Abstract. Background/Aim: Breast cancer is characterized by a high rate of mortality and is considered one of the deadliest types of cancer. It is of note that (–)-epigallocatechin-3-gallate (EGCG), the principal catechin of green tea, is able to hinder the growth of MDA-MB-231 breast cancer cells by influencing different signaling pathways, including apoptosis. Furthermore, EGCG is also used in the treatment of bone cancer pain. Tapentadol, an opioid drug acting at the level of noradrenaline (norepinephrine) reuptake inhibition and μ-opioid receptor, is able to modulate bone cancer pain and influence cancer cell viability by regulating apoptosis. Materials and Methods: In vitro assays were performed on triple-negative MDA-MB-231 cells treated with tapentadol (1, 5, 10, 20, 40 and 80 μg/ml) and EGCG (1, 10, 20, 40, 80, 160 μmol/l), alone and in combination. The effects of EGCG and TAP on viability were determined by wound-healing and MTT assays, while cell migration was assessed by transwell migration. Results: Cell proliferation, viability and apoptosis of MDA-MB-231 cells were impaired by the combination of EGCG and tapentadol. Specifically, our data show that EGCG and TAP reduced the proliferation of MDA-MB-231 cells by impairing cell-cycle progression (p<0.05). These findings suggest that the combination of these substances may represent a new strategy for the treatment of patients suffering from triple-negative breast cancer.

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*These Authors contributed equally to this study.

Correspondence to: Sabrina Bimonte, Division of Anesthesia and Pain Medicine, Istituto Nazionale Tumori, IRCCS Fondazione G. Pascale, Naples, Italy. E-mail: s.bimonte@istitutotumori.na.it

Key Words: (–)-Epigallocatechin-3-gallate, tapentadol, triple-negative breast cancer, cell proliferation, apoptosis.
Materials and Methods

Materials. Tapentadol hydrochloride was obtained by Grünenthal (Aquisgrana, Germany). Different doses of tapentadol (1, 5, 10, 20, 40 and 80 mg/ml) and EGCG (1, 10, 20, 40, 160 μM) were employed for in vitro experiments on MDA-MB-231 cells. EGCG was purchased from Sigma. Antibody to p53 was purchased from Imgenex (San Diego, CA, USA). 3,3-Diaminobenzidine, antibiotics (penicillin and streptomycin), Dulbecco’s modified Eagle’s medium (DMEM), and fetal bovine serum were purchased from Invitrogen (Grand Island, NY, USA). Hydroxymethyl (Tris), glycine, sodium chloride (NaCl), sodium dodecyl sulfate (SDS), and bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies to p53, cyclin D1, and anti-actin were obtained by Imgenex (San Diego, CA, USA).

Cell culture. The human ER-negative breast cancer cell line MDA-MB-231 was purchased from the American Type Culture Collection (ATCC Manassas, VA, USA). Cells were cultured in DMEM (Invitrogen) supplemented with antibiotics (penicillin 100 U/ml; streptomycin 100 U/ml), 10% heat-inactivated fetal bovine serum, and L-glutamine (2 mmol/l) in a humidified atmosphere at 37˚C. Wound-healing assay. The assay was performed as previously described (21) to test the migration of MDA-MB-231 cells (~70-80% confluence as a monolayer and at density of 0.3×10^6).

Proliferation assay. The effect of EGCG and tapentadol on cell proliferation was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Trevena, Gaithersburg, MD, USA), as reported by Bimonte et al. (21). The cells (~70-80% confluence as a monolayer) were incubated at density of 2×10^3 per well.

Cell invasion. MDA-MB-231 cells (5×10^4 of density and ~70-80% confluence as a monolayer) were cultured in DMEM and cell invasion was performed as described by Justus et al. (26).

In vitro apoptosis assay by flow cytometry. Cells were washed and suspended in 0.5 ml of phosphate-buffered saline, fluorescein isothiocyanate-dextran, annexin V and propidium iodide (PI). The in vitro assay was performed as described by Bimonte et al. (15).

Cell-cycle analysis. The cells were seeded at 3×10^5 cells per well into 6-well plates and incubated overnight at 37˚C in a CO₂ incubator (15). The cells were then washed with phosphate-buffered saline and treated with medium as control or with EGCG (40 μM), tapentadol (20 mg/ml), or their combinations (40 μM EGCG plus 20 mg/ml tapentadol), and incubated for 24 and 48 h. Analysis was performed as described by Bimonte et al. (15).

Figure 1. Effects of (−)-epigallocatechin-3-gallate (EGCG) and tapentadol (TAP) on migration of MDA-MB-231 cells. MDA-MB-231 cells were incubated in Dulbecco’s modified Eagle’s medium alone as control (CTR) or with 40 μmol/l EGCG, 20 mg/ml TAP, or their combination. After 48 h, the inhibitory effect of the two substances was clearly evident with respect to single substances and to controls. Values are the means±SE for three independent experiments. *Significantly different from the control at p<0.05.
Data were analyzed by using one-way analysis of variance (ANOVA) test and expressed as the mean values of at least three independent replications (*p*<0.05).

## Results

The effects of EGCG and tapentadol on the migration and the proliferation of MDA-MB-231 cells. A wound-healing assay was performed to assess the effects of EGCG and tapentadol on MDA-MB-231 cell migration. The data showed that EGCG and tapentadol alone retarded the migration of MDA-MB-231 cells at 48 h (*p*<0.05). The effect was significantly potentiated when the substances were combined (*p*<0.05) (Figure 1). We assessed whether EGCG and tapentadol is able to inhibit the proliferation of MDA-MB-231 cells by performing an MTT assay. A reduction in cellular proliferation after 48 h of treatment with all combinations was observed, as reported in Figure 2.

Data obtained by the invasion test showed that treatment with EGCG plus tapentadol effectively inhibited migration of MDA-MB-231 cells, which was less than those of MDA-MB-231 cells treated with single substances and controls without substances (Figure 3; *p*<0.0001).

Our results show that EGCG and tapentadol reduced the proliferation and the invasion of MDA-MB-231 at high doses, thus suggesting that toxicity may have caused cell death.

### Table I. Analysis of cell-cycle distribution of MDA-MB-231 cells treated with (−) epigallocatechin-3-gallate (EGCG), tapentadol (TAP), and both agents together.

| Treatment                                    | G0/G1 | S   | G2/M |
|----------------------------------------------|-------|-----|------|
| Control                                      | 61    | 12  | 24   |
| DMSO                                         | 62    | 12  | 24   |
| 40 μM EGCG                                   | 40    | 26  | 26   |
| 20 μM TAP                                    | 63    | 9   | 21   |
| 40 μM EGCG plus 20 μM TAP                   | 49    | 10  | 20   |

DMSO: Dimethyl sulfoxide.

### Figure 2. (−)-Epigallocatechin-3-gallate (EGCG) and tapentadol (TAP) inhibit the proliferation of MDA-MB-231 cells. MTT assay showed a dose-dependent inhibition of the viability of cells treated for 48 h with 40 μmol/l EGCG or with 20 mg/ml TAP, or their combination and TAP. A stronger effect was observed using the combined treatment. Values are the means±SE for three independent experiments. Significantly different at concentration of 40, 80 and 160 μM for EGCG and 20, 40 and 80 mg/ml for TAP *p*<0.05, **p*<0.01 and ***p*<0.001 by analysis of variance.

### Figure 3. (−)-Epigallocatechin-3-gallate (EGCG) and tapentadol (TAP) are able to inhibit the invasion of MDA-MB-231 cells at 48 h. A stronger effect was observed in all groups of treatment ***Significantly different compared with control at *p*<0.001 by analysis of variance. Values are the means±SE for three independent experiments.
The effects of EGCG and tapentadol on MDA-MB-231 cell apoptosis and cell-cycle progression. Flow cytometry was also performed to test the apoptosis of MDA-MB-231 cells treated with EGCG alone, tapentadol alone and both substances combined. Data showed that the percentage of cell death was higher in the group treated with combinations compared with respect to that observed in the groups with single treatment (Figure 4 and Table I).

Discussion

Our experimental in vitro studies highlighted findings on the effects of EGCG and tapentadol on TNBC cell growth. It is of note that conventional therapies adopted to treat patients with ER-positive breast cancer cannot be successfully applied to patients with ER-negative or TNBC due to the lack of expression of ER, progesterone receptor, or human epidermal growth factor receptor type 2, thus suggesting an urgent need for alternative strategies for treatment of patients with breast cancer. Several studies shed light on new possible therapeutic approaches for breast cancer management, based on the combination of natural compounds and conventional drugs. EGCG is able to regulate breast cancer progression by inducing the apoptosis and by inhibiting cell cycle progression (27). On the basis of the results obtained by using morphine on MDA-MB-231 cells, we decided to select the TAP as alternative opioids to morphine in vitro experiments on MDA-MB-231 cells. Here we evaluated the efficacy and synergism of EGCG and tapentadol against breast cancer cells. Our findings indicate that the combination of EGCG and tapentadol potentiates the antiproliferative effects of the single substances by apoptosis induction in MDA-MB-231 cells and by affecting cell-cycle progression. It is important to underline that refinement of experiments performed in further breast cancer cell lines are desperately needed to set out the precise in vitro dosages of both substances, in order to be translated into in vivo animal models.

Several points can be extrapolated from the results of these in vivo studies in order to translate findings to clinical practice. For instance, although in our experiments, the effect of the combined treatments showed synergistic effects, significant inhibition of migration, proliferation and cell-cycle progression in MDA-MB-231 cells, was observed supra-physiological at concentrations. Consequently, other experiments conducted using different concentrations are needed in order to make it possible to translate our results into clinical practice. Furthermore, because tapentadol acts on mu-opioid receptors (MORs) it should be investigated whether or not MDA-MB-231 cells express MORs. In addition, another point of discussion regards the possibility that MORs are implicated in the cytotoxic effect. On these bases, the potential effects of EGCG (regulation/modulation) on MOR expression should be better studied, thus other studies should be conducted in order to explain the mechanisms underlying our results.

Conclusion

Our data suggest that EGCG and tapentadol reduced the proliferation of MDA-MB-231 cells by inhibiting the progression of the cell cycle. This combination could be viewed as a new positive strategy for TNBC treatment. To our knowledge, this is a unique report of the combinatorial inhibitory effect of EGCG and tapentadol on MDA-MB-231 cancer cell growth. More experiments are needed to elucidate the signaling pathways in the regulation of growth of breast cancer cells affected by EGCG and tapentadol.
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Authors’ Contributions

BS, MC and AB are the main authors of the study. AC and CA were responsible for the coordination of this study. All Authors read and approved the final article.

Conflicts of Interest

The Authors declare that they have no competing interests in regard to this study.

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