The Effects of Tetrahydrocurcumin on Adriamycin and Taxane resistance in Anaplastic Thyroid Cancer Cells

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Abstract

Purpose

Anaplastic thyroid cancer (ATC) is rare but one of the most lethal human malignancy. Despite the advances in cancer treatments, ATC remained incurable due to a lack of effective treatments. ATC cells display resistance against all cytostatics. Adriamycin and taxanes have been used with limited efficacy. There is an urgent need for new treatments and less toxic substances that will enhance the effectiveness of current treatments. One of the most effective curcumin derivative, tetrahydrocurcumin (THC), appears to have several anti-cancer and anti-MDR (multidrug resistance) actions. Thus, we aimed to compare the anti-cancer efficacy of THC with adriamycin and docetaxel, as well as evaluate the anti-MDR action of two drugs in ATC cell lines, if any.

Methods

We used MTT test to find the IC50 values for Adriamycin (Adr), docetaxel (Doce) and THC (Cur). The cells were treated with IC50 and half-IC50 (IC25) doses. IC25 doses were also used for drug combinations. Wound healing assay, spheroid formation in soft agar, oxidative stress analysis, flow-cytometrically apoptosis, and multidrug resistance activity factor analysis (MAF) for MDR1 (P-gp), MRP1/2, BCRP expressions were performed in both cell lines.

Results

THC was more potent than docetaxel and adriamycin on cell migration, spheroid formation, anti-oxidant capacity, and apoptosis induction at LD50 dose in both cell lines. THC-induced MAF suppression was found particularly for MDR1 in both cell lines. Whereas adriamycin and docetaxel treatments lead to increased MRP1/2 and BCRP expressions. THC suppressed these actions dose dependent manner.

Conclusion

THC can modify MDR protein expressions and stem cell properties, which can increase the efficacy of doxorubicin and adriamycin in treating anaplastic thyroid cancer. THC has anti-cancer potential that is comparable to these two cytostatics. Curcumin could be considered an adjunctive component of the ATC treatment.

1. INTRODUCTION

Thyroid cancer is the most frequently seen endocrine malignancy, accounting for 3% of all newly diagnosed cancers worldwide (1). The rising incidence has been observed, particularly in papillary thyroid cancer, over the last decades (2). Papillary and follicular thyroid cancers are considered differentiated

thyroid cancers (DTC) and account for 95% of all thyroid malignancies. These tumors have quite a good prognosis, and cure is commonly possible even in advanced stages. However, anaplastic thyroid cancer (ATC) is the least common (1–2%) but most aggressive type of thyroid cancer and is called undifferentiated cancer (3). Potentially, ATC arises from the full dedifferentiation of DTCs after gaining new mutations. Because over 50% of ATC patients have co-existent DTC at histopathological analysis (4). The mean survival rates of ATC cases have been given as 4–5 months after the diagnosis, and survival beyond 2 years was given at 12% after multimodal treatment including surgery, radiotherapy, and cytotoxic chemotherapy including adriamycin, docetaxel, or cis-platinum (5, 6). Despite advances in several anti-cancer treatment modalities, ATC remained incurable, and mortality is close to 100 percent (7). Patients mostly die due to airway obstruction due to trakeal compression, vocal cord paralysis, extensive pulmonary invasion, and brain metastasis (3, 8). According to the final edition of the American Joint Committee on Cancer (AJCC/TNM eighth edition), all anaplastic thyroid cancer cases were classified as stage 4 disease, even the tumor was limited to the thyroid gland due to its aggressive behavior (8, 9). Survival advantages, i.e., 8–10 months, can be obtained only with multimodal treatment, including surgery, high-dose accelerated radiotherapy, and combination chemotherapy (8, 10, 11).

Besides extremely high proliferation rates, epithelial-to-mesenchymal transition, and cancer stem characteristics, ATC cells display chemo-resistance to all cytostatics (12, 13). Taxanes and doxorubicine have been used with limited efficacy to improve survival (14, 15). Particularly for this type of cancer, much more effective treatment strategies are urgently needed. Recently, research focus and therapeutic target shifted to some thyrosine kinase receptor inhibitors, increasing chemotherapy efficacy via less toxic compounds, immunotherapies, and drugs that overcome multidrug resistance mechanisms (8, 16).

Accumulating data supported that two dysregulated signaling cascades involve the pathogenesis of differentiated thyroid cancers: mitogen-activated protein kinase (MAPK) activation plays a main role in PTC development through some mutations of the RAF and RAS genes or gene fusions of RET/PTC and TRK (17). On the other hand, phosphatidylinositol-3 kinase (PI3K)/AKT activation through RAS, AKT1, and PTEN mutations plays key roles in the development of follicular thyroid cancer (FTC) (17). Conversion to the undifferentiated stage involves several additional mutations affecting other signaling pathways, such as p53 and Wnt/B catenine (17, 18). Towards ATC progression, the main identified expresional alterations were related to epithelial-to-mesenchymal transition, negative regulation of p53, loss of thyroid differentiation, and activation of ERK and PI3K/mTOR signaling (18). Compared with PDCs, ATC cases are associated with remarkably higher mutation rates of the TERT promoter (10 vs. 73%), TP53 (1 vs. 59%), and the PIK3CA-PTEN-AKT-mTOR pathway (1,4 vs. 39%) (19). The frequency of oncogenic driver mutations involving BRAF, specifically BRAFV600E, the most common mutation seen in papillary thyroid cancer (PTC), can be found in up to 40% of ATC cases (20). Targeting receptor proein kinases (RTKs), which are involved in the activation of MAPK and PI3K/AKT pathways, has yielded very promising results in particularly differentiated thyroid cancer (21). However, one drug application received clinical approval. The combination of ATP-competitive inhibitor dabrafenib and MEK inhibitor trametinib was approved by the FDA in 2018 for BRAFV600E mutation-carrier ATC patients after a published phase II study (16, 22). Out of 16 patients, the overall response rate (ORR) and the one-year OS rate were 69% and

80%, respectively. The MEK inhibitor trametinib has been adding BRAF inhibitors because of paradoxal MAPK activation, and dabrafenib/trametinib treatment has started to use other aggressive BRAF-mutated cancers, including biliary tract cancers and gliomas (23). Therapies targeting other key players of the MAPK pathway, such as RAF and MEK, have shown encouraging results, but inhibitors targeting the PI3K/Akt/mTOR pathway have shown disappointing clinical results (24, 25). Unfortunately, multi-drug resistance is inevitable for many RTK inhibitors. ATC cells become resistant against these targeting agents via paradoxically re-activation of their own signaling pathways due to genomic instability, autocrine secretion of some growth factors, and non-clarified mechanisms (26). Also, some targeting agents may have potential for trans-membrane drug efflux proteins (ABC transporters). For example, a recently published study demonstrated that VCAM-1 is upregulated in thyroid cancer cells treated with BRAF-inhibitor vemurafenib contributes to drug resistance via increasing ABCG2 in BRAF-mutant thyroid cancer cells (27). Moreover, significant toxicities have been observed during BRAF and MAPK/RAS-targeted therapies in thyroid cancer patients (28).

Modulating the expression of multidrug resistance (MDR) proteins by using less toxic compounds and increasing the efficacy of cytostatics or targeting compounds has become a critical strategy for the curative treatment of ATC. One of the main mechanisms of MDR is thought to be the overexpression of ATP-binding cassette (ABC) transporters, which increases drug efflux and drastically lowers the intracellular concentration of antineoplastic drugs. Over the last decades, three groups of ABC transporters (ABCB1(P-gp), ABCG2(BCRP), and ABCC1(MRP1)) and diverse compounds with ABC inhibitory activity have been identified to sensitize drug-resistant cells. For example, the multi-kinase inhibitor Motesanib (AMG706), which has an anti-tumor effect on thyroid cancer, inhibits the ABCB1 transporter and increases paclitaxel concentration in the KB-C2 cell line, which overexpresses ABCB1 (29). In a systematic review evaluating overexpression of ABC transporters, ABCC1, ABCG2, and, to a lesser extent, ABCB1 play essential roles in ATC resistance against docetaxel, adriamycine (doxorobicine), and cis-platinum (30). Several naturally occurring compounds, including resveratrol, quercetin, epigallocatechin-3-gallate, genistein, and curcuminoids, have been shown to induce apoptosis, re-differentiation, and cell growth inhibition in all types of thyroid cancers (31, 32). These compounds are also capable of altering cellular signaling pathways and the expression of ABC transporters in drug-resistant cancer cells (33). Curcumin is a natural compound extracted from Curcuma longa rhisomes, and its multiple anti-cancer properties have been widely demonstrated for many types of cancer. Its derivatives have also been shown to have drug-sensitizing functions in some cancers. As a most studied phytochemical, curcumin and its derivatives have also been shown to have drug-sensitizing and MDR-suppressing functions in various drug-resistant tumors, including breast, prostate, pancreas, lung, and head and neck cancers (34). Recently, some complated clinical phase I/II trials investigating the efficacy of curcumin alone and combined with chemotherapeutic agents revealed encouraging results in the aspects of side effects of chemo/radiotherapy, QoL, and tolerability (35). However, no prospective clinical trial addressing the chemosensitizing impact of curcuminoids on ATC cases has been published yet.

In the present study, the efficacy of a curcumin derivative, tetrahydrocurcumin (THC), on adriamycin and docetaxel resistance in two ATC cell lines was investigated. Additionally, THC's anti-cancer effectiveness

was compared with these two chemotherapeutic agents in vitro.

2. MATERIAL AND METHOD

2.1. Cell Lines

Human ATC cell lines, CAL-62 and 8505C, were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The CAL-62 cells were kept in DMEM/F12 medium (Gibco), and the 8505C cells were kept in RPMI-1640 medium (Gibco). Both mediums contained 1% penicillin/streptomycin, 1% amphotericin (Bristol-Myers Squibb), 1% L-glutamine, and 10% fetal bovine serum (FBS-Gibco). The cells were kept in 75-cm^2 flasks (Corning Co.) at 37°C with 5% CO2 and 95% humidity. Tetrahidrocurcumin (THC) was purchased from Sigma.

2.2. Cell viability test (IC^{50}) and Docetaxel/Adriamycin/THC treatments

ATC cells were seeded into 96-well plates at a density of 8000 cells per well. After 24 h, the cultures were treated with increasing concentrations of Docetaxel (0,1-1000 nM), Adriamycine (1 nM-10000 uM), and THC (5-100 uM) for 48 h, and the percentages (IC50 and IC25) of viable cells were determined using the MTT assay (3-[4,5-dimetyl]-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Absorbances were measured at a wavelength of 450 nm using a microplate reader (Thermo Electron Corp.). The determined IC50 and IC25 doses were labeled as Adr50, Adr25, Dox50, Dox25, Cur50, and Cur25, respectively, for further analysis. Combinations with THC treatment were half-IC50 dozes (Cur25 + Adr25 and Cur25 + Dox25).

2.3. Wound Healing Assay

ATC cells were seeded into 6-well plates, and each well consisted of $5x10^5$ cells. The cells were then incubated at 370°C with 5% CO2 until 90% confluence. A lineer wound was made using a 200-ul pipette tip. Subsequently, the cells were treated with the previously described doses for 48 hours. The migration area was scanned at different time intervals by an inverted microscope. Captured images were processed and analyzed using Image J software.

2.4. Spheroid Formation in Soft Agar

Cells belonging to each group were seeded into 25 cm^2 flasks at a density of 3,000,000 cells per 10 ml of medium. When the cells reached confluence, the medium was aspirated. For each group, drugs at predetermined concentrations were added to the cells in the medium (with a total medium volume of 10 ml). The cultures were then incubated in a 5% CO2, 37°C environment for 72 hours. After completion of the incubation, cell suspensions were prepared at a concentration of 16,000 cells/ml of medium.

Bottom Layer Preparation: A 1.2% agarose solution was prepared and dissolved in a microwave oven. The prepared agarose was then added to each well of a 6-well plate at a volume of 2.5 ml per well. The

agarose was allowed to solidify in a Class 2 cabinet under sterile conditions.

Top Layer Preparation: A 0.7% agarose solution was prepared and dissolved in a microwave oven. It was then allowed to cool to 37°C. For each group, the respective cell suspensions were added, and pipetting was performed. A mixture of 0.7% agarose and 1 ml of cell suspension containing 16,000 cells from each group was added separately to each well over the bottom layer, with a total volume of 2.5 ml per well. The agarose was allowed to solidify in a Class 2 cabinet under sterile conditions. After solidification, 2 ml of medium containing predetermined doses of agents were added to each well. The culture media for each group were refreshed every 3 days, and images were taken on days 3 and 7. The spheroids in each group were counted. In each group, the five spheres with the largest diameter were sampled and recorded for statistical comparisons.

2.5. Oxidative Stress Index (OSI) Analysis

The oxidative stress index (OSI) was calculated as the ratio between the total oxidant status (TOS) and the total anti-oxidant status (TAS). Specifically, OSI (arbitrary unit) = TOS (μmol H2O2 Eq/L)/TAS (μmol Trolox Eq/L). The TOS and TAS values were determined using automated colorimetric analysis kits (Rel Assay Diagnostics, Gaziantep, Turkey), following methods developed by Erel O (36, 37).

2.6. Flow cytometric Apoptosis Analysis

The Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen, Heidelberg, Germany) was used for the apoptosis assay. In brief, 5×10^5 CAL-62 and 8505 cells/well were plated in six-well plates. When the cells were 80% confluent (72 h), they were treated with curcumin, adriamycin, and docetaxel at previously established IC50 and IC25 concentrations and were further incubated in fresh medium for 24 h. The cells were then harvested and resuspended in 400μL binding buffer and stained with 5μL annexin V and PI (50μL/mL) at room temperature for 15 min in the dark. After adding 400 μL of binding buffer, the tubes were placed in a double-laser flow cytometer for analysis (Coulter EpicsXL-MCL, Beckman Coulter).

2.7. MDR1 (P-glikoprotein), MRP1/2, and BCRP Expression Analysis

Transmembrane Multi-drug Resistance Proteins (ATP-Binding Cassette) were analyzed flow-cytometically. MDR1, MRP1/2, and BCRP-mediated drug effluxes were assessed by the Effuxx-ID Green assay kit (ENZO Life Sciences, L rrach, Germany) following the manufacturer's instructions. Briefly, control and drug-applied cells were harvested by trypsinization and washed with PBS. Single-cell suspensions were mixed with a diluted MDR1 inhibitor at 37°C for 5 minutes, and then all samples were incubated with a diluted EFLUXX-ID Green Detection Reagent at 37°C for 30 minutes. After incubation, 5 μl of propidium iodide was added to all samples, and fluorescence was immediately measured using a double-laser flow cytometer (Coulter EpicsXL-MCL, Beckman Coulter). Multi-Resistance Activity Factor (MAF) for each transporter was calculated using the formula (MAF = 100x MFIinh-MFI0/MFIinh) (MAF: MDR activity factor; MFI0, MFIinh: mean fluorescence intensity in the presence and absence of inhibitors).

2.8. Statistics

The triplicate data were analyzed using the SigmaStat 3.5 software (Systat, Santa Cruz, USA). For comparison of the groups, an ANOVA was used. Significance was evaluated with the Tukey test. The Kruskal-Wallis test was used for flow-cytometric data comparisons between control and treatments, and a value of $p < 0.05$ was accepted as statistically significant.

3. RESULTS

3.1. IC50 Values of Curcumin, Adriamycin, and Docetaxel

The IC50 values of curcumin (THC), adriamycin, and docetaxel for CAL62 cells were determined as 12 μM, 1100 nM, and 10 nM, respectively. The IC50 values of curcumin (THC), adriamycin, and docetaxel for 8505C cells were determined to be 41 μM, 360 nM, and 30 nM, respectively (Fig. 1). These concentrations (IC50, IC25) were used in further experiments.

3.2. Cell Migration

Curcumin significantly suppressed cellular motility when compared to the other two drugs and control, both when given alone and combined with other drugs in both cell lines ($p < 0.001$, ANOVA followed by Tukey Test).

In CAL-62 cells, all treatments except docetaxel (Doce50 and Doce25) significantly suppressed cell migration when compared with control and docetaxel ($p < 0.001$). Interestingly, docetaxel did not alter cellular motility (Fig. 2). Comparisons between two drug combinations (Adr25/Crc25 vs. Doce25/Crc25) and docetaxel dozes (Doce25 vs. Doce50) were statistically insignificant.

In 8505C cells, remarkable motility suppression was observed in both the combination and CRC50 groups. Curcumin treatment significantly suppressed cellular motility at both dozes when compared with other drug dozes (ANOVA followed by Tukey). Docetaxel alone (Doce 50 and Doce 25) was found to be ineffective against the motility of 8505C cells. Significance exists between Adr50 and Adr25 but does not exist between combinations (Fig. 3).

3.3. Spheroid formation in soft agar

Spheroid sizes and numbers in soft agar were counted on the 7th day of the experiment. Total spheroid numbers in the CAL-62 experiment were as follows: 203, 188, 166, 173, 132, 144, 161, 145, and 155 in the control, Adr25/Crc25, Adr50, Adr25, Crc50, Crc25, Doce25/Crc25, Doce 50, and Doce25 groups, respectively. In the 8505C experiment, total spheroid numbers were as follows: 136, 145, 129, 147, 115, 143, 119, 121, and 127 in the control, Adr25/Crc25, Adr50, Adr25, Crc50, Crc25, Doce25/Crc25, Doce 50, and Doce25 groups, respectively.

In the 8505C experiment, mean spheroid diameters were measured as 3.268 ± 0.62 (± SEM); 1.757 ± 0.295; 2.707 ± 0.25; 2.503 ± 1.154; 0.933 ± 0.144; 1.406 ± 0.131; 1.787 ± 0.26; 2.207 ± 0.152; 2.650 ± 0.201

μm in the control, Adr25/Crc25, Adr50, Adr25, Crc50, Crc25, Doce25/Crc25, Doce 50, and Doce25 groups, respectively. All curcumin treatments significantly reduced spheroid sizes when compared to other drug applications and controls ($p < 0.001$, ANOVA followed by Tukey test) (Fig. 4).

In CAL-62 Experiment, mean spheroid diameters were measured as 5.033 ± 0.18 (± SEM); 2.364 ± 0.07; 2.014 ± 0.24; 2.934 ± 0.35; 1.790 ± 0.14; 2.099 ± 0.22; 3.288 ± 0.33; 3.808 ± 0.47; 3.874 ± 0.23 μm in control, Adr25/Crc25, Adr50, Adr25, Crc50, Crc25, Doce25/Crc25, Doce 50 and Doce25 groups, respectively. The measured values obtained from the control group were significantly higher than those of other groups except docetaxel-containing groups ($p < 0.001$, ANOVA followed by Tukey). Docetaxel-group values were also higher than curcumine and adriamycin treatments (Fig. 4).

3.4. Total Oxidant Status (TOS), Anti-oxidant Status (TOS), and Oxidative Stress Index (OSI)

In CAL-62 cells, mean oxidant (TOS) values after treatment were found as 9.19 ± 0.2; 3.916 ± 0.25; 6,94 ± 0.3; 7.71 ± 0.5 (SEM), 4,59 ± 0.3; 4.53 ± 0.3; 9.51; 9.19 ± 0.05; 9.48 ± 0.06 μmol H2O2 Eq/L in Control, Doce25/Crc25, Doce50, Doce25, Crc50, Crc25, Adr50, Adr25, and Adr25/Crc25 groups, respectively. The lowest values were observed in curcumin treatment (Crc50, Crc25). Curcumin reduced the oxidant status of Docetaxel treatment at IC50 dozes but did not reduce Adriamycin values (ANOVA followed by Tukey test). Mean anti-oxidant values were found as: 0.246 ± 0.02; 0.277 ± 0.01; 0.21 ± 0.05; 0.331 ± 0.01; 0.365 ± 0.04; 0.354 ± 0.02; 0.216 ± 0.05; 0.364 ± 0.04; 0.146 ± 0.05 (SEM) μmol Trolox Eq/L in Control, Doce25/Crc25, Doce50, Doce25, Crc%0, Crc25, Adr50, Adr25, and Adr25/Crc25 groups, respectively. OSI values were determined as: 3757.8 ± 422 (SEM); 1300.1 ± 64; 4680.5 ± 930; 2264 ± 35; 1147.2 ± 79; 1152.8 ± 76; 5160.2 ± 1503; 2612.3 ± 348; 8429,6 ± 1632 in the Control, Doce25/Crc25, Doce50, Doce25, Crc50, Crc25, Adr50, Adr25, and Adr25/Crc25 groups, respectively. Significantly increased OSI values were observed in the adriamycin-curcumin combination when compared with curcumine alone and Doce25 treatments in CAL-62 cells (Fig. 5).

In 8505C cells, mean oxidant (TOS) values after treatment were found as 10.73 ± 0.4; 10.3 ± 0.15; 6.81 ± 0.3; 8.65 ± 0.8 (SEM), 5.05 ± 0.17; 5.33 ± 0.4; 7.53 ± .07; 3.65 ± 0.5; 4.27 ± 0.5 μmol H2O2 Eq/L in Control, Doce25/Crc25, Doce50, Doce25, Crc50, Crc25, Adr25/Crc25, Adr50, and Adr25 groups, respectively. The significantly lowest values were observed in the curcumin and Adriamycin treatment groups (except Adr50). Curcumin reduced the oxidant status of Docetaxel treatment but did not reduce Adriamycin values ($p < 0.05$, ANOVA followed by Tukey test). Mean anti-oxidant values were found as: 0.612 ± 0.06; 0.574 ± 0.09; 0.471 ± 0.05; 0.35 ± 0.05 (SEM), 0.323 ± 0.04; 0.481 ± 0.04; 1.071 ± 0.01; 0.38 ± 0.04; 0.495 ± 0.08 (± SEM) μmol Trolox Eq/L in Control, Doce25/Crc25, Doce50, Doce25, Crc50, Crc25, Adr25/Crc25, Adr50 and Adr25 groups, respectively. Significantly higher levels were observed in the Adr25/Crc25 group ($p < 0.001$). OSI values were found as: 1756.2 ± 87 (SEM); 1992.1 ± 41; 1512.8 ± 204; 2624.4 ± 504; 1624.63 ± 177; 1115.26 ± 36; 704.23 ± 81; 1008.75 ± 185; 900,5 ± 76 in Control, Doce25/Crc25, Doce50, Doce25, Crc50, Crc25, Adr25/Crc25, Adr50 and Adr25 groups, respectively. Doce25 values were

significantly higher than Adr/Crc, Adr25, Adr50, and Crc25 ($p < 0.001$). The difference between Doce25/Crc25 and Adr25/Crc25 was also significant ($p = 0.026$) (Fig. 5).

3.5. Apoptosis Results

In the CAL-62 experiment, apoptotic cell content was 10.9; 13.3; 10.2; 11.8; 20.8; 16.3; 16.6; 20.7; and 18.4% in the control, Adr25, Adr50, Doce25, Doce50, Crc25, Crc50, Adr25/Crc25, and Doce25/Crc25 groups, respectively. Relatively high necrotic cell content was observed in Adriamycin-treated groups (Fig. 5).

In the 8505C experiment, apoptotic cell content was 10.3; 18.5; 17.7; 4.7; 25.3; 33.4; 89.3; 17.1; and 46.4% in the control, Adr25, Adr50, Doce25, Doce50, Crc25, Crc50, Adr25/Crc25, and Doce25/Crc25 groups, respectively. Remarkably increased apoptotic cell content was observed in curcumin-treated groups (Fig. 6).

3.6. MDR1, MRP1/2, and BCRP expressions and MAF values

In both cell lines, curcumin-induced multidrug resistance activity factor (MAF) suppression was obtained for MDR1 when compared with control. Docetaxel and Adriamycin-induced MAF increases were obtained for MRP1/2 and BCRP. Curcumin suppressed drug-induced MAF values for MDR1 in 8505 cells and for BCRP in CAL-62 cells (Fig. 7,8). Curcumin also suppressed MAF for BCRP in CAL-62 cells. (Fig. 7).

4. DISCUSSION

Due to the lack of effective treatment, ATC cases are probably the most unfortunate group of patients, with mortality rates close to one hundred percent. Survival advantages can be achieved with multimodal treatments consisting of high-dose radiotherapy, highly toxic cytostatic protocols including taxanes, adriamycin, cis-platimun, and surgical interventions (8). Recently, several multitargeted inhibitors of tyrosine kinase receptors, including dabratinib, lenvatinib, and pazopanib, have shown promising results (16, 38, 39). Some targeting agents, such as dabrafenib and trametinib, have been added in order to enhance chemotherapy efficacy and overcome drug resistance (16, 40). However, ATC cells eventually develop resistance to targeted therapies and tyrosine kinase inhibitors, similar to classical cytostatics (26, 41). As the authors of this study, we believed that targeting ATC stem cells (CSCs) was the most effective approach to increasing chemotherapy efficacy by suppressing drug resistance mechanisms. According to this concept, CSCs are a relatively rare cell population in a bulky tumor (2–3%), but these cells have the capacity to self-renew, initiate new tumors, switch to a metastatic phenotype via EMT, maintain their undifferentiated state, and more importantly, activate multidrug resistance mechanisms (42, 43). Moreover, transmembrane receptor proteins P-gp (MDR1) and BCRP are regarded as CSC markers, and the proportion of CSC marker expressions such as CD33, ALDH1, MDR1, and BCRP progressively increases from a differentiated state towards poorly differentiated or undifferentiated thyroid tumors (44). In addition, CSC-targeted therapies have been found to be effective against MDR-thyroid-resistant tumors (43).

In our previous work, CD 90 (glycophosphatidyinositol-anchored cell surface protein), a CSC marker frequently observed in poorly differentiated tumors such as hepatocellular carcinoma and esophageal squamous carcinoma, was found to be as high as 70% in CAL62 cells (13). We also found that THC (Tetrahydrocurcumin) did not alter CD90 expressions, whereas the ATC-CSC phenotype was significantly suppressed by THC and combined with docetaxel at the IC50 dose. In the present study, we aimed to define three topics: comparing the anticancer efficacy of THC with other two cytostatics at LD50 and half LD50 doses (a), determining synergistic efficacy with other two drugs if any (b), and identifying alterations in surface transport proteins as an indicator of MDR (c). Although clinical trials aiming to increase chemotherapy efficacy in drug-resistant tumors with various curcumin dose applications were initiated at phases I/II for non-ATC tumors, There is an urgent need for less toxic compounds to improve the efficacy of existing drugs in the ATC. In the present study, THC was specifically selected among other curcuminoids. Tetrahydrocurcumin is less cytotoxic than curcumin and displays cytoprotective properties against ROS-induced cellular damage and death (45). In addition, tetrahydrocurcumin was more potent than curcumin in scavenging hydroxyl and superoxide radicals (46). In our study, tetrahydrocurcumin increased adriamycin-associated oxidative stress in CAL62 cells, while docetaxel-associated oxidational stress significantly increased in 8505C cells (Fig. 5). This result supports tumor cell-dependent necroptotic effects of THC besides apoptotic cell deaths. Because the highest necrotic cell contents were found in the Adr/Crc combination in CAL-62 cells (21%), and in the in the Doce/Crc combination in 8505C cells (12%) (Fig. 6), Studies that support this idea have been observed in different tumors, such as melanoma (47). In the aspect of apoptosis induction, THC alone showed more successful results than other cytostatics, particularly in CAL-62 cells. (Fig. 6).

Regarding the anti-cancer characteristics, the results of this study indicate that THC is just as effective as the other two compounds at LD50 and LD25 doses. We believe that the fact that curcumin does not exhibit antagonism rather than synergistic impacts when combined with other cytostatics is a far more significant finding. In other words, THC can be included in the treatment protocols of ATC as a complement to other compounds. For example, docetaxel remained insufficient to suppress the migration capacity of both ATC cell lines, and this effect was eliminated with THC (Figs. 2 and 3).

The study in which we evaluated MDR activities using flow cytometry revealed that trans-membrane transport proteins, which are composed of three main groups and a large number of subgroups, show dynamic variability with drugs and their dosages. As shown in Figs. 7,8 adriamycin and docetaxel pre-treatment are associated with obviously elevated MAF values for MDR1, MRP1/2, and BCRP in both cell lines. Heterogeneity and genomic polymorphism regarding expressing MDR protein groups have also been observed in other studies (30, 48). For example, adriamycin is able to stimulate MDR1, MRP1, MRP5, MRP7, ABCG1, and BCRP, while docetaxel is able to induce ABCA8, MDR1, and MRP7 (48). THC and several curcuminoids have heterogenic downregulatory potential on three groups of ABC transporters in a dose-dependent manner (49, 50). Sagnou and co-workers (51) have investigated the MDR reversal potency of 22 curcumin derivatives against adriamycin-resistant leukemia cells, and they found two pyrazolo dimethoxycurcumin derivatives as potent as verapamil in aspects of Pgp (MDR1) expression and intracellular adriamycin concentrations. In our study, the MDR reversal activity of THC against

adriamycin and docetaxel was found to be associated with MDR1 and MRP1/2 downregulation in both cell lines. This MDR alteration pattern and soft agar assay results (Fig. 4) relatively indicate the anti-CSC properties of THC in anaplastic thyroid cancer cells. Because spheroid formation in soft agar is considered a very reliable indicator of cancer stem cell properties (52). The sphere diameters were significantly lower in the groups treated with curcumin and adriamycin at the LD50 dose in both cell lines. Docetaxel did not alter spheroid sizes. These two cytostatics are likely to have poor effectiveness in suppressing stem cell characteristics, and curcumin positively contributes to their anti-cancer properties. Zheng and his colleagues (53) found that there were as many as 70% of drug-resistant stem cell groups in various ATC cell lines, that adriamysis killed non-CSC cells, but that it remained ineffective in the CSCs. They concluded that this ineffectiveness is due to MDR1 and BCRP expressions. Some potent curcumin analogues, i.e., PAC (piperidone analogue of curcumin), are capable of inhibiting EMT/CSC pathways and Akt/mTOR signaling simultaneously (54). The major limitation of the present study is the lack of analysis of the expression patterns of MDR proteins within the CSC subgroup of ATC cells. There may be differences in the CSC group's MDR expression patterns and curcumin's efficacy. Also, reliable and commonly accepted surface markers clearly indicate the CSC subgroup does not exist for ATC.

In conclusion, THC can modify MDR protein expressions and stem cell properties, which can increase the efficacy of doxorubicin and adriamycin in treating anaplastic thyroid cancer. THC has anti-cancer potential that is comparable to these two cytostatics. Curcumin could be considered an adjunctive component of the ATC treatment.

Declarations

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Ethics approval: The ethical approval for the use of the cell lines for research purposes was obtained from the institutional ethical committee (Dokuz Eylul University Non-interventional Research Ethical Committee, Ref. no:2012/26-19).

Study registration: This in vitro study, registered as DEU.HSI.PhD-2012970057

Data Availability: Full data will be available from the corresponding author upon reasonable request.

Author Contributions: Study design: MAK and HK, Literature search and data collection: YK, EB Figure generation: YK and MAK, Interpretation of flow cytometric data: HA, Integration of novel techniques into research: HK Manuscript writing: MAK.

Competing Interests: The authors have stated that there are no conflicts of interest in connection with this article.

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Figures

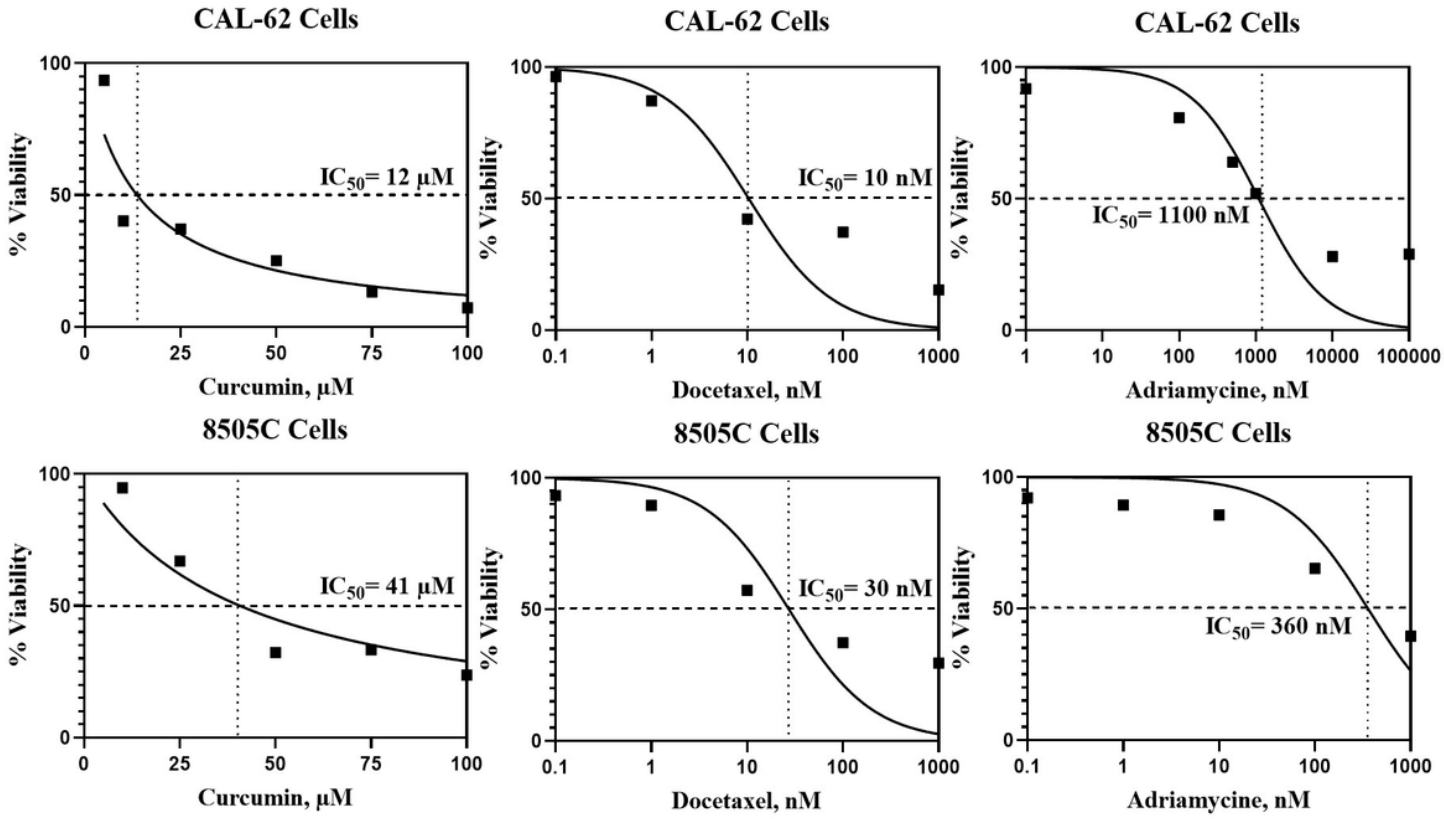

Figure 1

IC50 values of Curcumin, Docetaxel and Adriamycin against CAL-62 and 8505C cells.

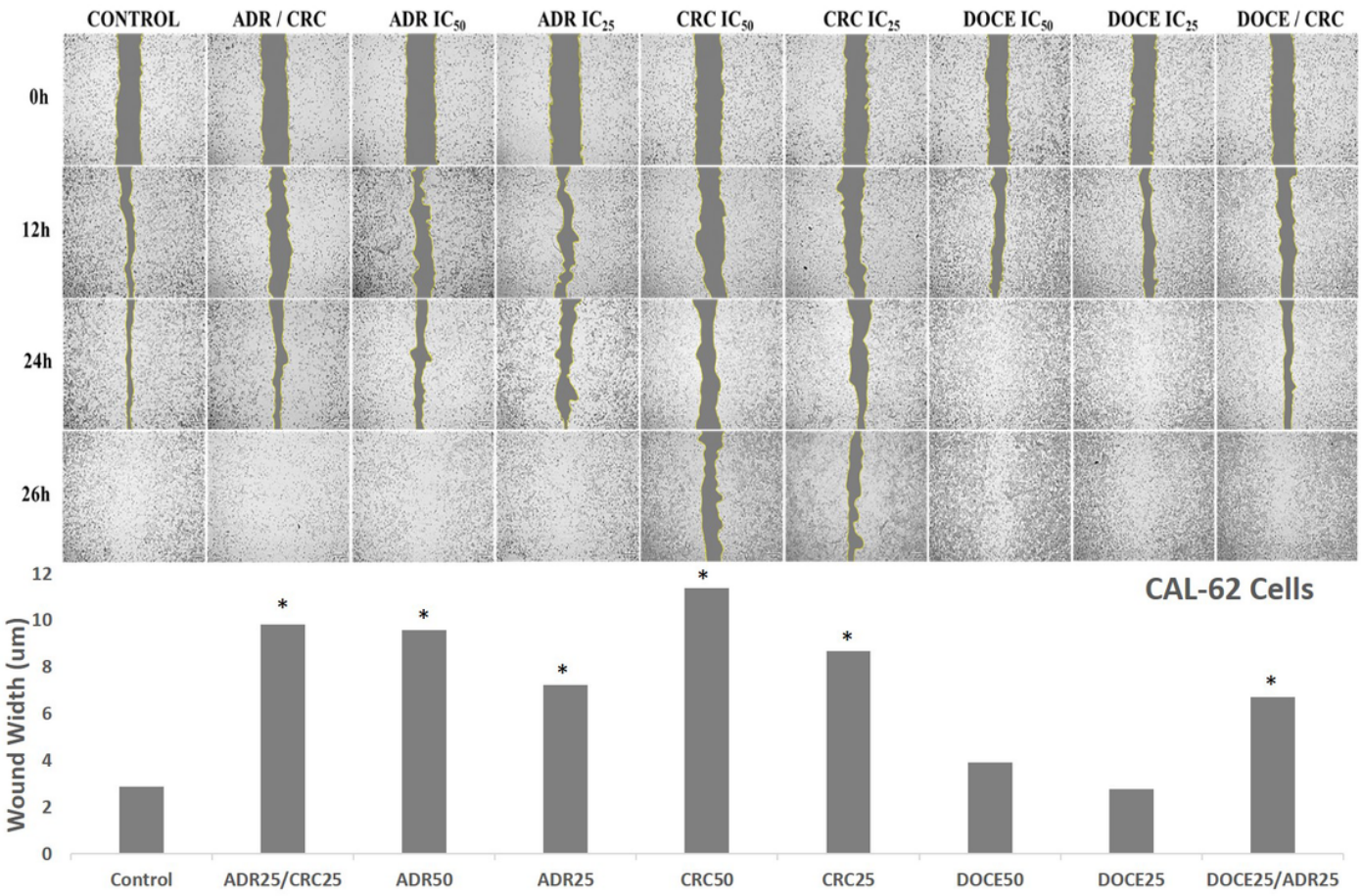

Figure 2

Wound healing assay. Docetaxel alone is not effective on migration capasity of CAL-62 cells. All treatments except docetaxel reduced CAL-62 cell kinetics significantly (* Indicates statistical significance between control and treatments). Significances were exist between docetaxel and other treatments including combinations (ANOVA followed by Tukey test).

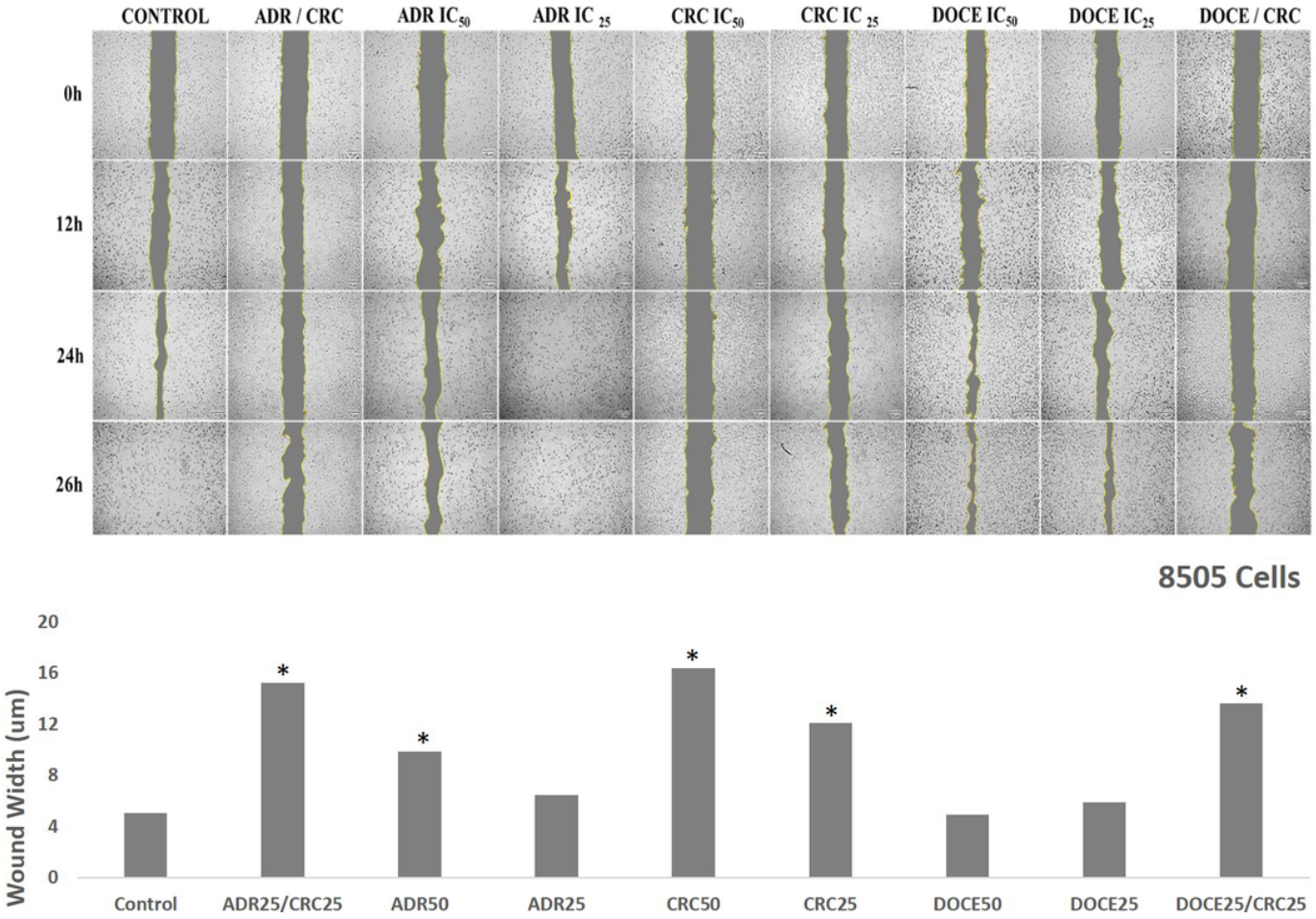

Figure 3

Wound healing assay. Docetaxel alone is not effective on migration capasity of 8505C cells. All treatments except docetaxel reduced 8505C cell kinetics significantly (* Indicates statistical significance between control and treatments). Significances were exist between docetaxel and curcumin contained groups (ANOVA followed by Tukey test).

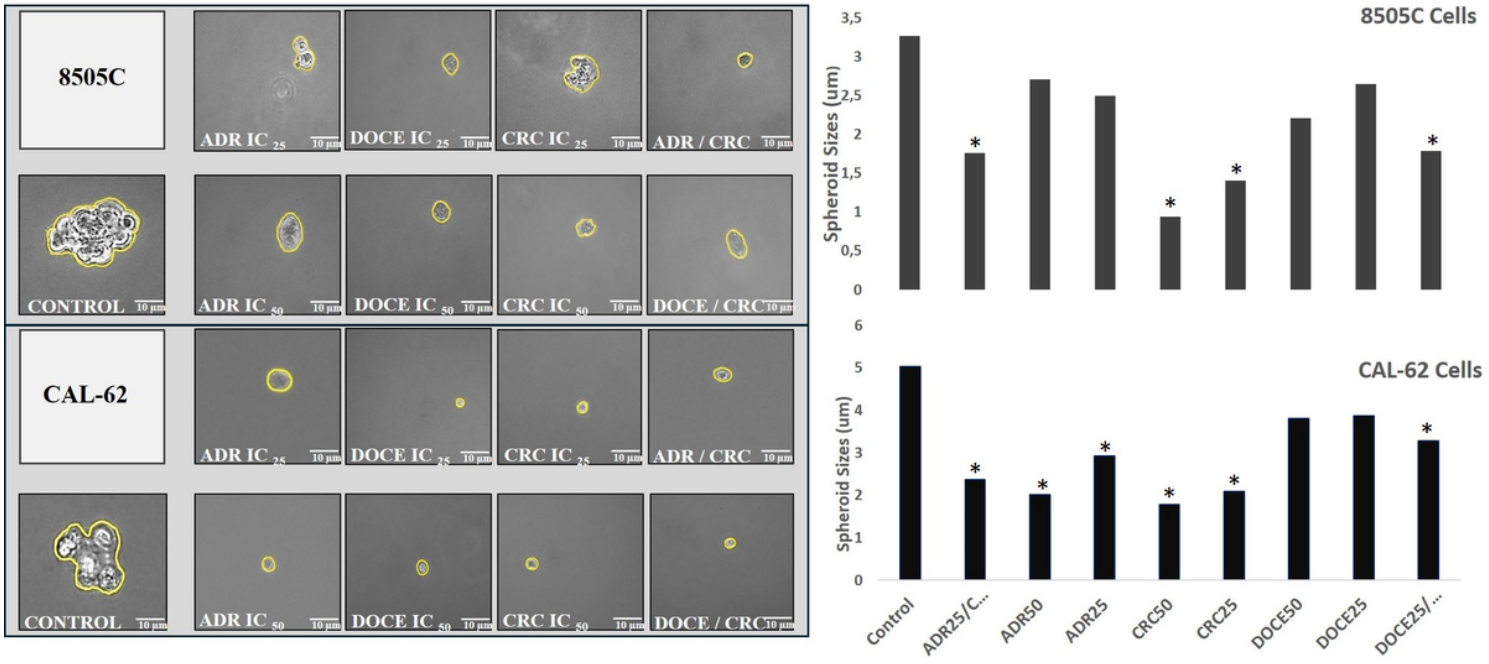

Figure 4

In vitro tumorigenicity in soft agar. Photographs indicate sample spheres in the groups. The five spheres with the largest diameter were sampled from the groups. Curcumin significantly reduced sphere diameters both alone and in combination with other drugs in both cell lines (ANOVA followed by Tukey test). (* Indicates statistical significance between control and treatments).

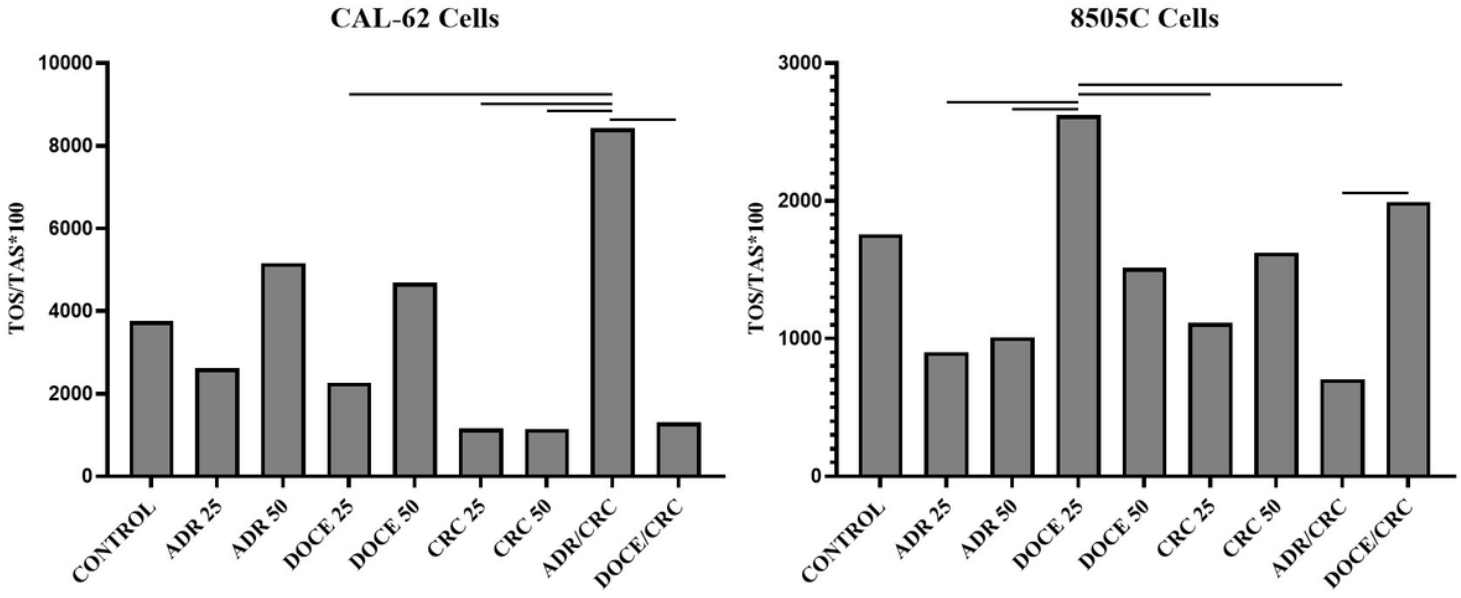

Figure 5

Oxidative Stress Index (OSI) values of treatment groups. Bars indicate statistical significance between treatment groups (ANOVA followed by Tukey Test)

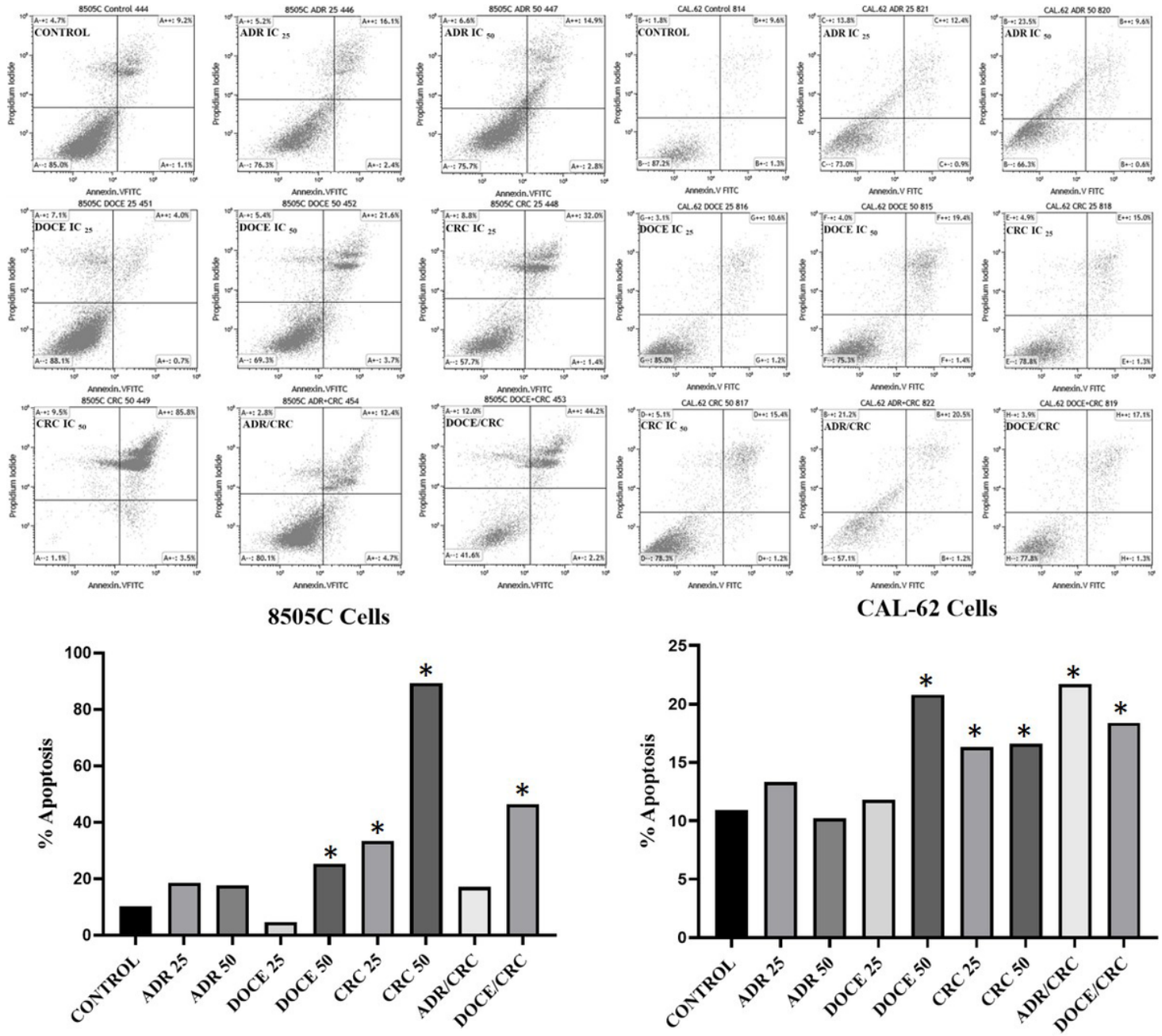

Figure 6

Flow cytometric analysis of apoptotic cell content in treatment groups (* Indicates statistical significance between control and treatments).

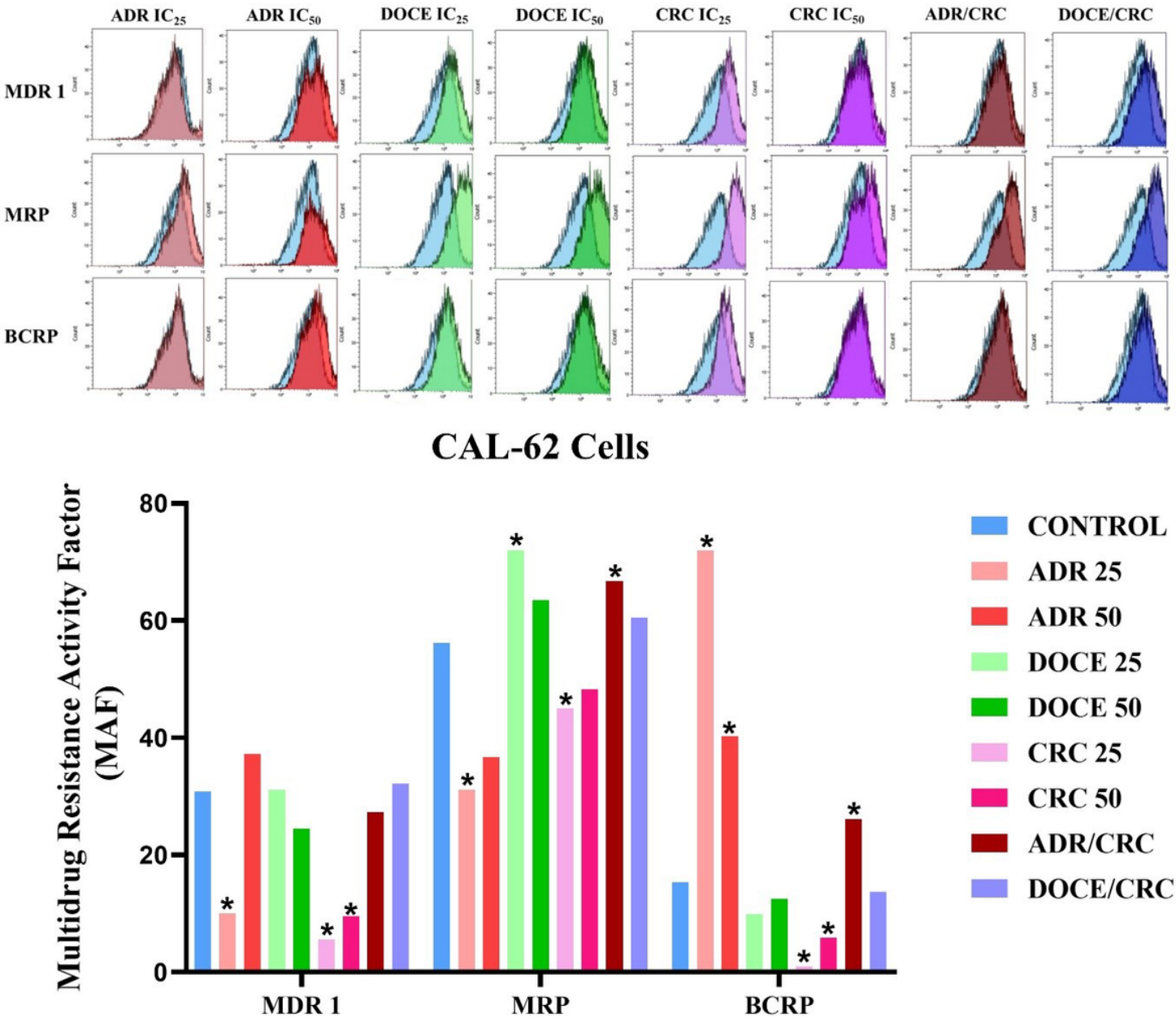

Figure 7

Multidrug resistance activity factor (MAF) values for MDR1, MRP1/2 and BCRP in CAL-62 cells after treatments (* Indicates statistical significance between control and treatments).

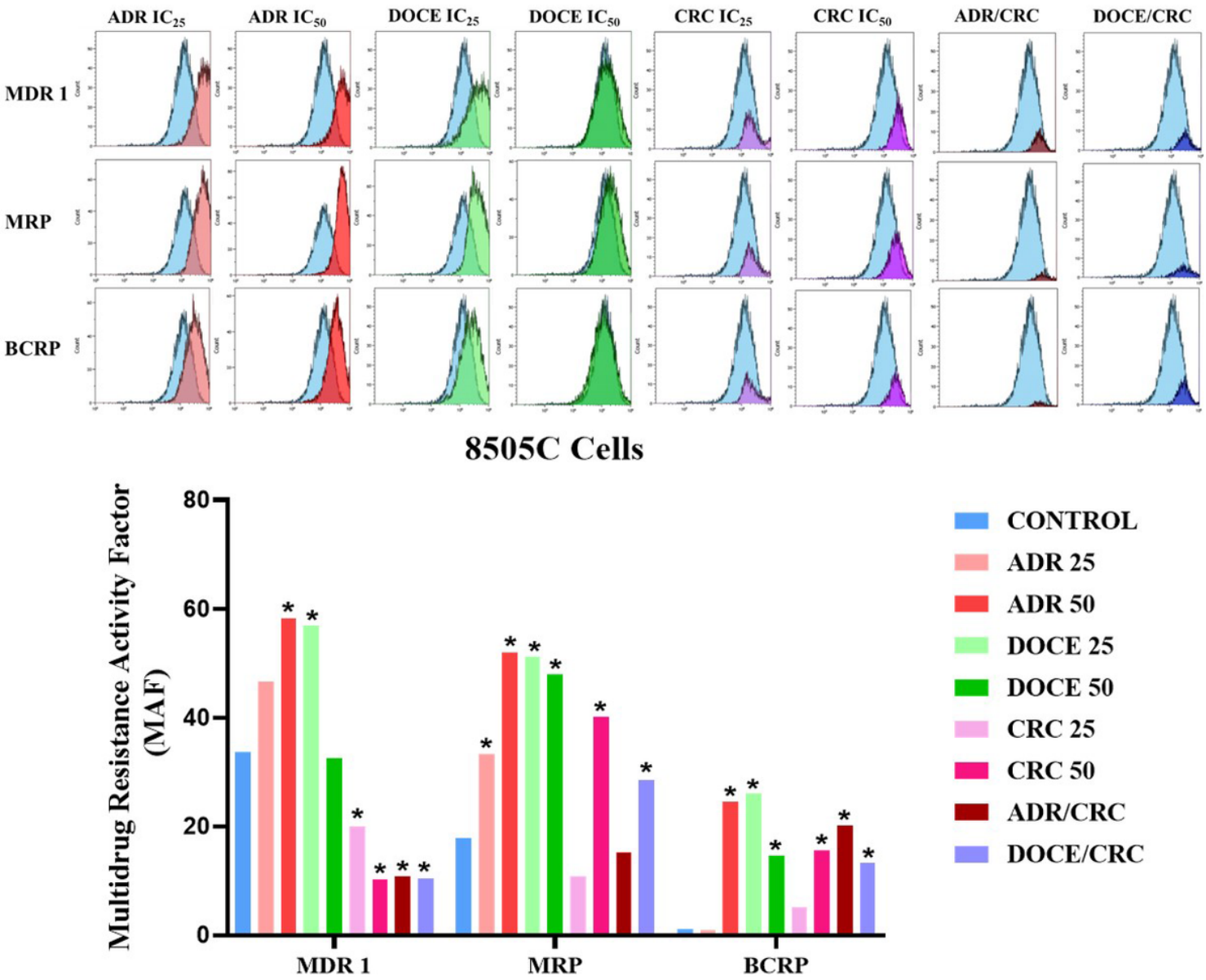

Figure 8

Multidrug resistance activity factor (MAF) values for MDR1, MRP1/2 and BCRP in 8505C cells after treatments (* Indicates statistical significance between control and treatments).