Methylnaltrexone, a Peripherally Acting Opioid Receptor Antagonist, Enhances Tumoricidal Effects of 5-FU on Human Carcinoma Cells

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Abstract. *Background: Methylnaltrexone, a novel peripherally acting opioid receptor antagonist, is used to treat opiate-induced constipation in cancer patients. Its effects on the activities of chemotherapeutic agents, however, have not been evaluated. In this study, the effect of methylnaltrexone on the action of 5-fluorouracil (5-FU) was tested in three human cancer cell lines. Materials and Methods: Treatment was for 72 h and the effects on cell proliferation were measured in human SW-480 colorectal cancer cells, MCF-7 breast cancer cells and non-small cell lung cancer cells in vitro. The apoptotic effect was analyzed by using flow cytometry. The cell cycle and expression of cyclin A were assayed after staining with propidium iodide and cyclin A-fluorescein isothiocyanate. Results: 5-FU decreased the cancer cell growth significantly in all three cancer cell lines in a concentration-dependent manner and methylnaltrexone enhanced the actions of 5-FU. Compared to 5-FU 10 μM alone on SW-480 cells (63.5±1.1%), on MCF-7 cells (58.3±3.1%), or on non-small cell lung cancer cells (81.3±1.6%), 5-FU 10 μM plus methylnaltrexone 1.0 μM reduced cancer cell growth in all three cell lines to 50.2±2.9% for SW-480 cells (p<0.05), 50.0±1.7% for MCF-7 cells (p<0.05) and 68.7±2.2% for lung cancer cells (p<0.01). Methylnaltrexone alone also showed anti-proliferative activity in the three cell lines. Methylnaltrexone at 1.0 μM, reduced SW-480 cell growth to 81.9±3.7% (p<0.01), MCF-7 cell growth to 85.9±2.4% (p<0.01) and lung cancer cell growth to 85.5±2.2% (p<0.01). Apoptosis was not induced by treatment of SW-480 cells with 1.0 or 10 μM methylnaltrexone for 48 h. However, methylnaltrexone increased the number of cells in the G$_1$-phase and decreased the expression of cyclin A. Conclusion: At its therapeutic concentrations for opioid-induced constipation, methylnaltrexone does not attenuate and in fact may enhance the tumoricidal activity of 5-FU. Enhanced 5-FU activity may be attributed to the distinct pathways of 5-FU and methylnaltrexone, an effect that could give methylnaltrexone a complementary role in the treatment of cancer with chemotherapeutic agents.*

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The opium poppy may have been used for several thousand years to control diarrhea and relieve pain. In 1803, Sertürner reported the isolation of a pure active alkaline substance from opium and named it "morphine" after Morpheus, the Greek god of dreams. Today, morphine and other opioids are widely used in different clinical settings, especially as analgesics to treat moderate to severe pain. Although opioids are effective in managing cancer pain, their most common and distressing adverse effect is bowel dysfunction. Opioid-induced constipation is found in 90% of patients treated with opioids and is a significant problem in 40%-45% of patients with advanced cancer (1, 2). For cancer patients using opioids for pain, the management of chronic constipation sometimes is more difficult than the control of pain (3). After repeated use of opioids, tolerance develops for analgesia, euphoria, sedation and respiratory suppression. However, tolerance does not appear to extend to the opioid effects on the gut (4, 5). Despite aggressive use of laxatives, patients find that constipation persists. In severe cases, patients may choose to limit or discontinue opioid pain drugs to reduce the discomfort of opioid bowel dysfunction (3, 6).

Naltrexone is a clinically prescribed opioid antagonist. Like other tertiary opioid receptor antagonists, such as naloxone and nalmefene, it is fairly lipid soluble and readily crosses the blood-brain barrier. These drugs block both the beneficial pain-relief as well as the adverse effects of opioids, thus significantly limiting their clinical utility in treating cancer patients with chronic opioid constipation (7-10). Methylnaltrexone, a quaternary derivative of naltrexone, is a novel peripheral opioid receptor antagonist (11, 12) (Figure 1). The addition of the methyl group at the ring amine of naltrexone forms a compound with greater polarity and lower lipid solubility than naltrexone. Because of these properties, the ability of methylnaltrexone to cross the blood-brain barrier is restricted in humans (13, 14). It reduces the undesired side-effects of opioids that are mediated by receptors in the periphery (*e.g.* in the gastrointestinal tract) without affecting analgesia or precipitating opioid withdrawal symptoms, which are predominantly mediated by receptors in the central nervous system (15, 16). Thus, methylnaltrexone offers a therapeutic potential to prevent or treat chronic opioid-induced constipation and improve the quality of life in cancer patients. In 2008, the U.S. Food and Drug Administration approved methylnaltrexone to help restore bowel function in patients with late-stage, advanced illness who are receiving opioids continuously to alleviate their pain (http://www.fda.gov/bbs/topics/NEWS/2008/NEW01826.html).

Surgery, chemotherapy and radiotherapy form the cornerstones of treatment of cancer. Because in advanced stages of the disease treatment relies on chemotherapy and radiation (17), patients with advanced cancer are likely to be receiving chemotherapy. Yet the effects of methylnaltrexone on the actions of chemotherapeutic agents have not been evaluated. In this study, the effect of methylnaltrexone on the activities of 5-fluorouracil (5-FU) was investigated in three human cancer cell lines, SW-480 colorectal cancer cells, MCF-7 breast cancer cells, and non-small cell lung cancer cells. The possible anti-proliferative mechanisms on cancer cells, such as apoptosis, cell cycle phases and cyclin A were also evaluated in SW-480 cells.

Materials and Methods

Drugs. 5-FU was obtained from American Pharmaceutical Partners Inc. (Schaumburg, IL, USA). Methylnaltrexone, a white, odorless powder, freely soluble in water, was obtained from Mallinckrodt Chemicals (St. Louis, MO, USA).

Cancer cell lines and cell culture. The three human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The SW-480 colorectal cancer cells (Leibovitz's L-15), MCF-7 breast cancer cells (RPMI-1640) and non-small cell lung cancer cells (DMEM) were grown in the indicated media supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (50 unit/ml; Invitrogen, Carlsbad, CA, USA). The cells were maintained in a tissue culture dish (100 mm in diameter) and
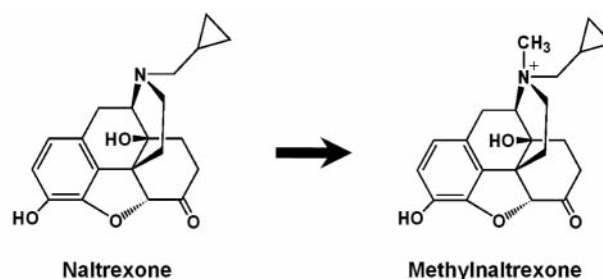

Figure 1. *Chemical structures of naltrexone and methylnaltrexone.*

kept in a humidified incubator (5% CO_2 in air at 37°C). The media were changed every 2-3 days. When the cells reached 80-90% confluence, they were trypsinized, harvested, and seeded into a new tissue culture dish according to previously described methods (18-20).

Cell proliferation analysis. To examine the anti-proliferative effects of the test agents, the cancer cells were seeded in 24-well tissue culture plates at approximately 10,000 cells/well with regular medium and allowed to adhere for 24 h. After adhesion of the cells, the culture medium was changed before the drugs were added. The cells were incubated with the test agents at different concentrations for 72 h. Control cultures were incubated in medium alone. At the end of the treatments, the incubation medium was removed and the cell monolayer was washed twice with phosphate-buffered saline (PBS). Then the cells were detached with trypsin and counted (Coulter Counter; Coulter Electronics, Hialeah, FL, USA) (21). All the assays were performed at least three times. The percentage of cancer cell proliferation was calculated as follows: Cell proliferation (%)=100 × (cell number in experimental well / cell number in the control well).

Apoptotic analysis. SW-480 cells were seeded in 24-well tissue culture plates. After one day, the medium was changed and methylnaltrexone was added. After treatment for 48 h, cells floating in the medium were collected. The adherent cells were detached with trypsin. Then culture medium containing 10% FBS (and floating cells) was added to inactivate the trypsin. After being pipetted gently, the cells were centrifuged for 5 min at 1500 xg. The supernatant was removed and the cells were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the manufacturer's instructions. Untreated cells were used as control for double staining. The cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, at least 20,000 cells were counted (22, 23).

Cell cycle and cyclin A analyses. SW-480 cells were seeded in 24-well tissue culture plates. After one day, the medium was changed and the cells were treated with methylnaltrexone for 48 h. The cells were harvested and fixed gently by adding 80% ethanol before they were placed in a freezer at –20°C for 2 h. They were then treated with 0.25% Triton® X-100 for 5 min in an ice bath. The cells were resuspended in 300 μl of PBS containing 40 μg/ml PI and 0.1 mg/ml RNase. Subsequently 20 μl of cyclin A-FITC
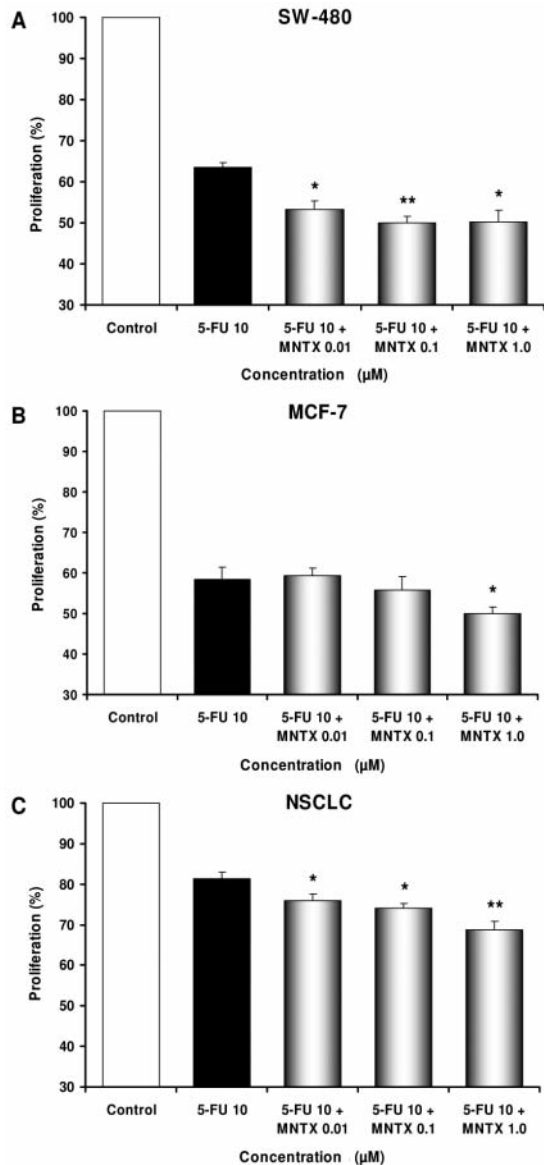
Figure 2. *Anti-proliferative effects of 5-fluorouracil (5-FU) and methylnaltrexone (MNTX) on (A) SW-480 colorectal cancer cells, (B) MCF-7 breast cancer cells and (C) non-small cell lung cancer (NSCLC) cells after 72 h treatment. Control is normalized to 100% . *p<0.05; **p<0.01, compared to 5-FU alone.*
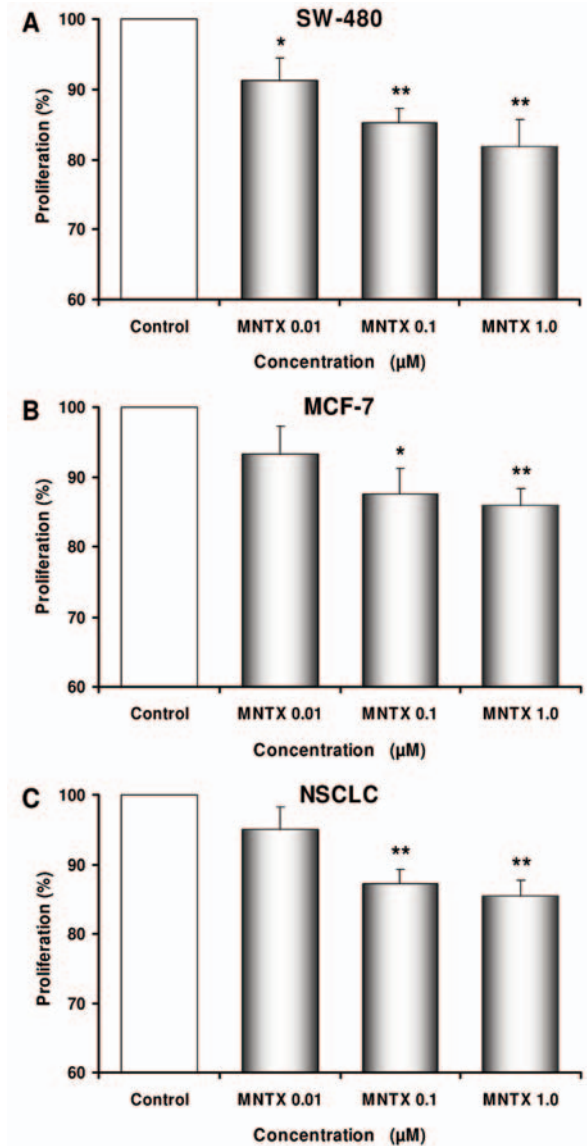

Figure 3. *Effects of methylnaltrexone (MNTX) on proliferation of (A) SW-480 colorectal cancer cells, (B) MCF-7 breast cancer cells and (C) non-small cell lung cancer (NSCLC) cells after 72 h treatment. Control is normalized to 100% . *p<0.05; **p<0.01, compared to control (100%).*

was added to the cell suspension. Untreated SW-480 cells were stained with the isotype control antibody. The cells were incubated in a dark room for 20 min at room temperature and then subjected to cell cycle and cyclin A analysis with a FACScan flow cytometer.

Statistical analysis. The data are expressed as mean±standard error (S.E.). Statistical analysis was performed using analysis of variance followed by a *post hoc* test for comparison of means. Differences were considered significant if $p<0.05$.

Results

Effects of 5-FU on human carcinoma cells. 5-FU reduced the carcinoma cell growth of the SW-480, MCF-7 and non-small cell lung cancer cells significantly, in a concentration-dependent manner. Compared to the control group (100%), 10 μM 5-FU reduced SW-480 cell growth to 63.5±1.1% ($p<0.01$), MCF-7 cell growth to 58.3±3.1% ($p<0.01$) and non-small cell lung cancer cell growth to 81.3±1.6% ($p<0.01$).
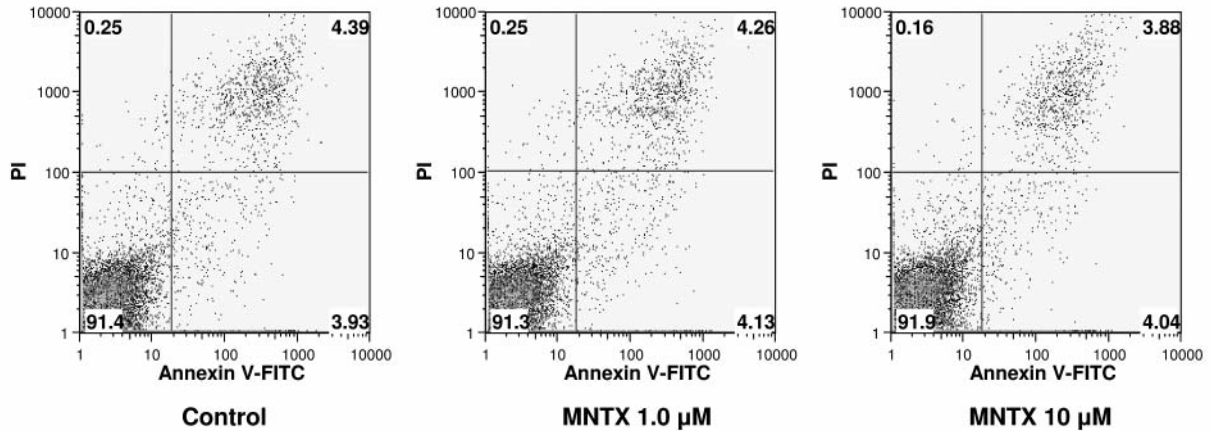
Figure 4. *Apoptosis assay using flow cytometry after annexin V-FITC/propidium iodide (PI) staining. SW-480 cells were treated with methylnaltrexone for 48 h. The percentages of differently stained cells are indicated. Apoptotic cells were positive for annexin V (lower right and upper right quadrants). MNTX=methylnaltrexone.*
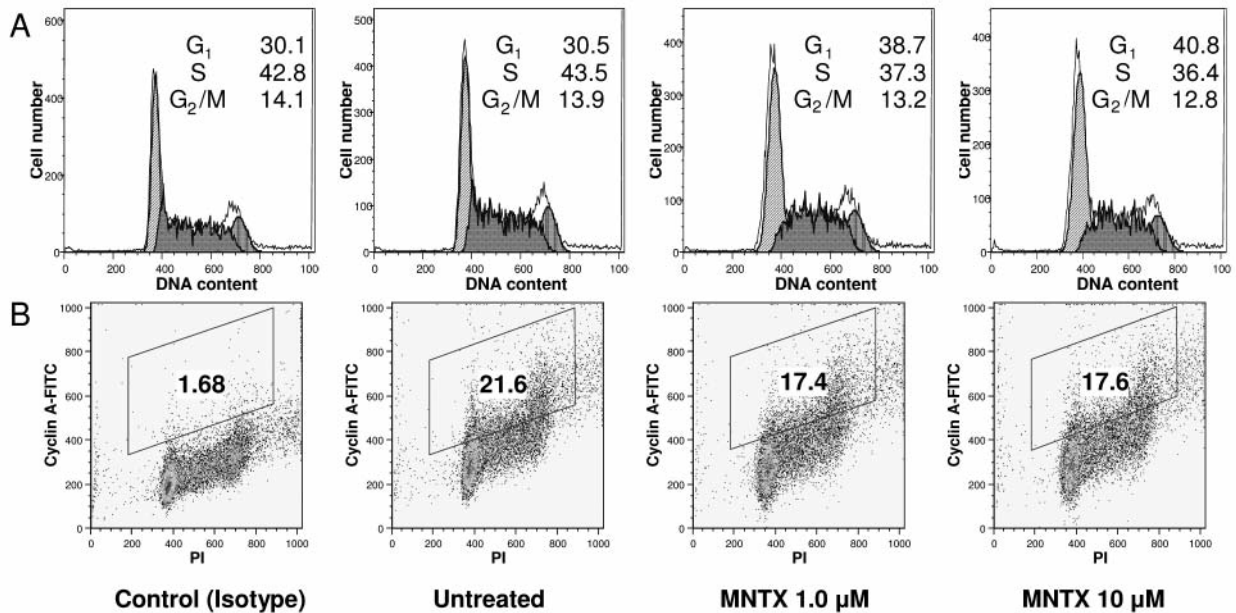

Figure 5. *Cell cycle and cyclin A analyses of SW-480 cells using flow cytometry. SW-480 cells were treated with 1.0 or 10 μM of methylnaltrexone for 48 h. (A) Cell cycle profiles. The percentages of cells in G_1-, S- and G_2/M-phases are indicated. (B) SW-480 cells stained with cyclin A-FITC and propidium iodide (PI). Untreated control cells were stained with isotype antibody and PI. The percentage of cyclin A positive cells is shown in the gate. MNTX=methylnaltrexone.*

This 5-FU concentration (10 μM) was selected for evaluating the interaction between 5-FU and methylnaltrexone.

Interaction between 5-FU and methylnaltrexone. Compared to 5-FU alone, 5-FU 10 μM plus methylnaltrexone 1.0 μM reduced cancer cell growth to 50.2±2.9% in the SW-480 cells ($p<0.05$), 50.0±1.7% in the MCF-7 cells ($p<0.05$) and 68.7±2.2% in the lung cancer cells ($p<0.01$ compared to 5-FU alone) (Figure 2). At concentrations from 0.01 μM to

1.0 μM, methylnaltrexone enhanced the effect of 5-FU in the three carcinoma cell lines.

Effects of methylnaltrexone on carcinoma cells. Methylnaltrexone alone had anti-proliferative effects in the three cell lines (Figure 3). Compared to control (100%), methylnaltrexone reduced SW-480 cell growth to 85.2±2.1% at 0.1 μM ($p<0.01$) and to 81.9±3.7% at 1.0 μM ($p<0.01$); reduced MCF-7 cell growth to 87.6±3.6% at 0.1 μM ($p<0.05$) and to 85.9±2.4% at

1.0 µM (p<0.01); and reduced lung cancer cell growth to 87.2±2.1% at 0.1 µM (p<0.01) and to 85.5±2.2% at 1.0 µM (p<0.01).

Apoptotic induction by methylnaltrexone. Compared to untreated control with early apoptosis of SW-480 cells of 3.93% and late apoptosis/necrosis of 4.39%, after treatment with 1.0 µM methylnaltrexone for 48 h, early apoptosis was 4.13% and late apoptosis was 4.26% (Figure 4). When the methylnaltrexone concentration was increased to 10 µM, apoptosis was similar to that in the control, suggesting that its anti-proliferative effect was not *via* the apoptotic induction pathway.

Effects of methylnaltrexone on SW-480 cell cycle and expression of cyclin A. Compared to the percentages in the untreated control of G_1 30.5%, S 43.5%, and G_2/M 13.9%, 1.0 µM methylnaltrexone increased the percentage of SW-480 cells in the G_1-phase to 38.7% and reduced the percentage of cells in the S-phase to 37.3% and the G_2/M-phase to 13.2%. With 10 µM methylnaltrexone, the G_1-phase increased to 40.8% (Figure 5A). As shown in Figure 5B, the percentage of cyclin A-positive cells in the untreated control was 21.6%. After treatment with 1.0 µM of methylnaltrexone for 48 h, the percentage of cyclin A-positive cells decreased to 17.4%. With 10 µM methylnaltrexone, the percentage of cyclin A-positive cells was 17.6%.

Discussion

At its therapeutic concentration for opioid-induced bowel dysfunction (24), methylnaltrexone did not interfere with the effects of 5-FU on the cancer cells in this study, suggesting that cancer patients should not be negatively affected by the co-administration of methylnaltrexone and 5-FU. It is possible that methylnaltrexone may even have an additive effect on 5-FU activity in the three tested cell lines.

Other studies have reported the relationship between opioids, cancer cells and cell proliferation (25-27), however, the effects of opioids on cancer cell growth are largely unknown.

Cell cycle progression is an important biological event in cancer cells. Targeting deregulated cell cycle progression and its modulation by chemotherapeutic agents to control the proliferation of cancer cells has gained widespread attention in recent years (28). As a thymidylate synthase inhibitor, 5-FU mainly interrupts the synthesis of pyrimidine thymine, which is important in DNA replication (29) and arrests cancer cells in the S-phase (30). In this study, methylnaltrexone increased the number of cancer cells arrested in the G_1-phase. It has been shown that when 5-FU is combined with other agents that arrest the cell cycle in the G_1-phase, its chemotherapeutic effect can be enhanced (31, 32). Since methylnaltrexone and 5-FU act at two distinct phases in the cell cycle, the combined use of these two compounds could enhance the effect of 5-FU. This study supports a complementary interaction between methylnaltrexone and 5-FU against cancer cells *in vitro*.

Cyclin A is a regulatory protein that functions in the S-phase of the cell cycle to induce mitosis. 5-FU increases cyclin A expression in the S-phase in cancer cells (30). Methylnaltrexone reduced the expression of cyclin A, suggesting that these two compounds have different anti-proliferative pathways. How the cyclin A level is affected after co-treatment with 5-FU and methylnaltrexone remains to be further evaluated.

It has been shown that angiogenesis, the formation of new blood vessels, promotes the growth and metastatic potential of various carcinomas (33, 34). Although the potential tumoricidal effect of methylnaltrexone is largely unknown, a recent study demonstrated the effects of methylnaltrexone on opioid-induced proliferation and migration of human endothelial cells, two key components in angiogenesis (35). Compounds that inhibit angiogenesis have therapeutic implications in numerous malignancies. In human dermal microvascular cells, opioids and vascular endothelial growth factor (VEGF) induced migration that was inhibited by pretreatment with methylnaltrexone (35). A recent report showed synergistic effects of methylnaltrexone with 5-FU and bevacizumab on the inhibition of VEGF-induced angiogenesis (36). Because opioids at therapeutic doses enhanced endothelial cell migration and proliferation in this model, methylnaltrexone treatment may have a benefit for patients taking regular doses of opioids for tumor-associated pain. In addition to exogenous opioids, endogenous opioids that are released in stress or pain may also affect endothelial cell migration and proliferation, which may make methylnaltrexone useful in anti-angiogenic therapy.

In summary, this study suggests that methylnaltrexone does not attenuate the tumoricidal activity of 5-FU and in fact may enhance it in three different human cancer cell lines. Although methylnaltrexone did not induce apoptosis in cancer cells at the concentrations used in this study, its cell cycle arrest profile was distinct from that of 5-FU. The underlying mechanisms of action of the inhibitory effect of methylnaltrexone on cancer cell growth and its additive effect to 5-FU remain to be elucidated in future experiments.

Acknowledgements

Disclaimer

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