Morphine can inhibit the growth of breast cancer MCF-7 cells by arresting the cell cycle and inducing apoptosis

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Running title: Morphine inhibit MCF-7 cells' progression

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Summary

Morphine is widely used for relieving cancer pain in patients with advanced cancer. However, whether morphine can suppress or promote the progression of cancer in breast cancer patients receiving morphine analgesia remains unclear. Therefore, we used an in vitro model treated with morphine and naloxone to investigate the effects of morphine on breast cancer cell line MCF-7. MCF-7 cells were cultured with different concentrations (0.01 to 10 µM) of morphine at 12th, 24th, 36th, 48th, 60th and 72th hours. Then, cell viability was measured through the MTT assay, and cell cycle and apoptosis assays were detected by flow cytometry (FCM). In addition, cell proliferation was conducted by colony formation assay. In this study, we have found that morphine (0.01 to 10 µM) could significantly reduce the cell vitality, growth and colony formation rate of MCF-7 cells, which has a certain relationship with cell cycle progression arrested at the G0/G1 and G2/M phase and MCF-7 cells apoptosis. Moreover, naloxone along with morphine could not reverse these effects, which indicates that the inhibition of MCF-7 breast cancer cell growth and proliferation by morphine could be its independent effect, not associated with opioid receptors. Morphine can inhibit cell growth by blocking the cell cycle and promote apoptosis in MCF-7 cells. Hence, morphine may be unable to promote the progression of cancer in breast cancer patients receiving morphine analgesia.

Key Words: Apoptosis, Breast cancer, Cell cycle, MCF-7 cells, Morphine
INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer among women.\textsuperscript{[1, 2]} In addition, younger women often present with more aggressive incidence of BC and leading to cause high mortality rate of BC, between the ages of 20 and 59.\textsuperscript{[3, 4]} Furthermore, most of the cancer patients are severely stressed by cancer pain, which in turn creates further psychological burden and affects patients outcome. Therefore, cancer pain has increasingly become the major focus of attention and opioids candidates are routinely used to treat cancer pain.

Morphine is the representative opioid and is widely used for treatment in advanced cancer.\textsuperscript{[5, 6]} Besides, its analgesic action, morphine has anti-nociceptive effect, and might even change tumor progression. In our earlier study, we found that opioid molecule fentanyl could inhibit the vitality of gastric carcinoma cells and cell cycle progression, the mechanism of which might be associated with inhibition of NF-κB nuclear translocation and PTEN tumor suppressor gene upregulation.\textsuperscript{[7]} We also demonstrated that morphine could suppress the growth and the cell cycle progression of MGC-803 cells, the mechanism of which might be related to activation of caspase-3 and caspase-9, and inhibition of NF-κB nuclear translocation.\textsuperscript{[8]}

Some studies have found that at a clinical concentration morphine (0.01 µM) can induce cell apoptosis or necrosis in MCF-7 human tumor cell lines in vitro and inhibit the growth of tumor cells.\textsuperscript{[9]} In addition, morphine (0.1 to 100 µM) and DAMGO (an µ-opioid receptor selective agonist) can inhibit the adhesive and migration of the HEK FLAG-MOP cells, the mechanisms of which are associated with mediation of an opioid receptor.\textsuperscript{[10]} However, other studies have claimed that Biphalin (an opioid receptor agonist) exhibits an inhibitory effect on human T98G glioma cell proliferation, while morphine (50 nM, 20 M, and 40 M) promotes T98G cell growth.\textsuperscript{[11]} These above opposite results may be related to different tumor cells, experimental conditions and different concentrations of morphine and time of
delivery under in vitro condition. The mechanisms of morphine-tumor interactions are very complex and remain to be fully understood, therefore requiring further research.

In this present in vitro study, we investigated the anti-proliferative effects of morphine, exposed to human breast cancer cell line MCF-7, in presence and absence of naloxone an opioid antagonist. MCF-7, breast cancer cell line isolated from 69 year old Caucasian women and routinely used for drug screening study rather than T-47D and MDA-MB-231. Therefore, use of MCF-7 is a suitable model for drug screening and development of new anti-cancer drug against breast cancer. Our aim is to understand whether morphine is safe for treatment of cancer pain and the possible mechanism of morphine effects on tumors. Therefore, to evaluate whether the observed effect is mediated by opioid receptor, naloxone was used to antagonize large doses of morphine in the present study.

MATERIALS AND METHODS

Cell culture and treatment

Human breast cancer cell line MCF-7 was obtained from Chinese Academy of Science, Shanghai Institute of cell biology, Shanghai, China. MCF-7 cells were maintained as a monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml). Cells were incubated in a 37°C 5% CO₂ environment, and medium was changed daily. When MCF-7 cells had reached logarithmic phase, cells were randomly assigned into 7 groups including control group, 0.01 µM morphine group, 0.1 µM morphine group, 1 µM morphine group, 10 µM morphine group, 10 µM naloxone group and (10 µM naloxone + 10 µM morphine) group. The cells in the experimental groups were cultured with 0.01 µM morphine, 0.1 µM morphine, 1 µM morphine, 10 µM morphine, 10 µM naloxone, respectively, while the cells in the compound
group were cultured with 10 µM naloxone for 30 min and then morphine was added to the culture medium with a morphine concentration of 10 µM. The cells in the control group were incubated normally.

**Cell proliferation and vitality assay**

To detect the proliferation and vitality of cells, a MTT assay and Cell Titer 96 AQueous assay system (Promega, Madison, WI, USA) was used, in accordance with the manufacturer’s instructions. MCF-7 cells were grown in 96-well plates at the density of $1 \times 10^3$ cells/well. Cells were treated with various concentrations (0.01 to 10 µM) of morphine, and cell viability was detected by the MTT assay at 12th, 24th, 36th, 48th, 60th and 72th h after treatment. MTT (20 µl, 5 mg/ml) was appended to each well, followed by incubation for 4 h in CO$_2$ incubator at 37°C. Four hours later, the culture medium was discard and 200 µl of dimethyl sulfoxide was appended to each well and oscillated for 10 min. Proliferation was defined as increasing the number of cells for cell growth and division. The cell proliferation rate was obtained by measuring the changes in the absorbance at 490 nm of viable cells on a plate. Optical density (OD) was measured by a 96-well Opsys MRTM microplate reader (ThermoLab systems, Chantilly, VA, USA) and RevelationTM Quick Link Software.

**Cell colony formation assay**

MCF-7 cells were digested into individual cell with 0.25% trypsin digestion and suspended in DMEM contained 10% FBS, streptomycin (100 µg/ml), and penicillin (100 U/ml). The cell suspensions were diluted as gradient multiples and plated in 6-well plates at 20 cells/cm$^2$, 6 wells for each group. Then, the cells of the plates were cultured until they developed into sufficiently large colonies in the control wells after 7 days. The colonies were fixed with pure methanol and stained with Giemsa solution. Digital photographs of the plates were taken for manually counting the number of colonies. The colony formation rate was calculated as follows: the colony formation rate = (colonies / seeded cells) \times 100\%.
Flow cytometry for cell cycle and apoptosis analysis

MCF-7 cells of each group were cultured for 24 h and then washed twice with cold PBS, treated with trypsin, and soaked in 4°C/70% ethanol for 30 min. The cell pellet was treated with PBS including 100 mg/L RNase and 10 mg/L propidium iodide for 30 min. The cells were analyzed by flow cytometry, and the cells were treated using an EPICS XL-MCL FACScalibur system (Becton Dickinson, Mountain View, CA, USA) for monochrome fluorescent cell counting. The cell cycle was analyzed by the Multiplus Software for Windows (Phoenix Flow Systems, San Diego, CA, USA). Ten thousand cells were collected for each sample.

MCF-7 cells of each group were incubated for 24 h and then digested with trypsin without EDTA. After centrifugation, the cell pellet was used for analysis of cell apoptosis. Apoptotic cells were measured using the Annexin-FITC/PI Apoptosis Detection Double Marking Kit (Jingmei Biotech Co., Shenzhen, China) and an EPICS XL-MCL FACScalibur (Becton Dickinson, Mountain View, CA, USA) following the manufacturer’s instructions. Fluorescent signal of twenty thousand cells was collected for each sample.

Statistical analysis

Data were presented as mean ± standard deviation (M ± SD). Statistical analyses were performed using the SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Multiple comparisons were performed with a one-way analysis of variance (ANOVA) followed by the Dunnett’s post hoc test. Differences in the MTT cell viability were analyzed using the general linear model, followed by the repeated measures. P values < 0.05 were considered to be significant.

RESULTS

The effects of morphine on cell growth and proliferation of MCF-7 cells

MCF-7 cells incubated with morphine showed significant reduction in cell vitality and growth, as measured with MTT assay (Fig. 1). The mean proliferation rate of MCF-7 cells
incubated with 0.01 to 10 µM morphine and 10 µM naloxone + 10 µM morphine were significantly lower than that of in the control group (repeated measures, all \( P < 0.05 \)). The differences of the mean proliferation rate of MCF-7 cells between the control group and 10 µM naloxone group were not statistically significant (repeated measures, \( P > 0.05 \)). The mean proliferation rate of MCF-7 cells incubated with 10 µM naloxone + 10 µM morphine was lower than that in the 10 µM naloxone group (\( P < 0.05 \)).

The effects of morphine on colony formation of MCF-7 cells

To further confirm the growth inhibiting potential of morphine on MCF-7 cells. Colony formation assay was conducted to observe the proliferation of the MCF-7 cells at various treatment conditions. Interestingly, we noticed the number and size of the cell colonies in all groups were significantly inconsistent. Furthermore, the concentrations of morphine (0.1 to 10 µM) were seemed to be better for MCF-7 colony growth inhibition. The colony formation rate of MCF-7 cells, which was incubated in all morphine groups (incubated with 0.01 to 10 µM morphine, and 10 µM naloxone + 10 µM morphine) was significantly lower when compared with that in the control group (all \( P < 0.05 \)), which shown well developed colony. Next, we wanted to investigate whether the growth inhibiting potential of morphine was mediated through its effect on opioid receptor. To do this, MCF-7 cells were co-treated with naloxone, an opioid antagonist. Remarkably, we found that naloxone treatment does not affect colony inhibiting potential of morphine, which indicates independent anti-proliferative effect of morphine. In addition, no significant difference was shown in the colony formation rate according to statistical analysis between the control group and the 10 µM naloxone alone exposed group (\( P > 0.05 \)). The colony formation rate of MCF-7 cells incubated with 10 µM naloxone + 10 µM morphine was lower than that in the 10 µM naloxone group (\( P < 0.05 \)) (Fig. 2). Finally this data suggested that morphine effectively restrained the growth and proliferation of MCF-7 cells.
The effects of morphine on cell cycle and apoptosis of MCF-7 cells

On the basis of inhibition rate on tumor cell viability and colony development, we aimed to describe the flow cytometry based cell cycle distribution to confirm whether anti-proliferative effect of morphine was associated with cell cycle arrest. We found that compared with the control group, all morphine treated groups revealed a higher number of MCF-7 cells remaining stagnate in the G0/G1 and G2/M phase (all $P < 0.05$), while the proportion of the stagnation in the S phase in all morphine groups was fewer compared in the control group (all $P < 0.05$). The differences in the cell cycle distribution between the control group and 10 µM naloxone group were not statistically significant ($P > 0.05$). Compared with the 10 µM naloxone group, the proportion of the stagnation in the G0/G1 and G2/M phase of MCF-7 cells incubated with 10 µM naloxone + 10 µM morphine were significantly higher ($P < 0.05$), while the proportion of the stagnation in the S phase in 10 µM naloxone + 10 µM morphine group were significantly lower ($P < 0.05$) (Fig. 3). The data showed that morphine inhibited cell growth and the cell cycle, which was significantly associated with the cell cycle arrest at the G0/G1 and G2/M phase. In addition, these data suggested that morphine suppressed cell growth and proliferation by preventing cell cycle progression from the G1 to the S phase.

To further understand whether morphine had the effect on promoting MCF-7 cells apoptosis, control and treated cells from different group were stained with Annexin V/FITC and PI, followed by flow cytometry analysis was performed. The viable cells appeared in the lower left quadrant of the dual parameter florescent dot plots, while the apoptotic cells existed in the right quadrant. As seen in Fig. 4, the apoptosis rates of MCF-7 cells in all morphine groups were higher than in the control group (all $P < 0.05$). No statistically significant difference was found in the apoptosis rates between the control and the 10 µM naloxone alone treated group ($P > 0.05$). The apoptotic rates in the 10 µM naloxone + 10 µM morphine group were significantly higher than those in the 10 µM naloxone group ($P < 0.05$). These findings
suggest that growth inhibition and anti-proliferative effect of morphine was associated with impairment of cell cycle and stimulation of apoptotic cell death.

Naloxone did not reverse these effects

According to results obtained from above findings and the statistical analysis did not show any apparent significant difference in the MCF-7 mean proliferation rate, colony formation rate, cell cycle distribution, and apoptotic cell death between the control group and the 10 µM naloxone alone treated group (P > 0.05). On the other hand, the mean proliferation rate, colony formation rate, cell cycle distribution and apoptosis rates of MCF-7 cells incubated with 10 µM naloxone + 10 µM morphine did not reverse these effects as those of MCF-7 cells incubated with 0.01 to 10 µM morphine. Thus, a more pronounced beneficial effect was observed in morphine treatment could be its independent effect and may not associated with opioid receptors.

DISCUSSION

Despite, continuous improvements in the cancer diagnosis and management, breast cancer patients is still thought to be predominantly incurable due to continuous drug resistance, severe side effect and pain mediated physiological stress. Therefore, searching predictable target based agent to control breast cancer pain and growth, has recently attracted much attention. Morphine is optional for most cancer patients to relieve from advanced cancer pain. In addition, recent findings have reported that Morphine may have some beneficial effects along with analgesic effect. However, its mechanism of action is not clearly understood. Hence, the effect of Morphine on health benefits is a debatable topic.

Our current study provide evidence that Morphine treatment could inhibits rate of MCF-7 cancer cell proliferation, which was clearly observed in accordance with various time and doses. Furthermore, Morphine effectively suppress the development of MCF-7 colonies, this effect was correlated with our MTT findings and revealed that Morphine could have beneficial effect to control cancer cell proliferation along with its antalgic effect. Indeed, an opioid antagonist naloxone failed to reverse the anti-proliferative effect of Morphine.
Therefore, Morphine has anti-proliferative effect and it was independent effect of Morphine and its effect not associated with opioid receptors. In consistence with our findings, Ge et al, have reported that Morphine combination with 5-Fluorouracil has substantial therapeutic effect. They found that the combination of 5-Fluorouracil and Morphine effectively suppress the viability of MCF-7 cells by activating apoptotic signaling cascade, whereas individual treatment of those compounds had little effect on MCF-7.\textsuperscript{[12]} In addition, a growing body of evidence also conclude that an opioid molecule Morphine has potent pharmacological activity along with its analgesic efficacy by regulating the molecular signaling network. Matrix metalloproteinases (MMPs) are a class of structurally related proteolytic enzymes and could stimulate degradation of extracellular matrix proteins. Critically, increased MMP activity is detected in a wide range of cancers and seems correlated to their invasive and metastatic potential. Gach et al., who found that, Morphine exposure to MCF-7 cells at the concentrations of 0.001 to 1 µM altered the expression of MMP-2 and MMP-9 mRNA overexpression and also increased its endogenous inhibitor, altered the proteolytic profile and then inhibited breast tumor cells growth and proliferation.\textsuperscript{[13]} Further, Morphine (> 500 µm for 24 hours) induced significant apoptotic death in MCF-7 and MDA-MB231 cells by blocking β-arrestin-2 through inhibition of caspase-8 pathway.\textsuperscript{[14]} In agreement with these reports, we found potent inhibition on MCF-7 viability and colony formation, which all together indicates better independent anti-proliferative effect of Morphine.

Generally, cancer cells exhibit deregulation of the cell cycle and frequently display cell cycle abnormalities and accumulate mutations, leading to uncontrolled proliferation and genomic instability. Therefore, destruction of cell cycle progression is an appreciated target for cancer treatment. Remarkably, our present study has shown significantly cell cycle arrest at the G0/G1 and G2/M phases and this effect on MCF-7 cells were more pronounced at various dose point. Furthermore, naloxone does not shown any effect on contentment with Morphine. Therefore, the effect of Morphine on G0/G1 and G2/M cell cycle destruction may be its independent effect. Further our findings was consistent with previous report, who shown that
morphine has anti-cancer potential in both in vitro and in vivo findings.\textsuperscript{[15]} Thus our present findings conformed that Morphine induced growth and colony inhibition was associated to cell cycle arrest. Moreover, other studies have also demonstrated that the anti-cancer mechanism of some agents was associated with cell cycle arrest and apoptosis induction.\textsuperscript{[16]} Further, recent study have suggested that cell cycle destruction by exogenous agent was more promising effect rather than its apoptotic potential.\textsuperscript{[17]} Furthermore, growing body of evidence provides evidence that Morphine could inhibit various type of cancer cell growth, which were mostly regulated through inhibition of angiogenesis\textsuperscript{[18]}, promoting apoptotic death via c-Jun N-terminal kinase (JNK)/caspase pathway\textsuperscript{[19]} and modulating VEGF-A expression\textsuperscript{[20]}, which all leads to control tumor micro environment and metastasis.\textsuperscript{[21, 22]} Thus, our present findings give a conclusion that Morphine has potent anti-proliferative, growth inhibition and anti-tumor activity, these effects were mediated through an induction of cell cycle arrest.

Actually, most anti-cancer compounds targets cell cycle and apoptotic pathway and being frequently evaluated in several preclinical trials. Hence, to confirm the anti-proliferative effect of Morphine, we examined apoptotic cell death through fluorescence based flow cytometric analysis and found significant apoptotic cell death, which reveals better anti-cancer effect of morphine. Further, to confirm whether its apoptotic effect was associated to opioid receptors, naloxone was co-treated with morphine, but naloxone has no effect on Morphine activity.

Morphine decreased µ-opioid receptor expression in MCF-7 cells while naloxone had the opposite effect. However, naloxone did not inhibit the MCF-7 cell proliferation at concentrations of 0.01 to 100 µM. In addition, up- or down-regulation of µ-opioid receptor mRNA levels did not have any direct effect on cell proliferation.\textsuperscript{[23]} Low doses (0.01 µM) morphine promoted cell proliferation, while higher doses (1 µM) of morphine would inhibit
cell proliferation. These effects were a joint regulation of miRNA networks and the specific characteristics of the target tissue. Naloxone could not fully block the effects; therefore, the observed effects may be mediated by non-opioid mechanisms.[24] Both expression of NET1 and cell migration were increased by Morphine. The NET1 gene, not opioid receptors, promoted the direct impact of morphine on breast cancer cell migration.[25] In our present study, 10 µM naloxone did not reverse the inhibition on MCF-7 cell growth and proliferation of morphine. These results suggest that the inhibition on MCF-7 cell growth and proliferation of morphine were not associated with the opioid receptor.

Some have reported that a possible direct effect of µ-opioid receptor on morphine (0.1 µM) and growth factor signaling and consequent proliferation, migration and EMT transition during lung cancer progression.[26]

However, other studies found that morphine might increase tumor progression and promote tumor angiogenesis, cancer cell invasion and metastasis in vivo.[27-29] Morphine promotes breast cancer stem cell properties and tumor growth via activating Wnt/β-catenin signaling.[30] Naloxone reversed the inhibition on tumor cell adhesion of morphine, but did not influence the inhibitory effect on the production of matrix metalloproteinases (MMPs) from tumor cells.[31] Bortsov[32] et al found that opioid pathways may be involved in tumor growth and suggested that genetic variants influenced the relationship between the opioid system function and the survival of cancer.

Based on the two different opinions, some studies advocated that new pharmaceuticals with anticancer properties or methods might be an alternative solution for cancer pain treatment.[33] However, if morphine has the anticancer effects, it will provide a better analgesic anti-cancer prospect for cancer patients.

In summary, our findings provide a novel beneficial information that, analgesic agent Morphine could be effective therapeutic option to control cancer pain and growth.
Particularly, its anti-proliferative effect was attained through inhibition of cell cycle progression from the G1 to the S phase and promoting apoptotic cell death in MCF-7 cells. Moreover, the study showed that naloxone an opioid antagonist, failed to inhibit anti-proliferative and apoptotic effect of morphine. This signifies that, morphine may not stimulate tumor progression in breast cancer patients, could be beneficial candidate for cancer patients owing to its anti-proliferative effect. However, inhibition on MCF-7 cell growth and proliferation by morphine may be regulated through cell signaling pathways. Therefore, further molecular studies are warranted to know the mechanisms underpinning morphine efficacy.

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Conflict of Interest (COI)

The authors declare no conflicts of interest.
References


**Figure legends**

Fig. 1. Cell survival as assessed by MTT assay in MCF-7 cells.

*P < 0.05, versus the control group. #P < 0.05, versus the 10 µM naloxone group. Mean ± SD (n = 12).

Fig. 2. Results of colony formation assay.

*P < 0.05, versus the control group; #P < 0.05, versus the 10 µM naloxone group. Mean ± SD (n = 6).

Fig. 3. Cell cycle analysis of MCF-7 cells.

*P < 0.05, versus the control group. #P < 0.05, versus the 10 µM naloxone group. Mean ± SD (n = 6).

Fig. 4. Results of the cell apoptosis rate.

The cell apoptosis rate was calculated by B2 + B4. The data showed in the histograms was the sum of early apoptotic cells and late apoptotic cells, i.e. B2 + B4. *P < 0.05, versus the control group. #P < 0.05, versus the 10 µM naloxone group. Mean ± SD (n = 6).
Figure 1

[Graph showing cell viability (A490 nm) over time (h) for different treatments including control, morphine 0.01 μM, morphine 0.1 μM, morphine 1 μM, morphine 10 μM, naloxone 10 μM, and naloxone + morphine (10 μM + 10 μM). The graph includes error bars and statistical significance markers (*, **, ***).]
Figure 2

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Figure 3
Figure 4

Control  Morphine 0.01 μM  Morphine 0.1 μM  Morphine 1 μM

Morphine 10 μM  Naloxone 10 μM  Morphine + naloxone

Cell apoptosis rate (%)