Morphine counteracts the effects of paclitaxel in triple-negative breast cancer cells

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Background & objectives: Several studies have provided evidence that opioids may play a role in cancer recurrence and metastasis. Multiple research data indicate that morphine can act as a proliferative or suppressive agent on tumour cells depending on the applied concentration. Therefore, this study was aimed to investigate whether the presence of clinically relevant concentrations of morphine has any effect on the efficacy of paclitaxel, a widely used chemotherapeutic drug, on the viability and apoptosis of human triple-negative breast cancer cell line.

Methods: MDA.MB.231 cells were treated with paclitaxel in the presence or absence of morphine and examined for cell proliferation by the MTT assay. In addition, the effect of morphine on paclitaxel-induced apoptosis was investigated by flow cytometric assay and by the ratio of Bax/Bcl-2 mRNA expression levels with quantitative real-time (qRT)-PCR.

Results: Morphine significantly increased the proliferation of breast cancer cells at low concentrations (0.1-2.5 µM) but higher concentrations showed cytotoxic effect. Pre-treatment with 0.1 or 1 µM of morphine decreased the paclitaxel-induced cytotoxicity, the proportion of apoptotic cell, and the ratio of Bax/Bcl-2 mRNA expressions.

Interpretation & conclusions: Our data suggest that morphine promotes breast cancer cell viability at clinically relevant plasma concentrations and reduces the apoptotic effect of paclitaxel. This interaction may be very important in clinical settings; however, more studies are needed to explore the plausible mechanisms of interaction and to correlate such findings through *in vivo* animal studies as well as clinically.

Key words Apoptosis - *in vitro* - MDA.MB.231 - morphine - opioid - paclitaxel - triple-negative breast cancer

Incidence of breast cancer, the leading cause of cancer related deaths, is increasing worldwide[1]. Cancer patients frequently suffer from pain. Morphine, an opiate analgesic, is considered one of the most effective clinically available analgesics for managing moderate-to-severe pain associated with advanced cancer and cancer surgeries[2]. Opioids exert their analgesic effects mainly through activation of µ-opioid receptors, which

are widely distributed throughout the central nervous system[3]. It has been shown that morphine exerts noticeable effects in the regulation of neoplastic cells by promoting angiogenesis[4] and immunosupression[5,6]. The presence of opioid peptides and opioid receptors has also been demonstrated in a variety of primary tumour cells and cell lines[7,8]. In a study, it was shown that transplantation of Lewis lung cancer cells did not cause cancer in μ-opioid receptor knock-out mouse[9]. Unfortunately, the role of morphine in the regulation of tumour cell growth is still controversial. Some studies have found that morphine can induce cell apoptosis or necrosis in human tumour cell lines *in vitro*[10,11], but others have shown that morphine enhances tumour cell growth *in vivo*[4,12-14] and *in vitro*[13-15].

Evidence is accumulating that anaesthetic and analgesic interventions can affect risk of cancer recurrence or metastasis[16]. In the light of these studies, the possibility of morphine and other opioid analgesics used for pre-medication or post-operative pain relief may affect tumour regeneration and metastasis has increased the interest in this field.

The present study was aimed to investigate the effect of relevant plasma concentrations of morphine on highly aggressive breast cancer cell line in the presence of paclitaxel, a commonly used chemotherapeutic agent.

Material & Method

Experiments were performed in the laboratories of Genkok Genome and Stem Cell Center, University of Erciyes, Kayseri, Turkey. Human triple-negative breast carcinoma cell line MDA.MB.231 (ATCC® HTB26™) was cultured in a culture medium RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 per cent FBS (Gibco BRL, USA), one per cent glutamax (Gibco, Paisley, UK) and one per cent penicillin-streptomycin (Gibco, Paisley, UK) in a humidified atmosphere of five per cent CO_2 at 37°C. Morphine sulphate was obtained from Turkish Grain Board (TMO), Afyon, Turkey and freshly prepared just before the experiment by dissolving in complete medium. Paclitaxel (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and kept at −20°C as an aliquoted stock solution. Paclitaxel freshly diluted in culture media just before the experiment, and concentration of DMSO was less than 0.05 per cent in culture plates. Sterile 0.22 μm syringe filters (Millex GV Filter, Germany) were used to sterilize solutions of morphine and paclitaxel.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay: The effects of the drugs on cell proliferation was determined by using MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA] colorimetric assay. Briefly, $2×10^3$ cells/100 μl were seeded into 96-well microplates (Corning Costar, Corning, NY, USA) with three replicates. The next day, various concentrations of morphine, paclitaxel or combinations were applied to the wells, including the respective control groups (medium or DMSO), and incubated for 24 h and 48 h. Morphine or medium was administered 4 h before paclitaxel or DMSO in the combined groups. After the treatment period, cells were exposed to 10 μl of MTT solution (5 mg/ml) for 3 h. At the end of the incubation period, the supernatant was removed, 100 μl of DMSO was added to each well, and the absorbance was measured at 560 nm using a microplate reader (Promega Multireader Glomax, USA). The mean optical density (absorbance) of three wells in the indicated groups was used to calculate the percentage of the viable cells using the following formula;

$$\% \text{ cell viability} = \frac{(\text{Atreatment} - \text{Ablank})}{(\text{Acontrol} - \text{Ablank})} \times 100$$

(where, A=absorbance). Data were represented as mean per cent cell viability of three independent experiments.

Apoptosis detection: Analysis of apoptosis was carried out using the Muse Annexin V and Dead Cell Assay (Merck Millipore, Hayward CA, USA) according to the manufacturer's instructions. Briefly, $1×10^5$ cells/well were seeded into six-well plates (Corning Costar) and allowed to attach overnight. After 24 h of seeding, cells were pre-treated with 0.01, 0.1 or 1 μM morphine or complete medium alone, and after 4 h, 28 nM paclitaxel or DMSO was added to wells and incubation continued at 37°C. After 24 h of paclitaxel treatment, cells were collected by trypsinization and diluted to a concentration of $4×10^5$ cells/ml with phosphate-buffered saline (PBS) containing one per cent FBS. Then, 100 μl of cell suspension was added to 100 μl Muse Annexin V and Dead Cell reagent, incubated for 20 min and analyzed using Muse Cell Analyzer (Merck Millipore). The apoptotic ratio was determined by the identification of four populations: (*i*) living cells; Annexin V (−)

and 7-AAD (−), (*ii*) early apoptotic cells; Annexin V (+) and 7-AAD (−), (*iii*) late apoptotic cells; Annexin V (+) and 7-AAD (+), (*iv*) non-apoptotic dead cells; Annexin V (−) and 7-AAD (+).

Quantitative real-time polymerase chain reaction (qRT-PCR): Briefly, 3×10^5 cells/well were seeded into six-well plates and allowed to attach overnight. After 24 h of seeding, cells were pre-treated with 0.1 or 1 μM morphine or complete medium, and 4 h after the morphine treatment, 28 nM paclitaxel or DMSO was added to wells and continued to incubation at 37°C. After 48 h administration of paclitaxel, total RNAs were isolated using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany). The quantity and purity of total RNAs were determined by the NanoDrop spectrophotometer (BiospecNano, Shimadzu, Japan); cDNA was synthesized by the 0.5 μg of RNAs using RT^2 First Strand Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Forward and reverse primers (2 μl each), 0.2 μl cDNA, 7.8 μl nuclease-free water and 10 μl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) were mixed to obtain the final volume of 20 μl and distributed to each 96 well PCR plate. RT-PCR was performed in a Roche LightCycler 480 (Roche, Switzerland). The expression levels of Bax and Bcl-2 mRNAs were normalized to GAPDH mRNA. Primer sequences for Bax mRNA (NM_001291429.1); (F) 5' TCTGACGGCAACTTC AACTG 3', (R) 5' AGTCCAATGTCCAGCCCATG 3' and Bcl-2 mRNA (NM_000633.2); (F) 5' CCAACATTCTCTCCACAGCTC 3', (R) 5' TGGGCCAGAGCTACATCTTTA 3', for GAPDH mRNA; (F) 5' ATGGGTGTGAACCATGAGAAG, 3' (R) 5' AGTTGTCATGGATGACCTTGG 3'. The qRT-PCR programme was configured as; 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Following the analysis, the relative expressions ($2^{-\Delta\Delta CT}$) of the genes was calculated using the expression levels (CT)[17]. Three independent experiments were performed, and cDNA from each sample were run in duplicate.

Statistical analysis: Results were presented as mean±SD of three independent experiments for each assay. The normality distribution was analyzed by Shapiro-Wilk test, and the homogeneity of variance was tested using Levene's test. Statistical comparisons between multiple groups were determined by deploying one-way ANOVA followed by Tukey HSD *post hoc* test, using the IBM SPSS Statistics 22.0 software (IBM Corp., Chicago, IL, USA). GraphPad Prism software version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA) was used for graphics.

Results

Morphine increased the proliferation of cells: Morphine significantly increased the proliferation of breast cancer cells with concentrations of 0.1-2.5 μM, 24 and 48 h after application (*P*<0.05). When applied at 100 μM or higher concentrations, a significant cytotoxic effect of morphine was observed (*P*<0.05). Cytotoxicity induced by the chemotherapeutic agent paclitaxel was significant at concentrations of 28-3100 nM at 24 and 48 h (*P*<0.05, Fig. 1A and B).

Pre-treatment of breast cancer cells with 0.1 or 1 μM morphine not only increased cell proliferation but also significantly reduced the paclitaxel (28 and 56 nM)-induced cytotoxicity 24 and 48 h after application (*P*<0.05, Fig. 2A and B). 10 μM morphine had no significant effect on paclitaxel-induced cytotoxicity (*P*>0.05).

Morphine decreased the apoptotic effect of paclitaxel: As depicted in Figure 3, morphine did not cause any significant apoptotic effect when administered alone but reduced the apoptotic effect of paclitaxel. Flow cytometric analysis showed that 1 μM morphine significantly countered the apoptotic effect of paclitaxel (Fig. 3A, B and Table I, *P*<0.05).

*Morphine decreased the Bax/Bcl-2 mRNA expression ratio*: The mRNA expression ratios of the apoptotic gene *Bax* was analyzed relative to the antiapoptotic gene *Bcl-2* in the groups administered with morphine, paclitaxel and their combinations. Bax/Bcl-2 ratio was decreased with 1 μM morphine treatment, while it increased significantly with 28 nM paclitaxel (*P*<0.05). Increased Bax/Bcl-2 ratio with paclitaxel decreased with 0.1 or 1 μM morphine pre-treatment, significantly (*P*<0.05). The increased Bax/Bcl-2 ratio with paclitaxel was significantly reduced with 0.1 or 1 μM morphine pre-treatment (*P*<0.05, Fig. 4 and Table II).

Discussion

Morphine is considered one of the most effective analgesic drugs available clinically for anaesthetic pre-medication and management of cancer pain with cancer metastasis[2]. Morphine acts directly on the central
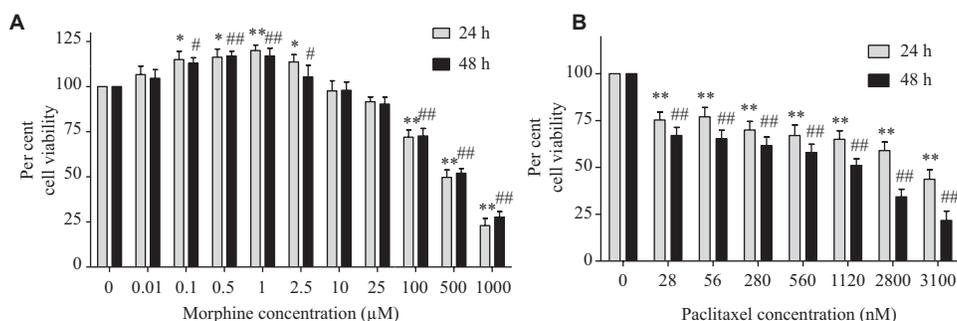
Fig. 1. Cell viability evaluation of MDA.MB.231 breast cancer cells treated with (**A**) morphine, and (**B**) paclitaxel by MTT assay. Bars represent the mean±SD of three independent experiments. P *<0.05, **<0.01 compared to control at 24 h; P # <0.05, ##<0.01 compared to control at 48 h treatment
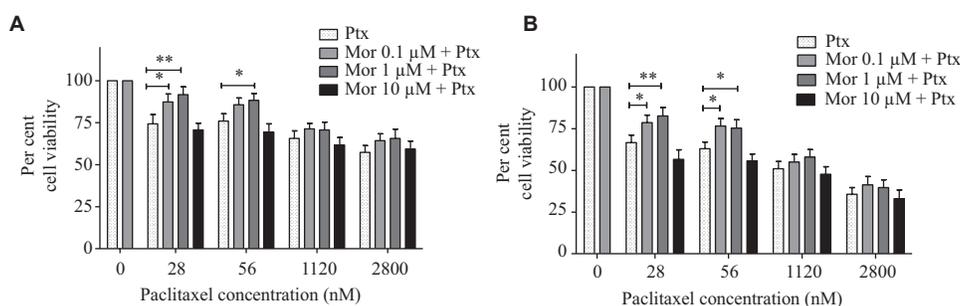

Fig. 2. Evaluation of cell viability of MDA.MB.231 breast cancer cells by the MTT assay. Cells were treated with Ptx (0, 28, 56, 1120, 2800 nM) 4 h after the various concentrations of Mor (0, 0.1, 1, 10 μM) and incubated for (**A**) 24 h and (**B**) 48 h. First two bars represent the control solvent groups; dimethyl sulphoxide (Ptx) and complete medium (Mor). Bars represent the mean±SD of three independent experiments. P *<0.05 compared to Ptx-treated group. Ptx, paclitaxel; Mor, morphine

nervous system and activates μ-receptors to ameliorate pain[3]. However, the presence of opioid peptides and receptors has been demonstrated in a variety of tumours and cell lines, including human breast carcinomas[7,8]. In this study, clinically relevant plasma concentrations of morphine have been shown to increase breast cancer cell viability and reduce the apoptotic effect of paclitaxel. The present study provides evidence that the presence of clinically relevant low morphine concentrations (0.1 and 1 μM) reduces the ratio of Bax/Bcl-2 mRNA expressions induced by paclitaxel in triple-negative breast cancer cells. Since morphine is frequently used in managing cancer pain, this interaction might have clinical implication and relevance.

In addition, several studies have demonstrated the central and peripheral immunosuppressive effects of opioids[18,19]. It was shown that morphine could reduce the activity and number of natural killer cells[20]. Furthermore, pre-clinical studies have shown the proangiogenic effects of morphine. Morphine (1 μM) has been shown to induce endothelial cell proliferation but is cytotoxic at higher concentrations. In mice breast tumour xenograft model, morphine

increased tumour progression by promoting tumour neovascularization[4,13]. In animal studies, morphine has been shown to promote the growth of tumours and reduce overall survival in mice[12,13]. Fentanyl, a synthetic opioid, has also been shown to induce stemness in human breast cancer cell lines[21]. The effects of morphine are mediated by several mechanisms, but the opioid receptor antagonist naloxone has been shown to inhibit the effects of morphine on MDA.MB.231 cells *in vitro*[22]. Consistent with these results, it has recently been shown that very low morphine concentrations can protect human primary glioblastoma and rat adrenal medulla pheochromocytoma cell lines from methamphetamine-induced cytotoxicity[23]. In the present study, morphine was applied in a wide range of concentrations and triggered proliferation of triple-negative breast cancer cells at low concentrations while inducing cytotoxicity at ≥100 μM. In addition, morphine in the range of clinical plasma concentrations inhibited paclitaxel-induced cytotoxicity and apoptotic cell ratio.

Similar to our results, it was earlier shown that morphine at a concentration of 3.5 μM reduced the
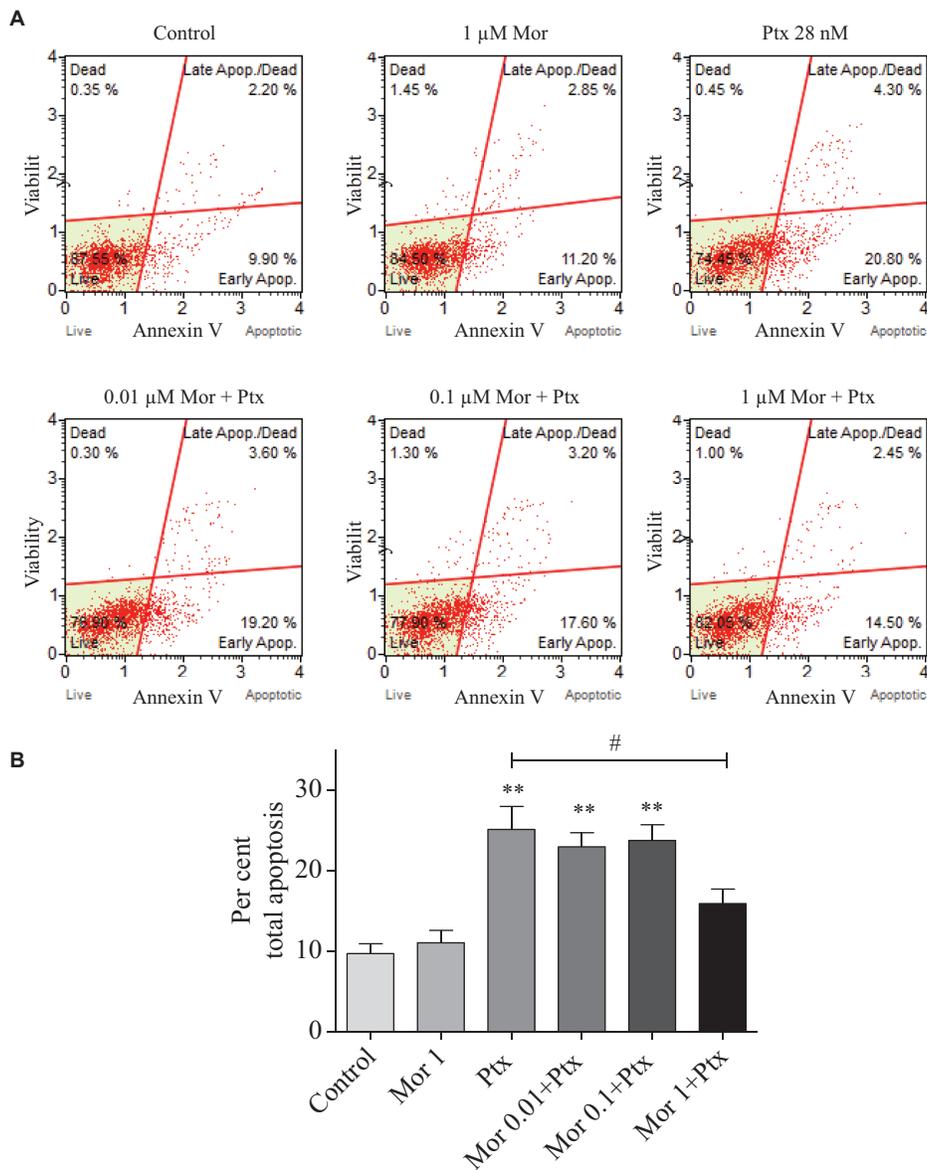
Fig. 3. The effect of Mor, Ptx or combinations on the apoptotic cell death of MDA.MB.231 breast cancer cells. Cells were pre-treated with Mor (0, 0.01, 0.1 and 1 μM) and 4 h after treatment with 28 nM Ptx for 24 h. (**A**) Apoptotic cells were determined by flow cytometric analysis with Muse™ Annexin V and Dead Cell Assay. (**B**) Bar graph showing the percentage of apoptotic cells including the sum of early and late apoptosis of cells following treatments. Bars represents the mean±SD from three independent experiments. P *<0.05, **<0.01 compared to control, P #<0.05 compared to Ptx. Ptx, paclitaxel; Mor, morphine

cytotoxic effect of cisplatin on human nasopharyngeal carcinoma cells by decreasing the mRNA expression of Bax while increasing Bcl-2[24]. In addition, low-dose morphine decreased cisplatin-induced apoptosis and increased tumour angiogenesis in mice[24]. Another study[14] reported that 1 or 10 μM morphine treatment reduced the apoptotic effects of doxorubicin or paclitaxel by decreasing the cleavage of PARP and caspase. In a recent study by Yu *et al*[25], it has been shown that the apoptotic effects of common chemotherapeutic drugs, paclitaxel, cisplatin, and 5-FU, on cervical

cancer cells are significantly reduced by morphine. On the contrary, some studies have shown that morphine has cytotoxic effects on various human cancer cell lines[10,11]. Morphine was reported to induce cell apoptosis or necrosis in human tumour cell lines when administered at concentrations higher than the clinical concentration ($\geq$100 μM)[10,11]. Morphine at 4 mM has been reported to induce 60 per cent apoptosis in SH-SY5Y neuroblastoma cells[11]. It was shown that high concentrations of morphine (250-1250 μM) improved antitumour effects of 5-fluorouracil on estrogen receptor-

Table I. Percentage of total apoptotic MDA.MB.231 cells following treatment with morphine, paclitaxel, or combinations

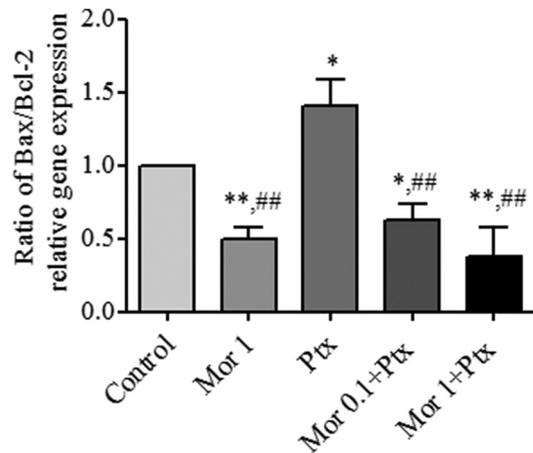| Treatment	Percentage of total apoptosis (mean±SD, n=3)
Control	9.70±2.12
Morphine 1 μM	11.04±2.64[##]
Paclitaxel 28 nM	25.13±4.95[**]
Morphine 0.01 μM+paclitaxel 28 nM	22.30±3.05[**]
Morphine 0.1 μM+paclitaxel 28 nM	23.76±3.35[**]
Morphine 1 μM+paclitaxel 28 nM	15.91±3.13[#]

P [**] <0.01 compared to control; P [#] <0.05; [##] <0.01 compared to paclitaxel.

Table II. Bax/Bcl-2 mRNA expression ratio of MDA.MB.231 cells treated with morphine, paclitaxel, or combinations

Treatment	Ratio of Bax/Bcl-2 mRNA expression (mean±SD)
Control	1.00±0.00
Morphine 1 μM	0.49±0.08[**, ##]
Paclitaxel 28 nM	1.40±0.18[*]
Morphine 0.1 μM+paclitaxel 28 nM	0.62±0.11[*, ##]
Morphine 1 μM+paclitaxel 28 nM	0.38±0.19[**, ##]

P [*] <0.05; [**] <0.01 compared to control; P[#] <0.05; [##] <0.01 compared to paclitaxel. Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein

Fig. 4: The ratio of Bax/Bcl-2 mRNA expressions of MDA.MB.231 breast cancer cells treated with Mor (1 μM), Ptx (28 nM), or combinations (Mor 0.1 μM+Ptx 28 nM, Mor 1 μM+Ptx 28 nM). Control group treated with DMSO 4 h after complete medium treatment. Bars represent the mean±SD of three independent experiments. P [*] <0.05, [**] <0.01 compared to control, P [#] <0.05, [##] <0.01 compared to Ptx. Ptx, paclitaxel; Mor, morphine; DMSO, dimethyl sulphoxide.

positive breast cancer cells[26]. These results indicate that the concentration of morphine is an important factor in explaining different effects of morphine on cancer cells and the efficiency of chemotherapy. However, such concentrations are much higher compared to clinical plasma morphine concentrations achieved in cancer patients. Median serum concentrations of cancer patients receiving oral and subcutaneous morphine have been reported to be 60 (range 1-2560 nM) and 179 nM (range 3-1680 nM), respectively[27]. In another study, the median and mean serum concentrations of oral morphine received by cancer patients were determined as 268.8 nM and 5.6 μM, respectively[28]. Hence, in the present study, proliferation assay was performed with wide range of concentrations of morphine (10 nM-1 mM), and then, apoptotic assay was carried out with 0.1 and 1 μM morphine. There are two main limitations in our study that can be addressed in future. Firstly, the study was performed on a single cancer cell line, MDA.MB.231. This cell line was used as it

is known as a highly metastatic triple-negative breast cancer and has limited treatment options. Secondly, it needs to be investigated in *in vivo* conditions such as with appropriate animal models.

To conclude, morphine used in cancer patients may possibly affect tumour proliferation and lead to the development of chemotherapeutic drug resistance. The present study uncovered the direct proliferative effect of morphine on breast cancer cells and its interaction with the apoptotic effect of paclitaxel, but more studies are needed to examine the interaction mechanisms and confirm our results *in vivo*.

***Conflicts of Interest*:** None.

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