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 autophagy. 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 platinum derivatives (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 G2/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% CO2. 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, Germany).
of the maximal effect (EC50Top), the concentration needed to achieve 50% of the maximal effect (EC50) and the sigmoidicity of the curve (HillSlope) values were used to determine the null interaction surface by effect summation following equation 1 (Prism5; GraphPad Software, Inc., La Jolla, CA, USA):

\[
y = 1 - \frac{\text{Top} \times x^{\text{HillSlope}}}{(\text{EC}_{50}^{\text{HillSlope}} + x^{\text{HillSlope}})}
\]

the maximal effect, (Top), the concentration needed to achieve 50% of the maximal effect (EC50) 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):

\[
z = 1 - \frac{\text{Top}_A \times x_A^{\text{HillSlope}_A}}{(\text{EC}_{50-A}^{\text{HillSlope}_A} + x_A^{\text{HillSlope}_A})} - \frac{\text{Top}_B \times x_B^{\text{HillSlope}_B}}{(\text{EC}_{50-B}^{\text{HillSlope}_B} + x_B^{\text{HillSlope}_B})}
\]

where TopAB is the maximal effect of drug A/B, xAB is the concentration of drug A/B, HillSlopeAB is the sigmoidicity of the curve of drug A/B, and EC50-AB 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 VUPobserved/VUPexpected 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 × 10⁵ 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. IC50 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 IC50 concentrations were then used to define fixed ratios (equi potency ratio) for subsequent interaction studies and CI evaluation.

F2 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
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
allowed us to determine Top, EC50 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 VUPobserved/ VUPexpected 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).
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


Received March 7, 2013
Revised April 16, 2013
Accepted April 17, 2013