The Indole-3-carbinol Cyclic Tetrameric Derivative CTet Synergizes with Cisplatin and Doxorubicin in Triple-negative Breast Cancer Cell Lines

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Abstract. *Background/Aim: The indole-3-carbinol cyclic tetrameric derivative (CTet) inhibits breast cancer cell proliferation by endoplasmic reticulum stress and autophagy-related cell death induction, AKT/PKB (protein kinase B) activity inhibition and p53-independent overexpression of cyclin-dependent kinase inhibitor-1A (p21/CDKN1A). In the present study we evaluated the synergistic activity of CTet in combination with cisplatin and doxorubicin in triple-negative breast cancer cell lines. Materials and Methods: Synergisms were evaluated in terms of cell viability, induction of autophagy and overexpression of microtubule-associated protein-1 light chain-3 beta (MAP1LC3B) autophagy-related gene in MDA-MB-231 and BT-20 triple-negative breast cancer cells. Results: We demonstrated that CTet in combination with both cisplatin and doxorubicin synergistically inhibits cell viability and induces autophay. The MAP1LC3B gene was synergistically overexpressed in MDA-MB-231 cells treated with CTet-cisplatin combination. Moreover, the cytotoxic activity of CTet was improved in cells pre-treated with cisplatin and doxorubicin. Conclusion: This preliminary in vitro study confirms the potential of CTet as a chemopreventive agent or chemotherapeutic in combination with standard approaches for triple-negative breast cancer.*

The triple-negative (TN) breast cancer subtype is defined by lack of expression of estrogen receptor, progesterone receptor and human epidermal growth factor receptor-2 (HER2) (1). Chemotherapeutic options for TN breast cancer are limited because endocrine (*e.g.* tamoxifen) or anti-HER2 (*e.g.* trastuzumab) therapies are not effective, and clinicians currently employ combinations of chemotherapeutic agents that include anthracyclines, taxanes and the DNA-damaging platinums (1-3). However, the biological heterogeneity within TN breast cancer underlines the presence of a chemosensitive subgroup, lacking in a standardized approach, and a cytotoxic-resistant subgroup that is in urgent need of new therapies (1).

Indole-3-carbinol (I3C), an anticancer agent produced from cruciferous vegetables, and its oligomeric derivatives have been widely studied (4, 5). We have shown that the I3C cyclic tetrameric derivative (CTet) inhibits cell proliferation of both estrogen receptor-positive and TN breast cancer cell lines (6, 7) and arrested tumor growth in a xenograft study (8). CTet also induced endoplasmic reticulum (ER) stress (9), followed by cell accumulation in the G_2/M phase and autophagy-related cell necrosis (8, 9). The up-regulation of key signaling molecules involved in the ER stress response and autophagy, the overexpression of cyclin-dependent kinase inhibitor-1A (p21/CDKN1A) and growth arrest and DNA-damage-inducible protein alpha (GADD45A) and the inhibition of AKT/PKB (protein kinase B) activity were identified as the main upstream molecular mechanisms through which CTet inhibits cell proliferation (8, 9).

Here we evaluated the synergistic activity of CTet in combination with molecules that inhibit cell proliferation by different mechanisms of action, namely cisplatin (DNA cross-linking) and doxorubicin (DNA intercalation, topoisomerase-II inhibition, iron-mediated oxidative damage) in TN breast cancer cell lines MDA-MB-231 and BT-20.

Materials and Methods

Cell cultures and agents. The TN human breast cancer cell lines MDA-MB-231 and BT-20 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 g/l non-essential amino acid, 50 mg/l streptomycin, 1,000 U/l penicillin at 37˚C in a humidified incubator with 5% CO_2. All cell culture materials were purchased from Sigma-Aldrich (St. Louis, MO, USA). CTet was synthesized and formulated with aqueous solution of γ-cyclodextrin (CAVAMAX®; Wacker, Munich,

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Germany), as reported by Lucarini *et al*. (7). In all experiments, anticancer agents were two-fold diluted in cell culture medium and directly used in cell treatments.

Cell viability assay. Triplicate samples of 5×10^3 cells in 96-well plates were treated for 72 h with increasing concentrations of CTet, cisplatin and doxorubicin, alone or in combination. Cell viability was evaluated using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA), as reported previously (9). The results are expressed as the relative number of viable cells in treated samples respect to controls (untreated cells).

Synergy quantification. Combination index: Combination Indices (CI) and inhibitory concentration of viability at 50% (IC_{50}) values were estimated using CalcuSyn software (Biosoft, Cambridge, UK) based on the method of Chou and Talalay (10, 11). Output results are represented by CI plots as a function of the fractional effect (F_a). CI values <1 indicate synergy, =1 additivity and >1 antagonism (11). Response surface analysis: Data from the cell viability assay (expressed as relative cell viability) in single-drug treatments were interpolated following equation 1 (Prism5; GraphPad Software, Inc., La Jolla, CA, USA):
(Eq. 1)

$$y = 1 - \frac{Top \times x^{HillSlope}}{\left(EC_{50}^{HillSlope} + x^{HillSlope}\right)}$$

the maximal effect, (*Top*), the concentration needed to achieve 50% of the maximal effect (EC_{50}) and the sigmoidicity of the curve (*HillSlope*) values were used to determine the null interaction surface by effect summation following equation 2 (Mathematica 5.2, Wolfram Research, Champaign, IL, USA):
(Eq. 2)

$$z = 1 - \frac{Top_A \times x_A^{HillSlope_A}}{\left(EC_{50-A}^{HillSlope_A} + x_A^{HillSlope_A}\right)} - \frac{Top_B \times y_B^{HillSlope_B}}{\left(EC_{50-B}^{HillSlope_B} + y_B^{HillSlope_B}\right)}$$

where $Top_{A/B}$ is the maximal effect of drug A/B, $x_{A/B}$ is the concentration of drug A/B, $HillSlope_{A/B}$ is the sigmoidicity of the curve of drug A/B, and $EC_{50\text{-}A/B}$ is the concentration of drug A/B needed to achieve 50% of the maximal effect. The volumes under the planes (VUP) of the expected and observed surfaces were computed by integration and interpolation, respectively (Mathematica 5.2, Wolfram Research). The drug interaction was computed as the ratio $VUP_{observed}/VUP_{expected}$, indicating synergy and antagonism at values <1 and >1, respectively (12).

Detection and quantification of acidic vesicular organelles (AVOs). As a marker of autophagy, the appearance and volume of AVOs were analyzed by acridine orange staining (13). Briefly, 5×10^5 MDA-MB-231 and BT-20 cells were seeded in a 60 mm diameter dish to attach overnight. Cells were then treated with CTet, cisplatin and doxorubicin, alone or in combination and stained with 1 μg/ml acridine orange for 15 minutes as described by Galluzzi *et al*. (9). Fluorescent micrographs were taken using a fluorescent microscope (Blue excitation filter). To quantify autophagy induction, the images were processed using Photoshop software (Adobe Systems, San Jose, CA, USA). To selectively quantify red signals (AVOs), the red channel of entire images was amplified and shadow red signal was removed using the channel mixer and color balance tools, respectively. AVOs were then quantified as red/green ratio by computing the red signal output (AVOs) normalized to the green signal output (cytoplasm and nucleus).

RNA extraction and cDNA synthesis. MDA-MB-231 cells were treated with CTet, cisplatin and doxorubicin, alone or in combination, for 24 h. Total RNA was extracted using the RNeasy plus kit (Qiagen, Hilden, Germany), and quantified using a spectrophotometer UV-2401 PC (Shimadzu, Kyoto, Japan). The cDNA was synthesized from 1 μg of total RNA using M-MuLV Reverse transcription Kit (Diatheva, Fano, Italy) with oligo-dT priming.

Real time-quantitative PCR (RT-qPCR). RT-qPCR primers for amplification of microtubule-associated protein-1 light chain-3 beta (*MAP1LC3B*), and the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and actin-β (*ACTB*), have been described elsewhere (9). RT-qPCR was performed in a final volume of 20 μl using Hot-Rescue Real-time PCR mix containing SYBR green, 0.025 U/μl Hot-Rescue Plus DNA polymerase (Diatheva), 200 nM each primer and 0.7 μl synthesized cDNA. PCR reactions were run in triplicate in a RotorGene 6000 instrument (Corbett Life Science, Sydney, Australia), with the following amplification conditions: 95˚C for 10 min, 40 cycles at 95˚C for 15 s and 60˚C for 50 s. A triplicate non-template control was included for each primer pair. Relative mRNA expression was calculated using the comparative quantification application of the RotorGene 6000 software, using *GAPDH* as a reference gene. *ACTB* was used as second housekeeping gene on selected samples to confirm results obtained with *GAPDH*.

Statistical analyses. Statistical differences were evaluated using the Mann-Whitney test ($p<0.01$). Statistical analysis of gene expression analysis was performed using an unpaired *t*-test with Welch correction (InStat; GraphPad software).

Results

Synergy quantification by the Chou and Talalay method. IC_{50} values were determined by the median effect equation (14), resulting in values of 3.23±0.26 (r=0.96), 12.00±0.36 (r=0.99) and 0.16±0.02 (r=0.98) μM for CTet, cisplatin and doxorubicin, respectively, in MDA-MB-231 cells and 5.66±0.11 (r=0.99), 5.27±0.13 (r=0.98) and 0.33±0.06 (r=0.99), respectively, in BT-20 cells. These IC_{50} concentrations were then used to define fixed ratios (equipotency ratio) for subsequent interaction studies and CI evaluation.

F_a-CI plots of CTet-cisplatin and CTet-doxorubicin combinations for MDA-MB-231 and BT-20 are shown in Figure 1A. The results show that all drug combinations gave

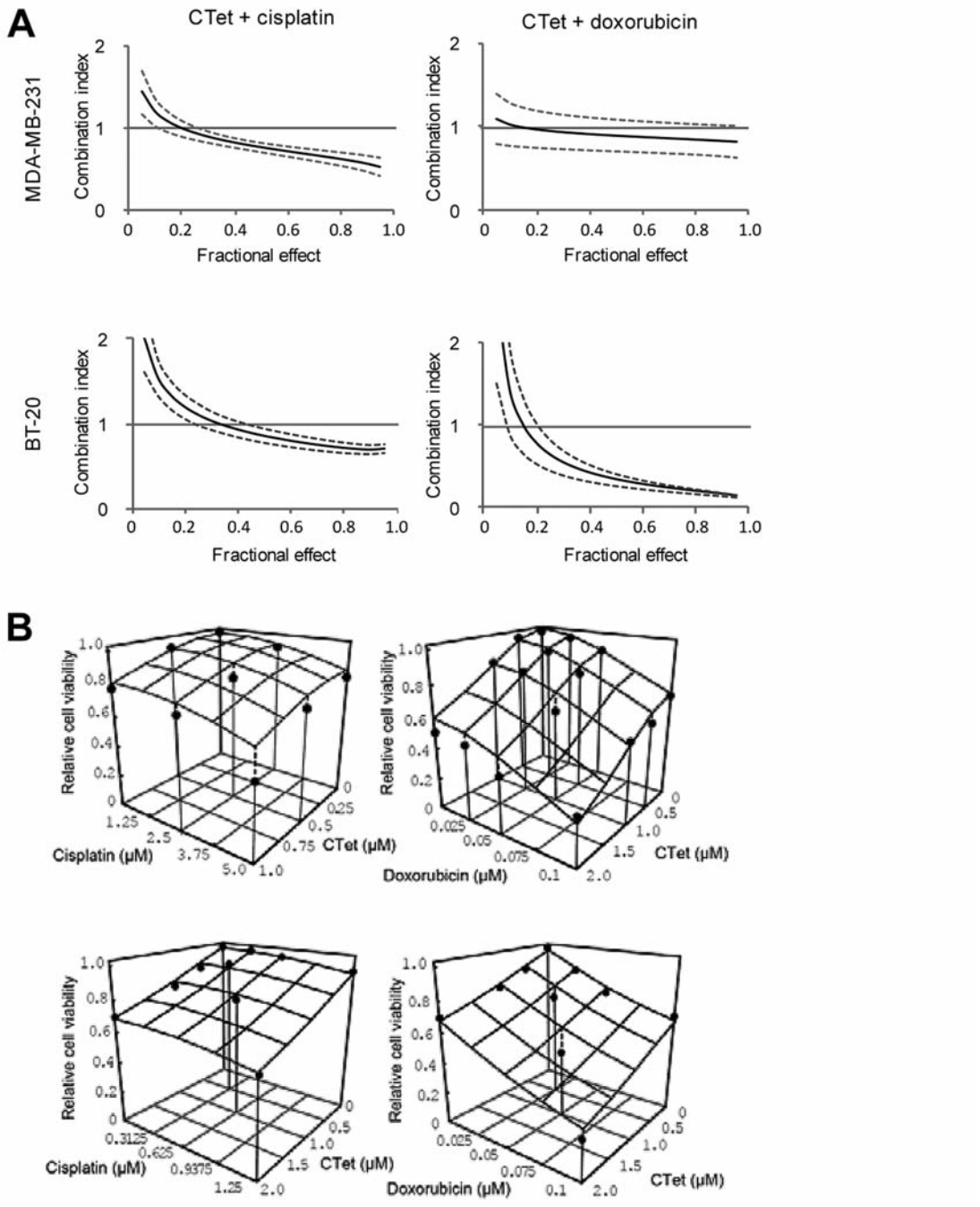

Figure 1. *A: Synergy quantification of CTet-cisplatin (1:5 ratio) and CTet-doxorubicin (1:0.05 ratio) combinations using the Chou and Talalay method. Data are expressed as combination indices (CI)±SD as a function of fractional effect (Fa). CI values <1 indicate synergy, =1 additivity and >1 antagonism. B: Synergy quantification using the response surface analysis. The expected combined effect of the drug combinations are represented as three-dimensional response surfaces, computed as described in Matherials and Methods. Data points (observed effect) below the response surface (observed activity is more than expected activity) indicate synergy.*

a synergistic effect in both MDA-MB-231 and BT-20 cells. A good synergy was observed in MDA-MB-231 cells treated with CTet-cisplatin (1:5 constant ratio) and in BT-20 cells treated with CTet-doxorubicin (1:0.05 constant ratio).

Synergy quantification by response surface analysis. To better-analyze the interaction between CTet, cisplatin and doxorubicin, we analyzed data using the null interaction surface approach (15). Non-linear regression analyses

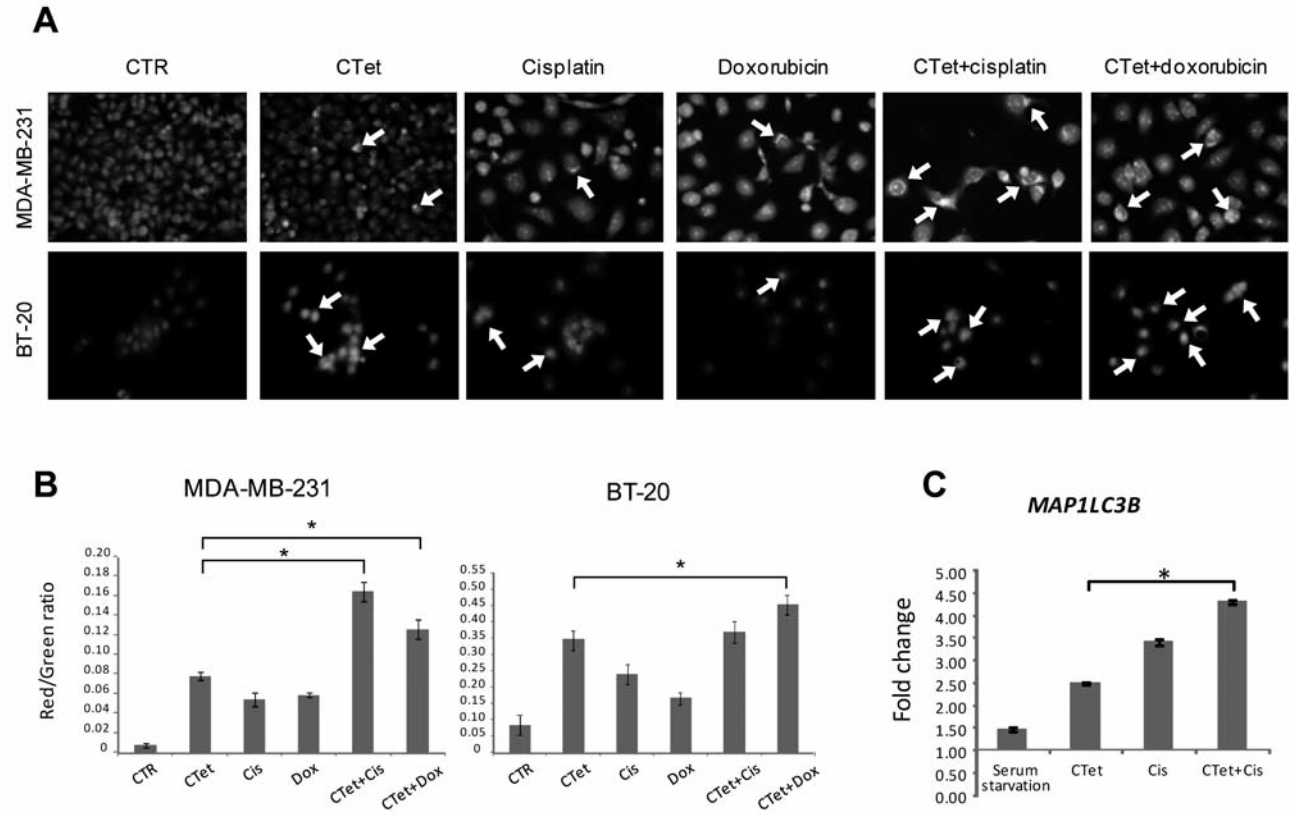

Figure 2. *CTet in combination with cisplatin (Cis) and doxorubicin (Dox) synergistically induce autophagy in triple-negative (TN) cell lines. A: Cells were treated with 2 μM CTet, 10 μM (MDA-MB-231) and 1.25 μM (BT-20) cisplatin, and 0.1 μM doxorubicin for 72 h and stained with acridine orange. Bright red granules (white arrows) are indicative of acidic vesicular organelles (AVO), considered as markers of autophagy. Images are representative of two experiments. B: AVO quantification. At least three images from each sample were selected and processed with Photoshop for AVO quantification and red/green ratio evaluation. Data are means±SEM; * Significantly different at p<0.01. C: Gene expression analysis of microtubule-associated protein-1 light chain-3 beta (MAP1LC3B) autophagy-related gene. Quantitative real-time polymerase chain reaction was carried out on MDA-MB-231 cells treated with 8 μM CTet, 40 μM cisplatin and the relative combination for after 24 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Data are shown as mean±SD. Asterisks indicate statistically significant values at p<0.01. CTR is Control.*

allowed us to determine Top, EC_{50} and *HillSlope* values for each drug effect, useful in calculating the response surfaces (not shown). The expected combined effect of the drug combinations are represented as three-dimensional response surfaces (Figure 1B) with two horizontal axes (individual drug concentrations) and a vertical axis (expected effect of drug combination). The observed effect of drug combinations are represented as data points in a three-dimensional space. Data points below the response surface (observed activity is greater than expected activity) indicate synergy. Results show that the experimental data point fell below the expected response surface markedly for CTet-cisplatin combinations in MDA-MB-231 cells and CTet-doxorubicin combinations in BT-20 cells (Figure 1B), confirming the synergistic interactions evaluated using the Chou and Talalay method. The computation of $VUP_{observed}$/ $VUP_{expected}$ confirmed the synergistic interactions, resulting in 0.97 and 0.99 ratios for CTet-cisplatin and CTet-doxorubicin combinations, respectively, in MDA-MB-231 cells.

Effect of CTet, cisplatin, doxorubicin and their combinations on autophagy induction. Synergistic activity of CTet-cisplatin and CTet-doxorubicin combinations were also evaluated in terms of autophagy induction in MDA-MB-231 and BT-20 cells. The results in Figure 2A confirm the induction of autophagy in CTet-treated cells (8, 9), while few autophagic cells were observed in cisplatin- and doxorubicin-treated cells. On the other hand, the combination treatments showed a marked induction of autophagy in both CTet-cisplatin- and CTet-doxorubicin-treated cells. The red/green ratio computation allowed us to quantify the AVO formation, showing that the autophagic response was significantly enhanced with the combination treatment with respect to CTet-alone (p<0.01) (Figure 2B).

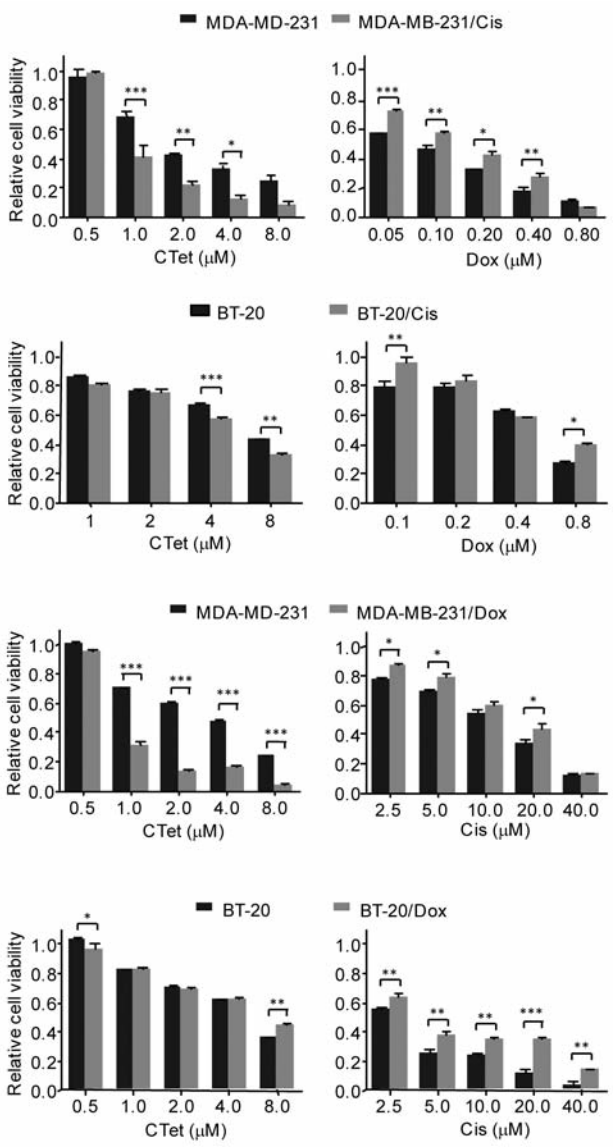

Figure 3. *CTet activity in triple-negative (TN) breast cancer cell pre-treated with cisplatin (MDA-MB-231/Cis; BT-20/Cis) and doxorubicin (MDA-MB-231/Dox; BT-20/Dox). Cells were treated with cisplatin (1 and 5 μM for MDA-MB-231 and BT-20 cells, respectively) and doxorubicin (0.1 μM) for 72 h, trypsinized and plated at 5×10^3 in 96- well plates. Cells were then treated with CTet, cisplatin (Cis), and doxorubicin (Dox) at the indicated concentration for 72 h, and cell viability was evaluated. Data are means±SEM. Differences were evaluated by two-way ANOVA followed by Bonferroni post-test; *p<0.05; **p<0.01; ***p<0.001.*

MAP1LC3B overexpression. The expression profiles of *MAP1LC3B* autophagy-related gene were assessed in MDA-MB-231 cells treated with CTet, cisplatin, or a combination of both, for 24 h. Untreated cells and cells exposed to serum starvation for 24 h were used as control. The experiment was repeated twice, and each RT-qPCR was run in triplicate. Figure 2C represents the mean±SD of the two experiments.

The *MAP1LC3B* transcript levels increased significantly with all treatments ($p<0.01$), whether compared to untreated cells or to cells exposed to serum starvation. Moreover, the expression in cells treated with both CTet and cisplatin was significantly higher ($p<0.01$) compared to the levels reached with CTet or cisplatin alone, confirming the synergistic effect of these drugs also at the transcription level. On the other hand, the overexpression of *MAP1LC3B* gene was not revealed in doxorubicin-treated cells and the CTet-doxorubicin combination did not increase *MAP1LC3B* overexpression induced by CTet (not shown).

Effect of CTet on cisplatin- and doxorubicin-pretreated cells. The activity of CTet was evaluated in TN breast cancer cells pre-treated for 72 h with cisplatin (MDA-MB-231/Cis, BT-20/Cis) or doxorubicin (MDA-MB-231/Dox, BT-20/Dox). As shown in Figure 3, CTet cytotoxic activity was improved in MDA-MB-231 and BT-20 cells pre-treated with both cisplatin and doxorubicin. Interestingly, a decrease of the activity of doxorubicin in MDA-MB-231/Cis and BT-20/Cis, and cisplatin in MDA-MB-231/Dox and BT-20/Dox was observed, underlining the importance of using drugs with different mechanisms of action in combined therapies.

Discussion

The current options for TN breast tumors include chemotherapeutic drugs such as anthracyclines, taxanes and the DNA-damaging platinums, and combination approaches have been described and currently ongoing in several clinical trials (1).

We showed that the I3C cyclic tetrameric derivative CTet is a potent anticancer agent against both estrogen receptor-positive and TN breast cancer cell lines (6-9). In this study, we demonstrated that CTet synergizes with cisplatin and doxorubicin in TN breast cancer cells. Since drug combination analysis is controversial (16), we analyzed the data using a dual mathematical approach. The most commonly used method, based on the median-effect equation derived from the mass-action law principle, provides the theoretical basis for the CI-isobologram equation that allows for quantitative determination of synergism (10,11, 14). The alternative approach based on the non-linear regression fitting and response surface analysis, has been described by Greco *et al*. (15) and, more recently, Boucher and Tam proposed a mathematical formulation of additivity based on surface response computation by individual drug effect summation (17). Synergisms reported here were evaluated using both methods, giving similar results.

Given that the main mechanism of CTet activity is the induction of ER stress and subsequent autophagic-related cell death (9), the synergy between CTet, cisplatin and doxorubicin was also demonstrated in terms of autophagy

induction. The quantification of AVO formation reflects the major synergism between CTet and cisplatin in MDA-MB-231 cells and between CTet and doxorubicin in BT-20 cells. Moreover, the synergistic effect in the up-regulation of autophagy-related gene *MAP1LC3B* was significant for CTet and cisplatin in MDA-MB-231 cells.

The enlarged and flattened cell morphology after doxorubicin and cisplatin exposure indicates the induction of senescence (18). Interestingly, combination treatments resulted in both autophagic and senescence morphological features that could represent the mechanism of synergistic inhibition of cell viability.

Triggering the autophagic pathway has been recently considered as a strategy for cancer prevention due to its role in limiting the accumulation of cellular damage to proteins, organelles and DNA, all of which can contribute to mutation and initiate transformation (19). Our results indicate that the TN breast cancer cells pre-treated with DNA-intercalating agents are more sensitive to CTet but not to cisplatin and doxorubicin. This suggests that autophagy-inducers such as CTet could have a role in the prevention of drug resistance and in chemoprevention of tumorigenesis.

In conclusion, this preliminary *in vitro* study demonstrates the synergistic activity of the I3C cyclic tetrameric derivative CTet in combination with cisplatin and doxorubicin in TN breast cancer cells in terms of cell viability, induction of autophagy and overexpression of *MAP1LC3B*, confirming CTet as promising chemopreventive and chemotherapeutic agent. Further studies are needed to evaluate the synergistic effect of CTet and DNA-intercalating agents in xenograft studies.

References

1 Oakman C, Viale G and Di Leo A: Management of triple negative breast cancer. Breast *19(5)*: 312-321, 2010.

2 Dawood S: Triple-negative breast cancer: Epidemiology and management options. Drugs *70(17)*: 2247-2258, 2010.

3 De Laurentiis M, Cianniello D, Caputo R, Stanzione B, Arpino G, Cinieri S, Lorusso V and De Placido S: Treatment of triple-negative breast cancer (TNBC): Current options and future perspectives. Cancer Treat Rev *36(Suppl 3)*: S80-86, 2010.

4 Aggarwal BB and Ichikawa H: Molecular targets and anticancer potential of indole-3-carbinol and its derivatives. Cell Cycle *4(9)*: 1201-1215, 2005.

5 Weng JR, Tsai CH, Kulp SK and Chen CS: Indole-3-carbinol as a chemopreventive and anticancer agent. Cancer Lett *262(2)*: 153-163, 2008.

6 Brandi G, Paiardini M, Cervasi B, Fiorucci C, Filippone P, De Marco C, Zaffaroni N and Magnani M: A new indole-3-carbinol tetrameric derivative inhibits cyclin-dependent kinase 6 expression, and induces G1 cell cycle arrest in both estrogen-dependent and estrogen-independent breast cancer cell lines. Cancer Res *63(14)*: 4028-4036, 2003.

7 Lucarini S, De Santi M, Antonietti F, Brandi G, Diamantini G, Fraternale A, Paoletti MF, Tontini A, Magnani M and Duranti A: Synthesis and biological evaluation of a γ-cyclodextrin-based formulation of the anticancer agent 5,6,11,12,17,18,23,24-octahydrocyclododeca[1,2-b:4,5-b':7,8-b'':10,11-b''']tetraindole (CTet). Molecules *15*: 4085-4093, 2010.

8 De Santi M, Galluzzi L, Lucarini S, Paoletti MF, Fraternale A, Duranti A, De Marco C, Fanelli M, Zaffaroni N, Brandi G and Magnani M: The indole-3-carbinol cyclic tetrameric derivative CTet inhibits cell proliferation via overexpression of p21/CDKN1A in both estrogen receptor-positive and triple-negative breast cancer cell lines. Breast Cancer Res *13(2)*: R33, 2011.

9 Galluzzi L, De Santi M, Crinelli R, De Marco C, Zaffaroni N, Duranti A, Brandi G and Magnani M: Induction of endoplasmic reticulum stress response by the indole-3-carbinol cyclic tetrameric derivative CTet in human breast cancer cell lines. PLoS One *7(8)*: e43249, 2012.

10 Chou TC and Talalay P: Quantitative analysis of dose effect relationships: The combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul *22*: 27-55, 1984.

11 Chou TC: Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev *58(3)*: 621-681, 2006.

12 Tam VH, Schilling AN, Lewis RE, Melnick DA and Boucher AN: Novel approach to characterization of combined pharmacodynamic effects of antimicrobial agents. Antimicrob Agents Chemother *48(11)*: 4315-4321, 2004.

13 Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D and Yahalom J: A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res *61(2)*: 439-444, 2001.

14 Chou TC: Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res *70(2):* 440-446, 2010.

15 Greco WR, Park HS and Rustum YM: Application of a new approach for the quantitation of drug synergism to the combination of cis-diamminedichloroplatinum and 1-β-D-arabinofuranosylcytosine. Cancer Res 50(17): 5318-5327, 1990.

16 Greco WR, Bravo G and Parsons JC: The search for synergy: A critical review from a response surface perspective. Pharmacol Rev *47(2)*: 331-385, 1995.

17 Boucher AN and Tam VH: Mathematical formulation of additivity for antimicrobial agents. Diagn Microbiol Infect Dis *55(4)*: 319-325, 2006.

18 Sullivan R, Pare GC, Frederiksen LJ, Semenza GL and Graham CH: Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity. Mol Cancer Ther *7(7)*: 1961-1973, 2008.

19 Steeves MA, Dorsey FC and Cleveland JL: Targeting the autophagy pathway for cancer chemoprevention. Curr Opin Cell Biol *22(2)*: 218-225, 2010.

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