synergistic interaction in 62.5 and 125  $\mu$ M esomeprazole + cisplatin or carboplatin treatments (data not shown).



**Figure 1.** A549 cells were incubated for 72 h with various concentrations of esomeprazole and the % viabilities of the cells were assayed using the MTT assay.



**Figure 2.** The % viability is shown after cisplatin and carboplatin treatments with or without esomeprazole for 72 h by using MTT assay on A549 cell line. (A) Cisplatin and esomeprazole combination, \*\*\*  $P < 0.001$  when compared to cisplatin treatment alone. (B) Carboplatin and esomeprazole combination, \*\*\*  $P < 0.001$  when compared to carboplatin treatment alone.

**Table.** Combination index values were calculated for 250  $\mu$ M esomeprazole in combination with various concentrations of cisplatin and carboplatin.

	Treatment	Combination index
250 $\mu$ M esomeprazole	6.25% TDC cisplatin	0.04 $\pm$ 0.01
	12.5% TDC cisplatin	0.08 $\pm$ 0.01
	25% TDC cisplatin	0.15 $\pm$ 0.02
	50% TDC cisplatin	0.20 $\pm$ 0.05
	100% TDC cisplatin	0.28 $\pm$ 0.10
	200% TDC cisplatin	0.54 $\pm$ 0.17
	6.25% TDC carboplatin	0.16 $\pm$ 0.05
	12.5% TDC carboplatin	0.24 $\pm$ 0.01
	25% TDC carboplatin	0.37 $\pm$ 0.04
	50% TDC carboplatin	0.65 $\pm$ 0.09

### 3.2. Monitorization of cell death by a real-time cytotoxicity assay

The cytotoxic effects of carboplatin and cisplatin combined with three different doses of esomeprazole were assessed in real time for 72 h by analyzing impedance produced by the A549 cells (Figure 3). All doses of cisplatin had antiproliferative effects except 100 TDC since only at this dose was the final cell index value lower than the initial value (cytotoxic effect). The combination with esomeprazole resulted in an increase in antiproliferative effects of cisplatin at lower doses. Although esomeprazole itself had antiproliferative effects at 250  $\mu$ M, the cell index after 72 h was the same as the initial index. However, its combination with cisplatin caused cytotoxic effects even with doses as low as 25% TDC. Treatment alone with carboplatin resided in an antiproliferative range at all doses after 72 h. Similar to its combination with cisplatin, esomeprazole increased the antiproliferative effects of carboplatin at lower doses and 50% TDC and 100% TDC combinations of carboplatin with 250  $\mu$ M esomeprazole demonstrated cytotoxic activity.

### 3.3. Assessment of apoptosis by M30 assay

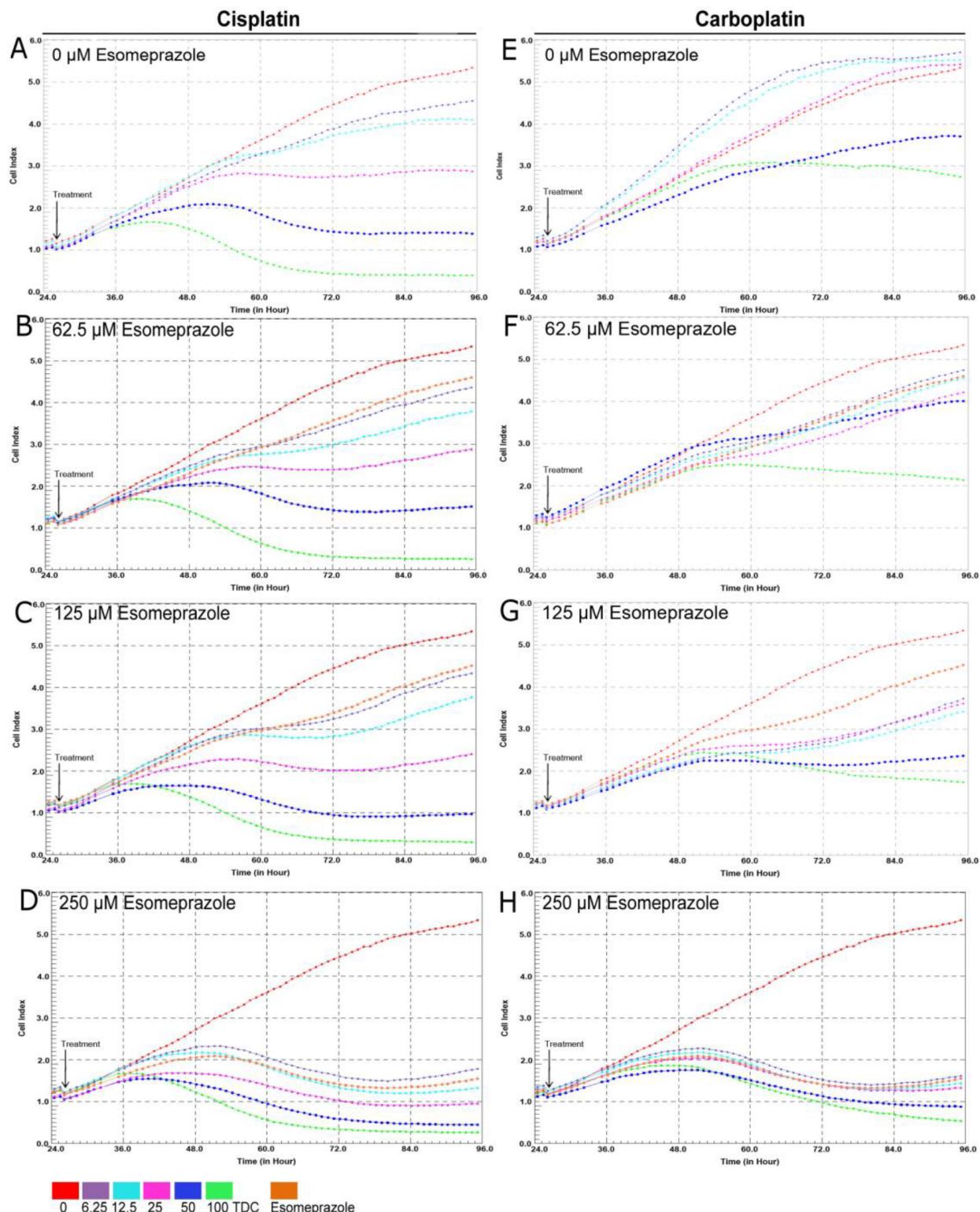
Levels of M30 (an apoptosis marker) were measured in the A549 cell line after 48 h of treatment with 100% TDC cisplatin and 100% TDC carboplatin alone and their combination with 250  $\mu$ M esomeprazole to determine the cell death mode. Cisplatin, esomeprazole + cisplatin, and carboplatin increased M30 levels, suggesting cell death by apoptosis. In contrast, no increase was observed in M30 levels in the esomeprazole + carboplatin combination (Figure 4).

### 3.4. Fluorescence microscopic examination of cell death

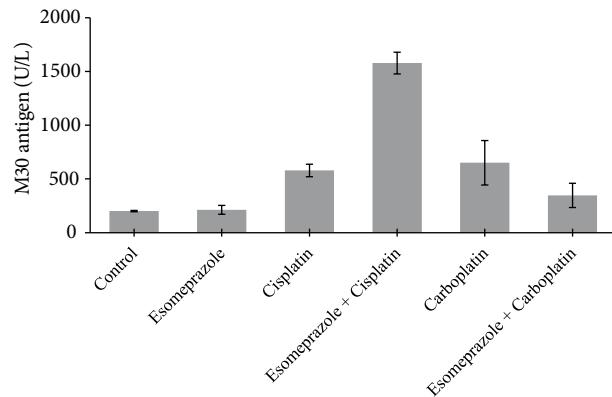
We examined the effects of carboplatin and cisplatin combination with esomeprazole microscopically to determine cell death mode. We did not observe remarkable Annexin-V-FITC staining after cisplatin and carboplatin treatments alone (data not shown). However, there was Annexin-V-FITC positivity in 250  $\mu$ M esomeprazole-treated cells reflecting the partial proapoptotic effect (Figure 5). Both Annexin-V-FITC and PI stainings were observed in 250  $\mu$ M esomeprazole combinations of 100% TDC cisplatin and 100% TDC carboplatin. Pyknotic nuclei were also observed in Hoechst 33342 staining in both combinations, although a higher fraction of nuclei were pyknotic in esomeprazole + cisplatin. The presence of PI in cells with pyknotic nuclei suggests that these cells had already lost their membrane integrity and underwent secondary necrosis, which is considered as a late-stage event in apoptosis (Figure 5).

### 3.5. SDS-PAGE and western blotting for confirmation of apoptosis

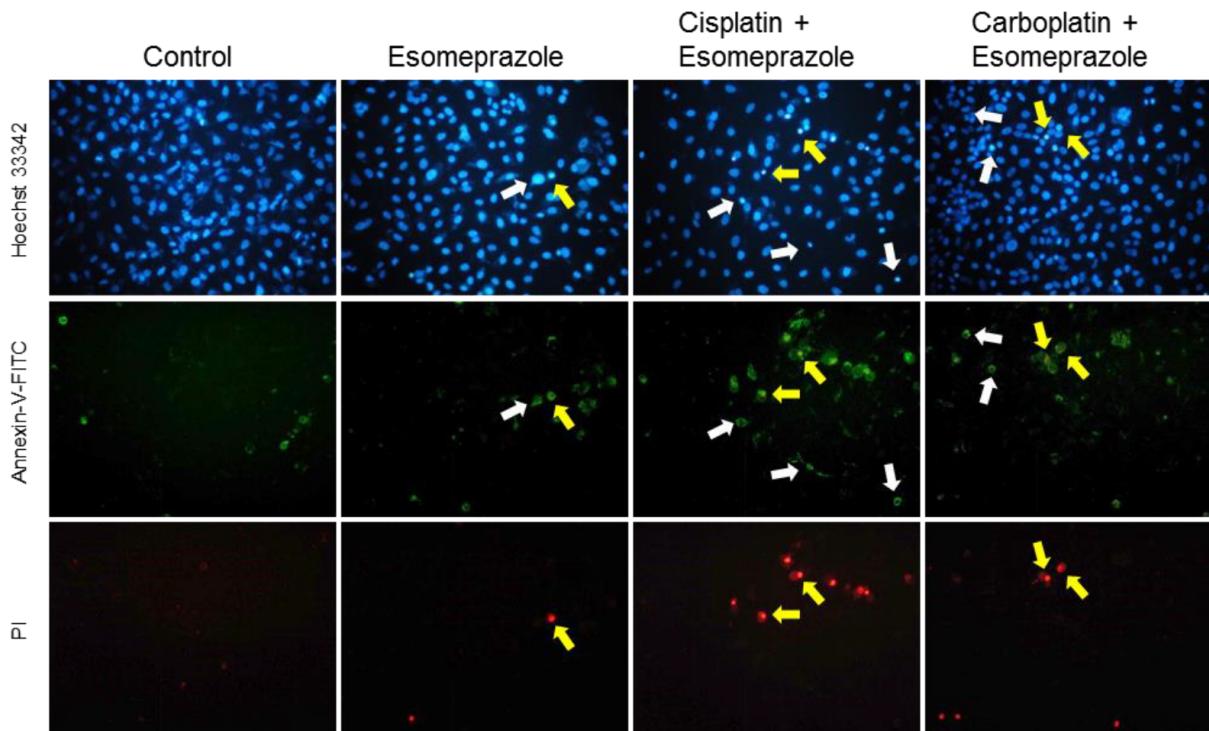
PARP cleavage is considered one of the hallmarks of apoptosis. Therefore, we performed western blotting in order to determine whether PARP was cleaved after carboplatin and cisplatin treatments and their combination with esomeprazole. Our aim was to clarify the cell death mode in carboplatin or cisplatin combinations with esomeprazole, as PARP cleavage can be considered to be a molecular marker of apoptosis. The results demonstrated in Figure 6 were obtained from the same membrane. Esomeprazole and carboplatin treatments alone caused a slight cleavage of PARP. However, cisplatin alone and



**Figure 3.** Cell viability was monitored for 72 h with real-time cytotoxicity assay. A549 cells were incubated with cisplatin alone (A), cisplatin in combination with increasing doses of esomeprazole (B-D), carboplatin alone (E), and carboplatin in combination with increasing concentrations of esomeprazole (62.5, 125, and 250 µM) (F-H). Note the shift from antiproliferative effect to cytotoxic effect of the lower doses of drugs when used in combination with 250 µM esomeprazole.



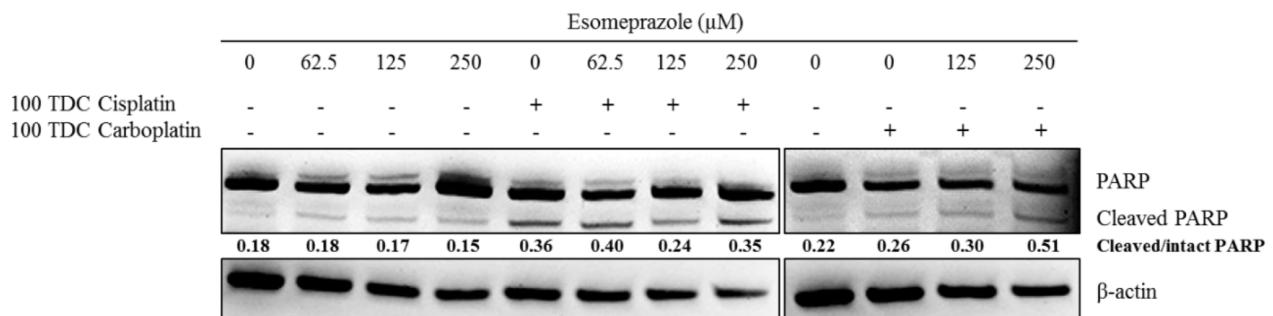
**Figure 4.** M30 levels (U/L) were measured 48 h after treatment with cisplatin (100% TDC), carboplatin (100% TDC), and their combinations with esomeprazole (250  $\mu$ M). M30 detection was performed in the cell culture medium by ELISA as explained in Section 2. Note the apoptosis-inducing effect of cisplatin, but not of carboplatin, which may induce another type of cell death modality.



**Figure 5.** Fluorescence images of cells were taken after staining with Hoechst dye 33342 (upper row), Annexin-V-FITC (middle row), and PI (lower row) following treatment with esomeprazole (250  $\mu$ M), cisplatin (100% TDC) + esomeprazole (250  $\mu$ M) combination, and carboplatin (100% TDC) + esomeprazole (250  $\mu$ M) combination for 12 h. White arrows denote early-stage apoptotic nuclei while yellow arrows denote late-stage apoptotic nuclei. Thus, a mixture of early- and late-stage apoptotic nuclei is seen in the treated population.

its combination with esomeprazole clearly resulted in PARP cleavage. Cleaved PARP levels did not change in carboplatin and esomeprazole combinations despite there being a slight increase in the 250  $\mu$ M esomeprazole

combination that might be attributed to the combined effects of individual carboplatin and esomeprazole treatments.



**Figure 6.** Changes in cleaved PARP levels determined by western blotting 24 h after 100% TDC cisplatin or carboplatin treatments in combination with increasing concentrations of esomeprazole (62.5, 125, and 250  $\mu$ M).  $\beta$ -Actin was used as the loading control.

#### 4. Discussion

The studies that revealed the presence of a distinct pH gradient between the intracellular and extracellular medium and the major role of the acidic microenvironment and acidic vesicles in the resistance of tumor cells to cytotoxic drugs brought forward the idea to increase chemosensitivity by changing the conditions of tumor microenvironment (Tannock and Rotin, 1989; Simon et al., 1994; Altan et al., 1998; Martinez-Zaguilan et al., 1999; Raghunand et al., 1999a, 1999b; Izumi et al., 2003; Mahoney et al., 2003). Our hypothesis was based on overcoming the pH gradients that were demonstrated as one of the reasons for chemoresistance with the usage of PPIs. One of the PPIs, esomeprazole, was used in this study to inhibit V-ATPases that are known to be highly expressed in tumor cells (Tannock and Rotin, 1989; Martinez-Zaguilan et al., 1999; Nishi and Forgac, 2002; Torigoe et al., 2002; Sennoune et al., 2004). There are examples of clinical trials aiming to increase the effectiveness of chemotherapy via a combination of a basic drug such as doxorubicin and a PPI. Pantoprazole in combination with doxorubicin was concluded to be feasible in a phase I trial in various solid tumors not including lung cancer (Brana et al., 2014).

Real-time cytotoxicity data were obtained from the xCELLigence system that gives information about whether a compound has antiproliferative, cytostatic, or cytotoxic effects at a particular dose and time point. In general, 62.5  $\mu$ M and 125  $\mu$ M esomeprazole combinations with carboplatin and cisplatin modified the antiproliferative effects of these drugs slightly. Importantly, despite the 250  $\mu$ M concentration of esomeprazole being cytostatic alone, its combination with cisplatin demonstrated cytotoxic effects at doses as low as 25% TDC. Combination with 250  $\mu$ M esomeprazole also modified the cytostatic effect of 50% TDC cisplatin treatment alone to become cytotoxic. Such a similar potentiation effect was observed when cisplatin was used in combination with another PPI, bafilomycin (Murakami et al., 2001). Using a lower dosage in carboplatin and cisplatin combinations with

esomeprazole might have implications in preventing the side effects of these therapeutics. Furthermore, the presence of a synergistic interaction at especially low-dose combinations of 250  $\mu$ M esomeprazole and cisplatin or carboplatin supports this approach. Cisplatin in combination with 250  $\mu$ M esomeprazole generated very strong or strong synergism in the 6.25%–100% TDC dose range (Chou, 2006). Taken together, combinations of 250  $\mu$ M esomeprazole and 25%–100% TDC cisplatin would be both cytotoxic and strongly synergistic.

Esomeprazole in combination with paclitaxel, docetaxel, gemcitabine, and vinorelbine did not show cytotoxic effects in the A549 lung cancer cell line. However, apparent and statistically significant cytotoxic effects were observed in combinations of cisplatin and carboplatin. In a recent phase II clinical trial conducted with metastatic breast cancer patients, it was proven that esomeprazole enhanced the effects of docetaxel followed by cisplatin combination treatment and improved the overall survival without any toxic effects (Wang et al., 2015). Luciani et al. (2004) showed a significant increase in chemosensitivity when omeprazole, another PPI, was used 24 h prior to treatment with chemotherapeutic agents in solid tumor cell lines other than lung cancer (22 melanoma, 2 colon adenocarcinoma, 2 breast cancer, 2 ovarian cancer, 1 endometrium cancer, and 1 T-lymphoblastoid cell lines). In contrast to our study, drug sensitivity disappeared when omeprazole was used simultaneously with chemotherapeutic agents. This finding suggests that there might be differences between cancer types and chemotherapeutic agents regarding the V-ATPase response to PPIs.

An increase in cell death was observed in combinations of weakly basic chemotherapeutic agents, cisplatin and carboplatin, with esomeprazole. However, the same outcome was not observed in combinations with other weakly basic therapeutics, namely gemcitabine and vinorelbine, which suggests that the enhanced cytotoxicity observed in cisplatin and carboplatin does

not solely depend on acidic or basic properties of the chemotherapeutic agents (Luciani et al., 2004; Booker et al., 2014).

After demonstrating the cell death-inducing effect of esomeprazole combination with cisplatin and carboplatin, we tried to determine the cell death mode by measuring cleaved cytokeratin 18 (M30) levels by using ELISA. Cisplatin alone and in combination with esomeprazole elevated M30 levels, suggesting apoptosis. The M30 increase in carboplatin treatments suggests apoptotic cell death; however, the carboplatin combination with esomeprazole lowered M30 levels, which illustrates a shift from apoptosis to another cell death modality. Western blotting confirmed the findings in the M30 assay for cisplatin and esomeprazole combinations with higher M30 levels in combination treatments corresponding to a higher amount of cleaved PARP, both suggesting enhanced apoptosis in cisplatin and esomeprazole combinations. On the other hand, PARP cleavage also occurred in carboplatin and esomeprazole combinations, although it was not as pronounced as in cisplatin combinations.

We performed fluorescence microscopy to decide conclusively about the cell death mode in both cisplatin and carboplatin combinations with 250  $\mu$ M esomeprazole. An early apoptotic event, translocation of phosphatidylserine to the extracellular layer of the cell membrane, was visualized by Annexin-V-FITC staining 12 h after

treatment. Annexin-V-FITC staining and the presence of pyknotic nuclei in both combinations supported apoptotic cell death. PI positivity in the majority of the cells also indicated that secondary necrosis following apoptosis had predominantly occurred at this time point. Thus, the findings from fluorescence microscopy support the results obtained from western blotting for cisplatin and carboplatin combinations with 250  $\mu$ M esomeprazole to trigger apoptotic cell death, but pyknosis and Annexin-V positivity was much more apparent with the combination of cisplatin and esomeprazole. This finding, combined with the unaffected M30 levels after carboplatin and esomeprazole combination, leads to the conclusion that apoptosis might not be the only cell death modality induced by carboplatin and esomeprazole combination.

The results of this study indicate that esomeprazole increases the sensitivity of tumors to chemotherapeutics and can protect from the side effects arising because of high doses of chemotherapeutics. Further *in vivo* investigation is needed to elucidate the mechanisms by which cisplatin and esomeprazole combinations can distinctively lead to a stronger cytotoxic activity in the A549 nonsmall-cell lung cancer cell line and other lung cancer cell lines.

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