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TITLE PAGE

Title:

UCP2 inhibition sensitizes breast cancer cells to therapeutic agents by increasing oxidative stress.

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ABSTRACT

Modulation of oxidative stress in cancer cells plays an important role in the study of the resistance to anticancer therapies. Uncoupling protein 2 (UCP2) may play a dual role in cancer, acting as a protective mechanism in normal cells, while its over-expression in cancer cells could confer resistance to chemotherapy and a higher survival through down-regulation of ROS production. Thus, our aim was to check whether the inhibition of UCP2 expression and function increase oxidative stress and could render breast cancer cells more sensitive to cisplatin (CDDP) or tamoxifen (TAM). For this purpose, we studied clonogenicity, mitochondrial membrane potential (ΔΨm), cell viability, ROS production, apoptosis and autophagy in MCF-7 and T47D (only the last four determinations) breast cancer cells treated with CDDP or TAM, in combination or without a UCP2 knockdown (siRNA or genipin). Furthermore, survival curves were performed in order to check the impact of UCP2 expression in breast cancer patients. UCP2 inhibition and cytotoxic treatments produced a decrease in cell viability and clonogenicity, in addition to an increase in ΔΨm, ROS production, apoptosis and autophagy. It is important to note that CDDP decreased UCP2 protein levels, so that the greatest effects produced by the UCP2 inhibition in combination with a cytotoxic treatment, regarding to treatment alone, were observed in TAM+UCP2siRNA-treated cells. Moreover, this UCP2 inhibition caused autophagic cell death, since apoptosis parameters barely increased after UCP2 knockdown. Finally, survival curves revealed that higher UCP2 expression corresponded with a poorer prognosis. In conclusion, UCP2 could be a therapeutic target in breast cancer, especially in those patients treated with tamoxifen.

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Keywords: *Oxidative stress, UCP2, ROS, cancer, cisplatin, tamoxifen, autophagic cell death, resistance.*

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INTRODUCTION

Increased oxidative stress plays a crucial role in a variety of pathologic conditions including cancer [1]. Cancer cells often exhibit high levels of intracellular reactive oxygen species (ROS) with complex and controversial biological effects. Previous studies in our lab have demonstrated the influence of ROS in breast cancer induction and progression [2-4]. ROS induce DNA damage, contributing to DNA instability and mutation, which in turn promotes cancer cell growth and survival [5]. However, excessive ROS levels can lead to cell growth arrest, senescence and cell death [5, 6]. Therefore, the effective regulation of endogenous and treatment-induced oxidative stress is an important factor to consider in both tumor development and the responses to anticancer therapies [7, 8] such as cisplatin (CDDP) or tamoxifen (TAM) treatments.

Several studies have suggested that mitochondrial uncoupling is a major mechanism in the regulation of oxidative stress [9-11]. Uncoupling proteins (UCPs) are a family of inner mitochondrial membrane proteins whose function is to allow the reentry of protons into the matrix, dissipating the proton gradient and, therefore, decreasing mitochondrial membrane potential and ROS production [9, 11]. It has been shown that UCPs are up-regulated in response to oxidative stress, acting as a feed-back mechanism to control ROS levels, suggesting that UCPs act as part of the antioxidant systems in the cell [10, 12].

Specifically, the role of uncoupling protein-2 (UCP2) in cancer has recently been recognized and has attracted more attention. Derdak *et al.* found that UCP2-null mice developed more colon tumors than the wild-type controls with increased oxidative stress [13]. On the other hand, the same authors found that that UCP2 promotes chemoresistance in colon cancer cells [14]. Consequently, UCP2 may have a dual role in cancer, acting as a protective mechanism in normal cells, while its over-expression in cancer cells may confer resistance to chemotherapy and a higher survival by the down-regulation of ROS levels [15].

This dual regulation of UCP2 expression has been also reported in breast cancer. Previous results in our group demonstrated that repression of UCP2 by estrogens may play a key role in estrogen-induced breast carcinogenesis [2, 4]. On the other hand, we

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found that breast tumors adapted to oxidative stress showed an increase in uncoupling proteins [3]. In addition, we also observed that ovarian cancer patients were more resistant to carboplatin/paclitaxel treatment when they had lower levels of antioxidant systems, including UCP2 and UCP5, prior to be treated, suggesting that these patients could increase their UCPs levels to counteract the increase of ROS levels produced by these treatments [16]. It is worthy to note that tumor aggressiveness is associated with the ability to acquire higher antioxidant defense [6, 7]. These studies, in addition to the association between UCP2 and tumor grade [17, 18], suggest that UCP2 over-expression in cancer progression could be a result of a long-term selecting procedure.

Recently, several studies have demonstrated that UCP2 is involved not only in cancer cell transformation, but also in chemoresistance [19-21]. In fact, in pancreatic cancer cells, mitochondrial uncoupling by UCP2 is a mechanism of resistance to the chemotherapeutic drug gemcitabine, working through the negative regulation of mitochondrial ROS production [19]. Moreover, UCP2 inhibition sensitized multidrug resistance acute promyelocytic leukemia cell lines to cytotoxic agents [21]. This evidence suggests that UCP2 targeting may be a novel therapeutic strategy for cancer in combination with drugs that promote oxidative stress.

To approach this aim, we analyzed whether inhibition of UCP2 by siRNA or the specific inhibitor genipin [21] increase oxidative stress and could render breast cancer cells more sensitive to cytotoxic agents. Specifically, we treated the cells with CDDP, one of the most widely used chemotherapeutic anticancer agents in many cancer types [22, 23], and TAM, which has been widely used for more than 30 years in breast cancer treatment and prevention [24]. Although the cytotoxic effects of CDDP are primarily mediated by the generation of nuclear DNA adducts [25] and TAM is an antagonist of the estrogen receptor (ER) [26]; CDDP targets the mitochondria, which stimulates the ROS production that contributes to its genotoxic effects [27], while ROS production is also involved in TAM cytotoxicity [8]. Therefore, the development of anti-cancer therapies based on UCP2 inhibition associated with traditional chemotherapeutic treatments could improve treatment efficacy, resulting in a higher oxidative stress and cancer cell death.

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MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's medium (DMEM) high glucose was from GIBCO (Paisley, UK). Genipin (methyl-2-hydroxy-9-hydroxymethyl-3-oxabicyclonona-4,8-diene-5-carboxylate), cisplatin (*cis-diamminedichloroplatinum II or CDDP)* and tamoxifen *(trans-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine or TAM)* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Routine chemicals used were obtained from Sigma-Aldrich, Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture

The MCF-7 and T47D human breast cancer cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and routinely cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were seeded in plates such that they were confluent when doing the measurements.

Cell transfection and treatments

For transfection, cells were cultured in 6-well or in 96-well culture plates overnight and then, at 60% confluence, transfected for 6 hours with a specific small interfering (si)RNA targeting UCP2 mRNA purchased in Santa Cruz Biotechnology (UCP2 siRNA (h): sc-42682). Lipofectamine 2000 reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) was used to perform cell transfection according to the manufacturer's protocol. After transfection the complexes were removed and cells were provided with normal growth medium. The day after, cells were exposed to vehicle (0.1% DMSO), 10 µM CDDP or 10 µM TAM for 48h.

For UCP2 inhibition, cells were exposed to 0-250 µM genipin for 24h prior the determination of the parameters.

Cell viability assay

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MCF-7 or T47D cells were seeded in 96-well plates and incubated overnight. The following day, cells were transfected with UCP2 siRNA or treated with genipin (0-200 µM), and cell density was measured over time (0-72 h) by crystal violet assay. Cytotoxic treatments were applied one day after transfection with UCP2 siRNA, when cells were exposed to the vehicle, CDDP and TAM for 48h, and cell viability was determined. Briefly, cells were stained with 0.5% (p/v) crystal violet in 30% (v/v) acetic acid for 10 min. After washing, the dye was solubilized in 100 µl of methanol and absorbance was measured photometrically (A595nm) using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) to determine cell viability.

Fluorimetric determination of H_2O_2 production (ROS production)

ROS production was measured fluorimetrically by using an Amplex® Red Hydrogen Peroxide/Peroxidase assay kit (Molecular Probes). MCF-7 or T47D cells were seeded in 96-well plates, and, the day after, incubated with UCP2 siRNA or genipin. For cytotoxic treatments, the day after UCP2 inhibition by siRNA, cells were exposed for 48h to the vehicle, CDDP and TAM. To chemically inhibit UCP2, cells were treated with different concentrations of genipin (0-250 µM) for 24h. Briefly, cells were exposed to 50 µM of Amplex Red reagent and 0.1 U/ml of horseradish peroxidase, in Krebs-Ringer buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4. Fluorescence was measured with an FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA), set at excitation and emission wavelengths of 571 and 585 nm, to detect the maximum slope of increment in the fluorescence within 1 hour of exposure to kit reagents. Thus, the measurement obtained is the H_2O_2 produced (related to ROS production) by the cells for one hour. Values were normalized per number of viable cells determined by crystal violet assay.

Mitochondrial membrane potential and autophagy

Mitochondrial membrane potential ($\Delta\Psi m$) and autophagy were measured fluorimetrically by using Tetramethylrhodamine methyl ester (TMRM) and Monodansyl cadaverine (MDC), respectively. MCF-7 or T47D cells (the latter only in MDC determination) were seeded in 96-well plates and, the next day, incubated with UCP2

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siRNA. The day after UCP2 inhibition by siRNA, cells were exposed for 48h to the vehicle, CDDP and TAM. Fluorescence measurement was performed in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 552 and 576 nm (ΔΨm by TMRM, 100 nM, 15 minutes of incubation), and 340 and 535 nm (autophagy by MDC, 50 µM, 15 minutes of incubation). Values were normalized per number of viable cells determined by crystal violet assay.

Apoptosis assay

Apoptosis was measured fluorimetrically by using Annexin V method, as described by Dando et al. [28]. Briefly, MCF-7 or T47D cells were seeded in 96-well plates and treated as mentioned in the previous section. At the end of the treatment, cells were fixed with 2% paraformaldehyde (BD Biosciences, NJ, USA) in PBS at room temperature for 30 min and washed twice with PBS. Cells were then stained with AnnexinV/AlexaFluor350 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) in annexin binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaOH, and 2.5 mM $CaCl_2$) for 10 min at room temperature in the dark. To finish, cells were washed once with annexin binding buffer. Florescence was measured in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 346 and 442 nm, with cells kept in 100 µl of annexin binding buffer. Values were normalized per number of viable cells determined by crystal violet assay.

Determination of cell survival using colony formation

For clonogenic assay, MCF-7 breast cancer cells were plated in 6-well plates and transfected the day after with UCP2 siRNA for 6h. Afterwards, the complexes were removed and cells were provided with normal growth medium. The following day, cells were exposed to the vehicle, CDDP or TAM for 48h. After removal of the medium containing cytotoxic agents, cells were trypsinized and plated at low density (5×10^3 cells per 60-mm plate). Cells were cultured for 14 days, with a change of the culture medium three times a week, and colonies were stained with crystal violet and counted for each condition at the end of the culture period.

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Real-time quantitative PCR

After transfection with UCP2 siRNA, total RNA was isolated from MCF-7 and T47D cultured cells by using TriPure® isolation reagent (Roche, Barcelona, Spain) following the manufacturer's protocol and then quantified using a spectrophotometer set at 260 nm. 1 µg of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10 µl volume of retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 µM random hexamers, 10 U RNase inhibitor, and 500 µM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−80°C) until the PCR reactions were carried out.

PCR was performed in triplicate samples by SYBR Green technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). The primers used were forward 5'-ggACACggACAggATTgACA-3' and reverse 5'-ACCCACggAATCgAgAAAgA-3' for the 18S ribosomal RNA gene, and forward 5'-ggTggTCggAgATACCAAAg-3' and reverse 5'- CTCgggCAATggTCTTgTAg-3' for the UCP2 gene. Total reaction volume was 10 µL, containing 7.5 µL Lightcycler® 480 SYBR Green I Master (containing 0.5 µM of the sense and antisense specific primers) and 2.5 µl of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95 °C), followed by 45 cycles consisting of a denaturation step (10 s, 95 °C), an annealing step (10s, 61 °C for 18S and 60 °C for UCP2), and an extension step (12s, 72 °C min). A negative control lacking cDNA template was run in each assay.

The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

Western blot analysis

MCF-7 cells were seeded in 6-well plates and transfected with UCP2 siRNA the following day for 6h. Complexes were then removed and cells were provided with normal growth medium, grown overnight and were treated the next day with vehicle,

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CDDP or TAM. Cell protein extracts were obtained scraping cells with 200 µl of RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 1 mM EDTA, 1mM NaF, 1 mM Na_3VO_4, 10 µM leupeptin and 10 µM pepstatin; finally, 1 mM PMSF was added just before harvesting the cells with the scraper). The lysate was sonicated three times at a 40% amplitude for 7 seconds. Then samples were centrifuged at 14000×*g* for 10 min at 4°C and protein content (supernatant) was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). A 20 µg protein aliquot from the cell lysate was separated on a 10% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. Membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20) for 1h. Antisera against UCP2 and Tubulin (Santa Cruz Biotechnology, CA, USA), and PARP and LC3A/B (Cell Signaling Technology Inc, Danvers, MA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

Measurement of 4-HNE adducts and carbonyl contents

MCF-7 cells were transfected with UCP2 siRNA and total protein extracted as previously described for *Western blot analysis.* For the analysis of 4-hydroxy-2-nonenal (4-HNE) and carbonyl groups, 40 µg and 10 µg of protein from cell lysate, respectively, were separated on a 10% SDS–PAGE gel and electrotransferred onto nitrocellulose membrane. Protein carbonyls were detected using the immunological method OxySelect™ Protein Carbonyl Immunoblot kit (Cell Biolabs, San Diego, CA, USA). For derivatization of carbonyl groups, the membrane was incubated with 2, 4-dinitrophenylhydrazine (DNPH) for 5 min, and then processed according to the manufacturer’s instructions. Unspecific binding sites on the membranes were blocked in 5% non-fat milk in TBS-T (Tris-buffered saline-with 0.05% Tween-20). Antiserum against 4-HNE (Alpha Diagnostic International, San Antonio, TX, USA) and DNP were used as primary antibodies. Bands were visualized using the Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad). The chemiluminescence signal was captured with a

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Chemidoc XRS densitometer (Bio-Rad Laboratories) and results were analyzed with Quantity One software (Bio-Rad Laboratories).

Kaplan-Meier survival curves

Kaplan-Meier plots were made using an online (www.kmplot.com) Kaplan-Meier plotter dataset [29], selecting the KM plotter for breast cancer. The analysis was performed taking into account the relapse-free survival (RFS) or the overall survival (OS) of patients. The patients were split by median (with the best cutoff) [30], and the follow up threshold was 10 years, censoring patients surviving over this threshold. The JetSet best probe set was used in order to select the optimal probe set for UCP2 gene [31]. The ER status was fixed in ER positive, as we know that our MCF-7 breast cancer cell line is ERα positive [32].

Statistical analysis

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are expressed as mean values ± standard error of the mean (SEM). Statistical significances between control and UCP2 inhibition were assessed by Student's t-test. The effects of UCP2 inhibition and cisplatin or tamoxifen treatment were analyzed using two-way analysis of variance (ANOVA), and when results reach significant combinatory effects, Student's t-test was performed in order to find out the significance between the experimental groups. Statistical significance was set at $P<0.05$. Kaplan-Meier statistical analyses were made using an online (www.kmplot.com) Kaplan-Meier plotter dataset [29].

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RESULTS

UCP2 levels after siRNA transfection

UCP2 inhibition by siRNA was determined by measuring the mRNA and protein levels 24h after siRNA transfection, as shown in Table 1, in order to ensure that UCP2 was at least partially inhibited. Indeed, UCP2 siRNA produced a 72% decrease in UCP2 mRNA levels and a 21% decrease in UCP2 protein levels in MCF-7 cells.

UCP2 inhibition reduces cell viability, and increases ROS production and macromolecules oxidative damage

To evaluate the effect of UCP2 on cell growth, a cell viability assay was performed using siRNA or the specific UCP2 inhibitor genipin. Figure 1A shows that UCP2 knockdown significantly decreased cell growth in a time-dependent manner (0-72h). UCP2 siRNA reduced MCF-7 proliferation by 10% at 24h, and 15% at both 48h and 72h. Furthermore, blocking of UCP2 activity with increasing doses of genipin (0-200 µM) reduced cell proliferation in a dose-dependent manner over the period 0-72h, reaching absolute inhibition at 200 µM (Figure 1B).

The ROS production was measured in UCP2 siRNA or genipin treated cells. As shown in Figure 2, UCP2 inhibition by both UCP2 silencing and genipin enhanced ROS production. Specifically, UCP2 siRNA increased ROS levels by 65% (Figure 2A), while genipin treatment for 24h induced ROS production with maximum levels being reached (298%) at 250 µM (Figure 2B).

In accordance with the great increase in ROS production triggered by UCP2 inhibition, it was decided to test whether UCP2 silencing and cytotoxic agents could increase oxidative damage to proteins and lipids. Table 2 shows that UCP2 silencing by siRNA for 24h increased levels of carbonyl groups and 4-HNE adducts in proteins (end markers of oxidative damage in proteins and lipids, respectively) by 13 and 19%, respectively.

Cisplatin and tamoxifen decreases cell viability and colony formation ability of MCF-7 breast cancer cells

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To investigate whether UCP2 inhibition could be a therapeutic target against breast cancer, its inhibition was tested in combination with CDDP and TAM in a cell viability assay. As shown in figure 3A, UCP2 knockdown improved the effectiveness of both cytotoxic agents. CDDP and TAM treatments decreased cell growth by 54% and 42% respectively, while UCP2 silencing reduced it by 32%. Interestingly, UCP2 silencing enhanced the antiproliferative effect of the cytotoxic treatments, especially in TAM+UCP2siRNA-treated cells (18% more than TAM-only treatment). Therefore, MCF-7 breast cancer cells were more sensitive to cytotoxic agent/UCP2 silent combination.

To evaluate the long-term effect of CDDP on the MCF7 breast cancer cell line, a clonogenic assay was performed (Figure 3B). UCP2 knockdown cells resulted in a significant reduction in their capacity to form viable colonies (19%), while CDDP and TAM treatments for 48h decreased it by 75% and 23% respectively. However, only the combination of UCP2 inhibition and TAM exposure reduced significantly (regarding to the treatment alone) the clonogenic survival (-27%).

Cytotoxic agents and UCP2 knockdown increase ROS production and mitochondrial membrane potential (ΔΨm)

Furthermore, the role of UCP2 knockdown was analyzed in the ROS production mediated by the cytotoxic agents. Figure 4A shows that cells presented higher ROS production after CDDP (122%) and TAM (66%) treatments, as well as a 97% increase with the UCP2 knockdown. Moreover, these increments in ROS production were enhanced when cells were treated with UCP2 siRNA in combination with cytotoxic agents, and, once again, the combination with the greatest increase regarding to cytotoxic alone was TAM+UCP2siRNA (+57%).

As UCP2 plays a role dissipating the proton gradient in the inner mitochondrial membrane, an analysis of ΔΨm was performed to determine whether it could be affected by UCP2 inhibition or the cytotoxic agents, and the results are shown in Figure 4B. As expected, the UCP2 knockdown produced an increase of 65% in ΔΨm, as well as did either of the cytotoxic agents acting alone (CDDP 64% and TAM 73%). The combination resulted in a rise of this parameter in the cytotoxic agents studied, CDDP (+55%) and TAM (+58%).

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Furthermore, in order to determine how CDDP and TAM treatments can affect UCP2 protein levels, Western blotting was performed. As expected, Figure 4C and 4D show that UCP2 silencing by siRNA produced 22% less UCP2 protein than the untreated cells. Remarkably, results also revealed that CDDP decreased UCP2 protein levels by 22%, while TAM treatment produced a slight increase in UCP2 protein levels in reference to control vehicle-treated cells, but did not reach any significant change.

Apoptosis is activated by cytotoxic agents, but not increased in combination with UCP2 inhibition

To assess the effect of UCP2 knockdown and cytotoxic agents in programmed cell death of cancer cells, an apoptosis assay was performed. Figure 5A shows that CDDP and TAM increased apoptosis of cancer cells by 69% and 45% respectively, and UCP2 inhibition resulted in only a slight increment of this parameter (16%). However, combination of the UCP2 knockdown with both cytotoxic agents did not result in any significant difference from the cytotoxic treatments alone. Additionally, the cleaved PARP/PARP ratio (apoptosis marker) was studied in CDDP and TAM treated cells in combination or without UCP2 inhibition. As it can be observed in Figure 5C, UCP2 knockdown did not cause any significant change in cleaved PARP ratio; however the cytotoxic agents did increase PARP cleavage by 97% (CDDP) and 247% (TAM). Interestingly, treatment of cancer cells with a combination of UCP2 siRNA and TAM reduced significantly the PARP cleavage with respect to TAM-treated cells (-152%), while CDDP-treated cells in combination with UCP2 siRNA did not present any significant change with respect to CDDP treatment alone.

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Activation of autophagic cell death is greater with cytotoxic agents in combination with UCP2 knockdown

As can be observed in Figure 5B, the inhibition of UCP2 triggered a 79% increase in the formation of autophagic vacuoles. Moreover, cytotoxic treatments exhibited a rise in autophagic vacuoles formation (by 224% CDDP and 226% TAM), and only TAM-treated cells showed an increase (+170%) when combined with UCP2 knockdown, since CDDP in combination with UCP2 inhibition had no any significant increase in comparison to the cytotoxic agent alone. Moreover, Figure 5D shows that UCP2

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knockdown increases the ratio LC3-II/LC3-I (autophagic marker) by 62%, as well as CDDP and TAM (by 124% and 178% respectively). Interestingly, combination of UCP2 siRNA with CDDP did not increase this LC3 ratio with respect to CDDP alone, although there was a rise in UCP2 siRNA and TAM combination with respect to cytotoxic treatment alone (+182%).

UCP2 knockdown affects cell viability, ROS production, apoptosis and autophagy in a similar manner in T47D cells after cytotoxic treatments

For the purpose of confirming the results obtained in MCF-7 cells, we have done some critical experiments in another breast cancer cell, T47D. In T47D the UCP2 specific siRNA reduced UCP2 mRNA by 48% (data not shown).

Cell viability assay in T47D (Figure 6A) revealed that UCP2 knockdown, although in a more subtle way than in MCF-7 cells, caused cell death (by 7%) and increased the effectiveness of the TAM treatment (-5% extra regarding TAM treatment alone).

Furthermore, figure 6B shows that in T47D cells the UCP2 knockdown increased the ROS production, even though milder (+11%) than in MCF-7 cells as well as the CDDP and TAM treatments (+46% and +29% respectively), and only the TAM treatment increased significantly the ROS production after UCP2 silencing (+28%).

Moreover, in figure 6C it can be observed that T47D apoptosis was increased with both cytotoxic treatments (+36% in CDDP-treated cells and +44% in TAM-treated cells) and the UCP2 knockdown resulted in a very slight raise in apoptosis (+10%); however, there were no significant changes in Annexin V fluorescence in the combination of cytotoxic and UCP2 siRNA regarding to the cytotoxic treatment alone.

Finally, figure 6D shows that UCP2 inhibition increased autophagy in T47D by 5%. In the same way, cytotoxic treatments caused an increase in autophagic vacuoles formation by 74% (CDDP) and 317% (TAM). Interestingly, only the TAM treatment in combination with UCP2 knockdown enhanced the autophagic vacuoles formation (+49%) in comparison with TAM treatment alone.

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High levels of UCP2 expression represent poorer prognosis in treated breast cancer patients

Figure 7 shows the Kaplan-Meier plots displaying the relapse-free survival (RFS) and the overall survival (OS) of patients expressing high or low levels of UCP2. As can be seen in the figure, a poor prognosis is significantly associated with high levels of UCP2 expression when all patients were taken into account (OS: P=0.0048; RFS: P=0.0073). Nevertheless, systematically untreated patients did not present any significant differences between high and low UCP2 expression patients. Finally, it is important to note a significant difference (P=0.0039) in RFS in TAM-treated patients, showing a poorer prognosis for those patients who had higher levels of UCP2 expression.

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DISCUSSION

In the current study, we inhibited UCP2 expression and function in two breast cancer cell lines and analyzed whether this specific inhibition increases oxidative stress and affect the effectiveness of ROS-based chemotherapeutic agents, such as cisplatin and tamoxifen. Definitely, UCP2 silencing provoked oxidative stress and compromised cancer cells viability. We show here that cisplatin (CDDP) decreased UCP2 protein levels while tamoxifen (TAM) did not do so. Both cytotoxic treatments generated oxidative stress in cancer cells and decreased cell viability, with greater changes resulting from combination with the UCP2 knockdown. Moreover, although CDDP and TAM promotes both apoptosis and autophagy, UCP2 inhibition increased mainly the autophagic cell death, alone or in combination with cytotoxic agents, especially TAM. Finally, we show here the importance of UCP2 expression levels for the prognosis of ER-positive breast cancer patients, showing that higher levels of UCP2 expression correspond to a poorer prognosis.

The role of ROS in cancer remains controversial, since the induction of severe cellular damage can lead to cell death, however, mild oxidative stress promotes cell survival. Mitochondria are linked to a complex adaptive response to protect cancer cells against ROS generation [33, 34], and UCP2 may contribute to this process [9, 10, 12]. The ability to control ROS through proton leak is considered to be the first line of defense against oxidative stress and is associated with increased cell survival [35]. We previously demonstrated that MCF7 breast cancer cells have high ROS levels and altered redox status which may contribute to cell growth [2, 4]. Moreover, the MCF7 and T47D cells express the UCP2 protein, which may be modulating oxidative stress [2, 4, 36], and therefore UCP2 inhibition plays an important role in modulating oxidative stress and cell viability. Inhibition of UCP2 expression and function compromised cell viability in a time and dose-dependent way, and reduced the capacity of cancer cells to form viable clones. These results are in agreement with others showing that UCP2 inhibition can be considered a valuable antiproliferative target in several cancers [17, 19, 37, 38]. Moreover, in a recent paper, it has been reported that genipin is able to decrease cell proliferation and clonogenic survival in UCP2 over-expressing breast cancer cells [17].

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The loss of cell viability induced by UCP2 inhibition may be due to higher ROS levels, since the increased ROS production observed with siRNA and genipin treatments seems to affect cancer cell viability, as it is dose-dependent in genipin-treated cells. However, the strong enhanced ROS production at high concentrations of genipin could not only be due to its effects on UCP2 but also to its protein crosslinking abilities [39]. For this reason, we decided to inhibit UCP2 just by using UCP2 specific siRNA in the experiments that followed.

These high levels of ROS produced by UCP2 knockdown triggered a rise of carbonyl groups and 4-HNE adducts, which supports that this UCP2 inhibition results in oxidative damage in cancer cells. These deleterious effects on the proteins and lipids of cancer cells may explain in part the antiproliferative effect of UCP2 silencing, as well as the role that mitochondrial uncoupling plays in the regulation of oxidative stress. In this regard, many studies have found that ectopic UCP2 improves control of oxidative stress in various cancer cells and promotes cell survival, leading to chemoresistance [14, 37].

ROS production is one of the cytotoxic effects of CDDP and TAM treatments [8, 27], so we suspected that inhibiting UCP2 could enhance treatment efficacy increasing ROS production leading to cancer cells death. UCP2 knockdown has greater impact when used in combination with TAM, because CDDP treatment reduced UCP2 protein levels, while TAM did not do so. Previously, it has been demonstrated that CDDP reduces UCP2 levels in colon cancer cells [38]. Furthermore, since MCF-7 cells are ERα-positive [32] and TAM is an antagonist of ERα [26], the addition of this cytotoxic agent could increase UCP2 levels because we have previously demonstrated that 17β-estradiol decreases the levels of UCP2 in MCF-7 cells [4].

UCP2 inhibition, as well as the cytotoxic treatments, produced an increase in mitochondrial membrane potential ($\Delta\Psi m$), which was accompanied by an increment in the ROS production (regarding to cytotoxic treatment alone), principally in TAM+siRNAUCP2-treated cells, in accordance with previous studies that have shown CDDP and TAM increase ROS levels in cancer cells [38, 40]. It is important to note that a little increase in $\Delta\Psi m$ give large stimulation of ROS production [41], suggesting that a

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small drop in UCP2 levels could mean a rise in the ΔΨm and, consequently, in ROS production. Likewise, this greater increase in ROS production could increase the drop in cell viability and the decrease in the capacity of cancer cells to form viable clones. This would be in agreement with Dalla Pozza et al., suggesting that the chemical inhibition of UCP2 has a synergistic antiproliferative effect with the cytotoxic treatment in pancreatic cancer cells [19]. Altogether, these findings suggest that UCP2 targeting may decrease cell growth, and enhance antiproliferative effects of cytotoxic agents, such as CDDP and TAM, modulating the oxidative stress in breast cancer cells. We can conclude that MCF-7 breast cancer cells are more sensitive to cytotoxic treatments in combination with UCP2 silencing due, at least in part, to an increase in ROS production.

Autophagy is a conserved evolutionary process that can enable cells to maintain homeostasis in unfavorable environmental conditions [42]. This process allows the cell to recover energy from damaged or unnecessary subcellular components (macromolecules or organelles) [42], therefore it has been considered as a process associated with cell survival. However, if the damage is too severe and a high level of autophagy persists, autophagic cell death or programmed cell death-2 will occur, which is a different phenomenon than apoptosis or programmed cell death-1 [43]. Therefore, our results indicate that inhibition of UCP2 causes autophagic cell death without a significant increase of apoptosis. CDDP and TAM treatments, nevertheless, promote both apoptosis and autophagy [44-47]. Although both treatments triggered an increase in both apoptosis and autophagy, their combination with UCP2 knockdown only increased the autophagic cell death, particularly in TAM-treated cells (probably due to UCP2 inhibition caused by CDDP treatment), suggesting that inhibition of UCP2 could be a future therapeutic target, especially in those patients with endocrine therapy with TAM. These results coincide with Dando et al., who recently demonstrated that UCP2 inhibition triggers autophagic cell death through ROS generation in pancreatic adenocarcinoma cells [28].

Results obtained in this study could be useful in clinical applications because the survival curves performed with data from over 1800 breast cancer patients revealed the importance of UCP2 levels in prognosis of breast cancer patients. Our results

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revealed that, when all cases are taken into account, high UCP2 expression was a poor prognosis indicator. The UCP2 dual role in cancer, which may have a protective mechanism in normal cells preventing malignant transformation although its over-expression in cancer cells may confer resistance to chemotherapy and a higher survival by down-regulation of ROS levels [15], could be explained by the Kaplan-Meier survival curves since untreated patients did not present any significant change in both disease-free patients or relapse-free survival (RFS), and overall survival (OS) parameters. In fact, in the RFS analysis of these patients, the higher UCP2 expression levels proved to be a better prognosis factor (non-significant data). Interestingly, TAM-treated patients had a poorer prognosis when they had greater UCP2 expression, which is not so obvious in chemotherapy-treated patients, probably due to the aforementioned effects of some cytotoxics such as CDDP over UCP2 protein.

In conclusion, we show here that the inhibition of the mitochondrial uncoupling protein 2 (UCP2) may cause the autophagic cell death in cancer cells through the ROS generation. Therefore, UCP2 could be a therapeutic target against breast cancer combined with cytotoxic agents which generate ROS, especially in combination with tamoxifen.

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CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Table 1. UCP2 mRNA and protein levels as the result of silencing by UCP2 specific siRNA in MCF-7 breast cancer cell line.

	Control	UCP2 siRNA
UCP2 mRNA levels (% AU)	100 ± 16	27.8 ± 0.4*
UCP2 protein levels (% AU)	100 ± 6	79.3 ± 9.7*

MCF-7 cells were transfected with UCP2 siRNA for 6h. Next, complexes were removed and cells were provided with normal growth medium for 24h, and then harvested for PCR and western blot analysis. Data represent the means ± SEM (n = 6). Values of Control were set at 100. AU: arbitrary units. * Significant difference between UCP2 siRNA-treated and LF-treated cells (Student's *t*-test; P≤0.05).

Table 2. Increase in carbonyl content and 4-HNE adduct formation after UCP2 silencing.

	Control	UCP2 siRNA
Protein carbonyls (% AU)	100 ± 2	113 ± 7*
4-HNE adducts (% AU)	100 ± 6	119 ± 7*

MCF-7 cells were transfected with UCP2 siRNA for 6h. Next, complexes were removed and cells were provided with normal growth medium for 24h. Whole-cell extracts were used for protein carbonyls detection (A) and 4-HNE adducts in proteins (B) by Western blot analysis. Values are expressed as means ± SEM (n=6) and normalized as percentage of the control value. * Significant difference between UCP2 siRNA-treated and Control cells (Student's *t*-test; P≤0.05).

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HIGHLIGHTS

1. Cell survival is decreased in breast cancer cells with UCP2 knockdown or inhibition.

2. UCP2 knockdown and inhibition increases oxidative stress in breast cancer cells.

3. UCP2 knockdown induces autophagic cell death, especially in tamoxifen-treated cells.

4. The UCP2 overexpression is a poorer prognostic factor in ER+ breast cancer patients.

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Figure 1

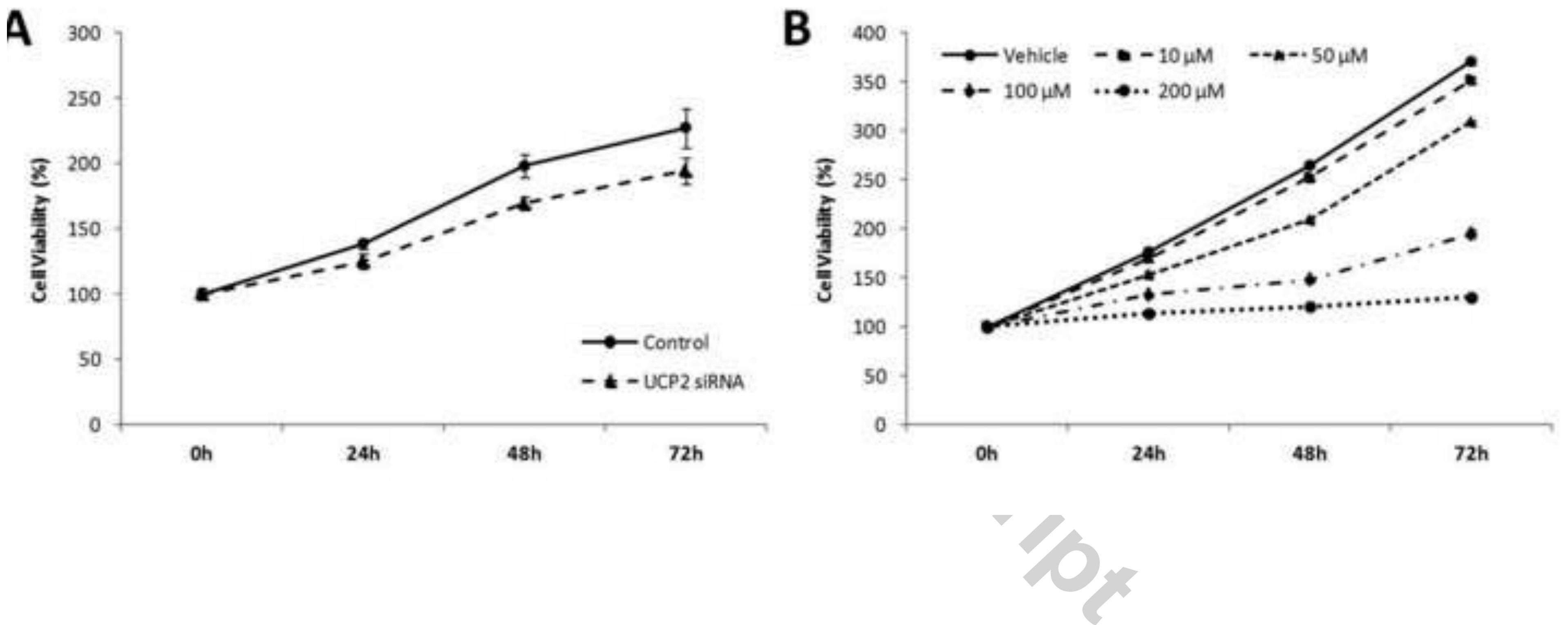

Figure 2

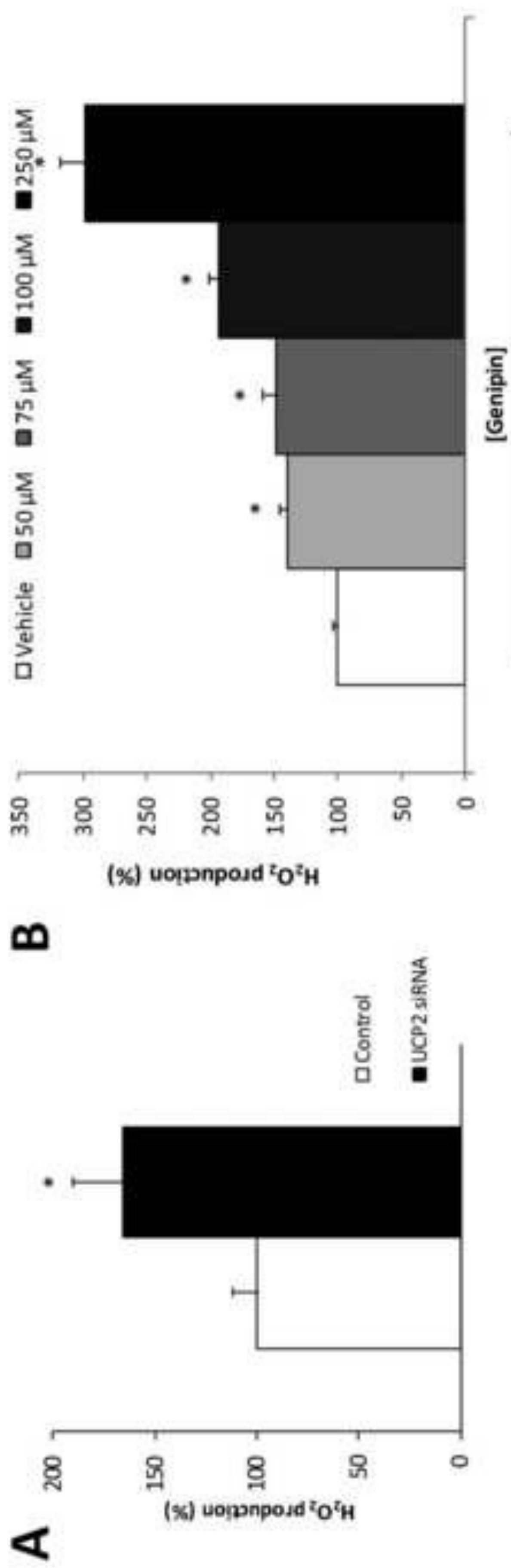

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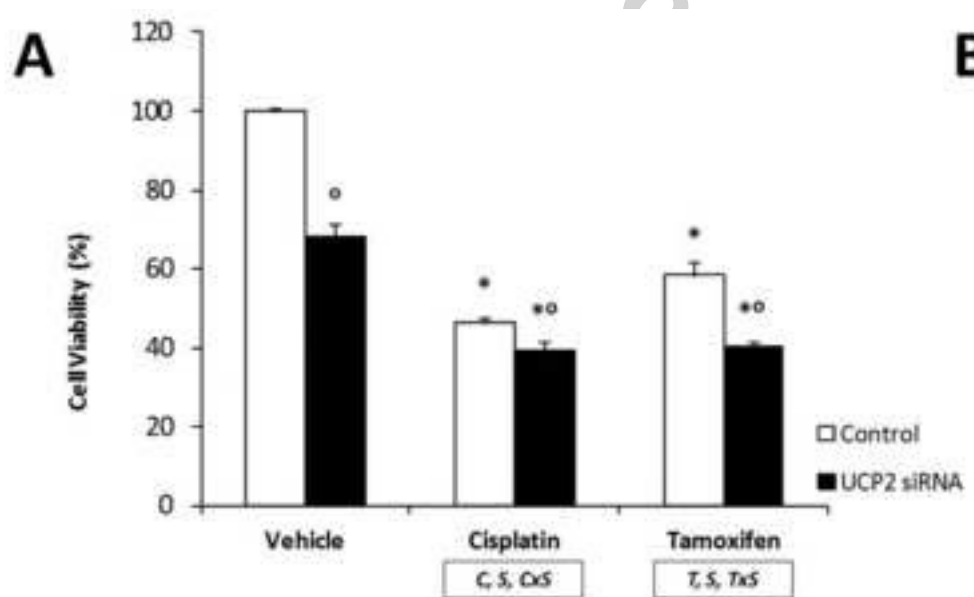
Figure 3



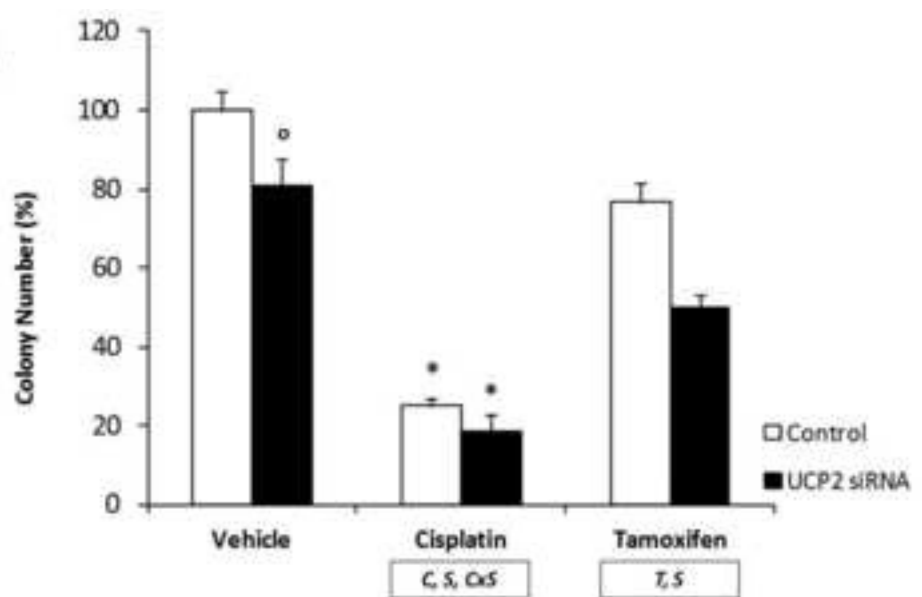


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Figure 4

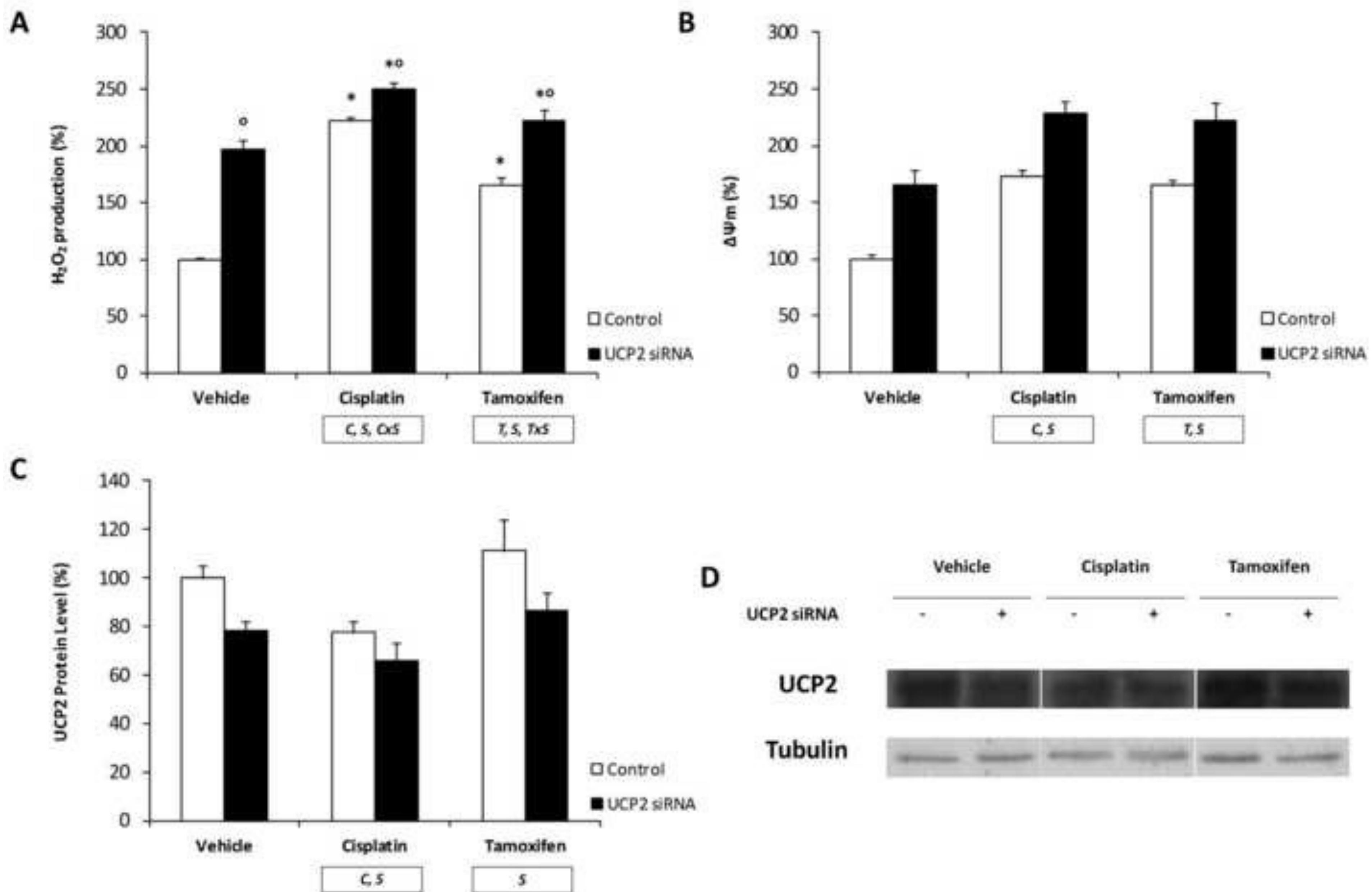

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Figure 5

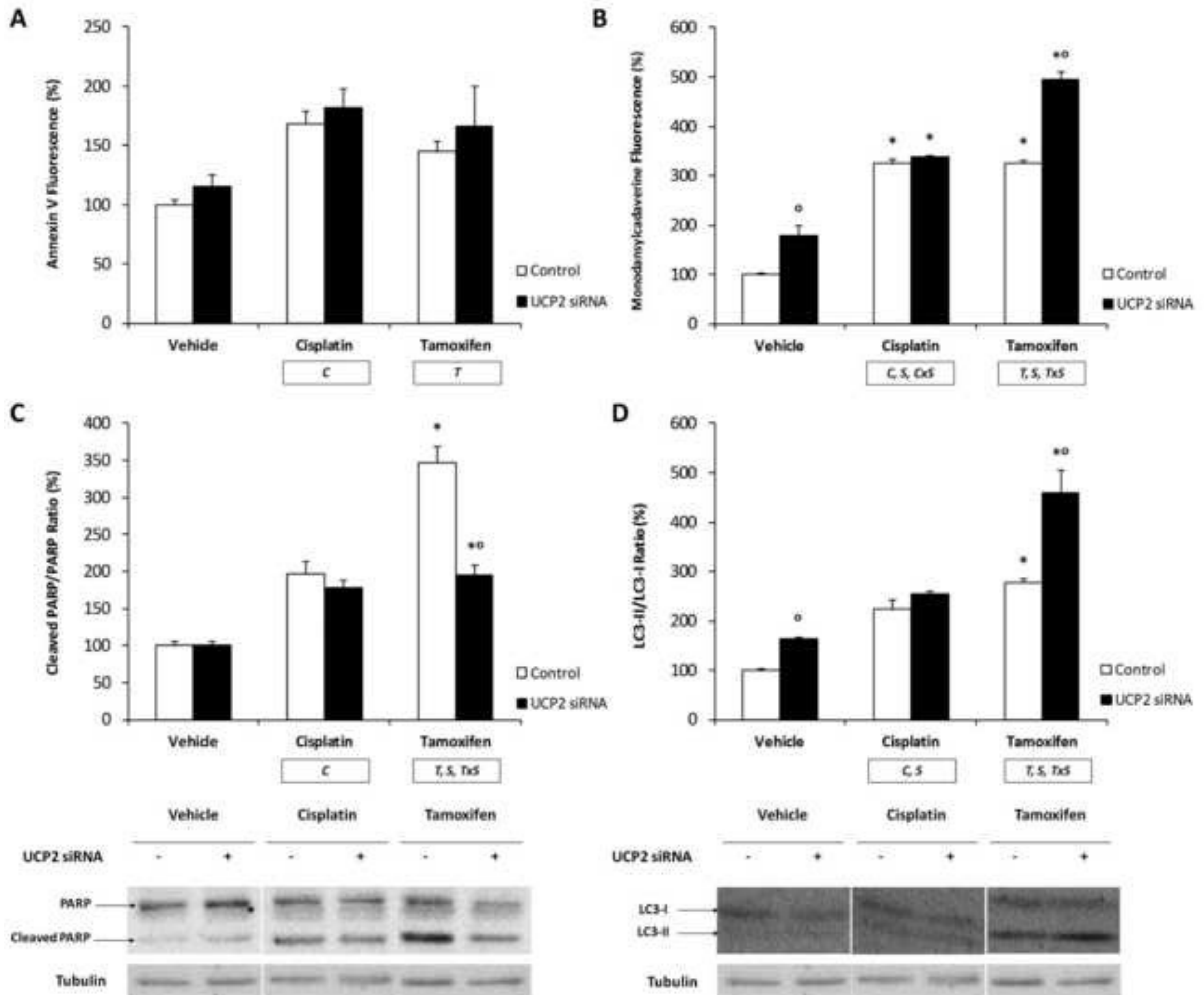

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Figure 6

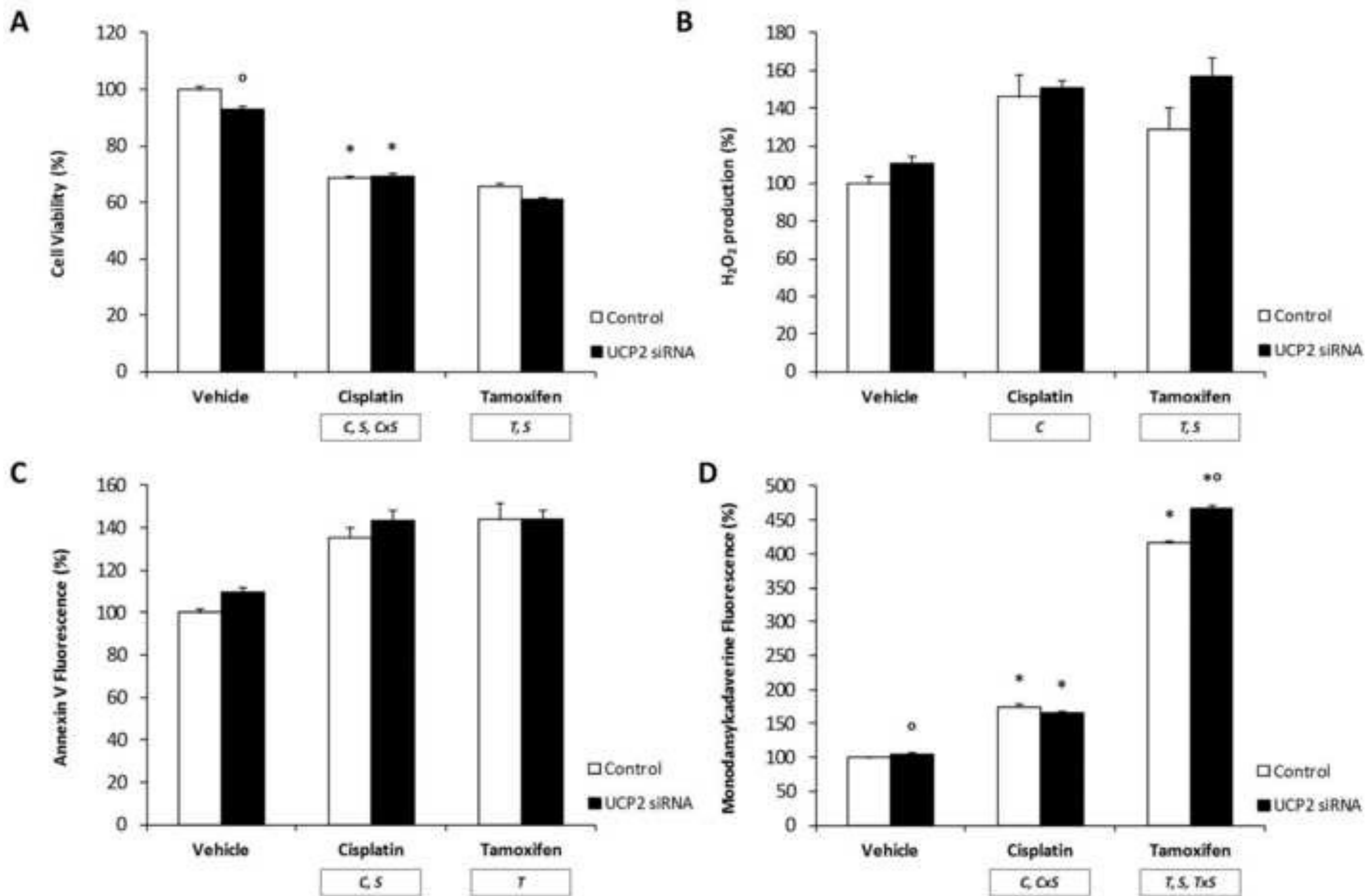

Figure 7

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