Cent. Eur. J. Biol. • 7(3) • 2012 • 411-418
DOI: 10.2478/s11535-012-0030-7

VERSITA

Central European Journal of **Biology**

Opioid-regulated pro- and anti-apoptotic gene expression in cancer cells

Communication

Katarzyna Gach[1], Anna Wyrębska[1], Janusz Szemraj[2], Anna Janecka[1,*]

[1]*Department of Biomolecular Chemistry, Medical University of Lodz, 92-215 Lodz, Poland*

[2]*Department of Biochemistry, Medical University of Lodz, 92-215 Lodz, Poland*

Received 13 July 2011; Accepted 08 March 2012

Abstract: Morphine, as well as opioid peptides, are well-known powerful analgesics. In addition to their use in the treatment of pain, opioids appear to be important in the growth regulation of neoplastic tissue. However, little is known on the influence of opioid peptides on apoptosis modulation in cancer cells. In the present study, we evaluated the effect of the μ-opioid receptor (MOR)-selective peptide, morphiceptin and its two synthetic analogs, on mRNA expression and protein levels of some crucial factors involved in apoptosis in three human cancer cell lines: MCF-7, HT-29, and SH-SY5Y. Using real-time PCR and ELISA assays, we have shown that the selected opioid peptides enhanced apoptosis of cancer cells by increasing the expression of pro-apoptoticc Bax and caspase-3, and decreasing expression of anti-apoptotic Bcl-2. Additionally, flow cytometry analysis performed on MCF-7 cells treated with annexin V/propidium iodide confirmed that the tested opioid peptides induced apoptosis in cancer cells. However, induction of apoptosis was not reversed by the opioid antagonist, naloxone, which suggests that this process is not mediated by the opioid receptors.

Keywords: *Opioid peptides • Apoptosis • Pro-apoptotic genes • Anti-apoptotic genes*

1. Introduction

Apoptosis is a complex regulatory process that involves changes in the expression of several genes. Deregulation of apoptosis is one of the hallmarks of cancer and can contribute to uncontrollable proliferation and tumor growth. Apoptosis can be mediated either by the death-receptor pathway [1] or by the mitochondrial pathway [2].

The mitochondrial-mediated pathway is controlled by the Bcl-2 family of proteins and involves the alteration of mitochondrial membrane permeability and the release of apoptotic proteins, such as cytochrome c, from the mitochondria to cytosol. Cytochrome c together with apoptosis protease-activating factor-1 (Apaf-1) work in coordination with caspase-9 to activate caspase-3 [3]. The death receptor-mediated pathway is initiated by the ligation of cell death ligands, such as tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL), with their death receptors. Ligation first triggers activation of the initiator caspase-8 and finally the effector caspase-3. Both pathways ultimately lead to the activation of caspase 3, which executes the apoptotic processes resulting in cell death [3,4].

Compounds that can induce apoptosis in tumor cells are important tools in cancer research and are sought as possible anticancer agents. Morphine, the exogenous ligand of the μ-opioid receptor (MOR), is one of the most effective drugs available clinically for the management of severe pain associated with cancer. In addition to its use in the treatment of pain, morphine appears to be important in the regulation of neoplastic tissue. Therefore, understanding the impact of morphine on cancer treatment is extremely important. Accumulating

* *E-mail: anna.janecka@umed.lodz.pl*

evidence suggests that morphine can affect proliferation, apoptosis and the migration of tumor cells. However, the effect of morphine on tumor growth is still contradictory, as both growth promoting and growth inhibiting effects have been observed [5].

In the present study, we investigated the influence of morphiceptin, a MOR-selective peptide, and its two very potent synthetic analogs (Figure 1), on apoptosis in cancer cells. The following cell lines were examined: breast MCF-7, colon HT-29 adenocarcinomas, and neuroblastoma SH-SY5Y. These lines have been previously reported to shown high levels of MOR [6].

2. Experimental Procedures

Naloxone hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Morphiceptin, [Dmt^1, D-Ala^2, D-1-Nal^3] morphiceptin and [Dmt^1, D-NMeAla^2, D-1-Nal^3] morphiceptin, were synthesized in our laboratory using a standard solid-phase method as previously described [7].

2.1 Cell cultures

The MCF-7 human breast adenocarcinoma, SH-SY5Y neuroblastoma and HT-29 colon cancer cell lines were purchased from the European Collection of Cell Cultures (ECACC). All cell lines were cultured according to protocols provided by the manufacturer. Culture mediums were supplemented with gentamycin (5 µg/ml) and 10% heat inactivated fetal bovine serum (both from Biological Industries, Haemek, Israel). Cells were maintained at 37°C in a 5% CO_2 atmosphere and grown until they were 80% confluent.

2.2 Incubation with opioids

Cells (MCF-7, SH-SY5Y, HT-29) were seeded in 25 mL cell culture flasks in 10 mL of standard growth medium. After 24 h, the growth medium was replaced by fresh medium supplemented with the opioid compound of interest in the desired concentration. Cells incubated without the addition of an opioid compound were used as control. After incubation, cells were washed twice with phosphate buffered saline (PBS, GIBCO, Invitrogen, Carlsbad, CA, USA) to remove added compounds and were then harvested by trypsinization. The cells were frozen then at -80°C prior to RNA isolation and protein analysis.

Tyr-Pro-Phe-Pro-NH_2	Morphiceptin
Dmt-D-Ala-D-1-Nal-Pro-NH_2	Analog **1**
Dmt-D-NMe-Ala-D-1-Nal-Pro-NH_2	Analog **2**

Dmt – 2',6'- dimethyltyrosine
D-1-Nal – 3-(1-naphthyl)-D-alanine)

Figure 1. The sequence of morphiceptin and its analogs used in the study.

2.3 Quantitative real-time PCR assay

Total RNA was extracted from the MCF-7, HT-29 or SH-SY5Y cells using Total RNA Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's protocol. The concentration and purity of isolated RNA was determined using a spectrophotometer to examine absorbance at 260 and 280 nm. cDNA was synthesized using the Enhanced Avian HS RT-PCR Kit and oligo(dT)$_{12-18}$ primers (Sigma-Aldrich, St. Louis, MO, USA). Caspase-3, Bax, and Bcl-2 transcripts were quantified by real-time PCR using the Mx3005P QPCR System (Agilent Technologies, Inc. Santa Clara, CA, USA) according to the manufacturer's protocol for the Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Inc. Santa Clara, CA, USA). The housekeeping gene, glyceraldehydes 3-phosphate dehydrogenase (GAPDH), was selected as the internal control. cDNAs were amplified with forward and reverse primers that were specific for human caspase-3, Bax, Bcl-2, and GAPDH transcripts. Caspase-3 primer sequences were 5'-TGAAGCTACCTCAAACTTCC-3' (forward) and 5'-CAGCATCACTGTAACTTGCT-3' (reverse). Bax primer sequences were 5'-ACCCGGTGCCTCAGGATGCGT-3' (forward) and 5'-GGCAAAGTAGAAAAGGGCGAC-3' (reverse). Bcl-2 primer sequences were 5'-CATGCTGGGGCCGTACAG-3' (forward) and 5'-GAACCGGCACCTGCACAC-3' (reverse). GAPDH primer sequences were 5′-GTCGCTGTTGAAGTCAGAGGAG-3′ (forward) and 5′-CGTGTCAGTGGTGGACCTGAC-3′ (reverse).

Real-time PCR reactions were run in triplicate using the following thermal cycling profile: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 58°C for 1 min and 72°C for 1 min. After 40 cycles, a standard dissociation protocol was applied to samples (*i.e.* melting curve analysis). Brilliant II SYBR Green fluorescence emissions were captured and mRNA levels were quantified using the critical threshold (Ct) value. A no-template control was included in each assay. Relative gene expression levels were determined using the $\Delta\Delta C_t$ method [8].

2.4 Protein determination

Cells collected after treatment with the test compounds were washed twice with phosphate buffered saline (PBS, GIBCO, Invitrogen, Carlsbad, CA, USA). Cell pellets were frozen at -80°C. Cells were then lysed and

analyzed for caspase-3, Bax and Bcl-2 protein levels using Human Caspase-3 (active) ELISA Kit (Invitrogen, Life Technologies, USA), Human Bax ELISA/EIA Kit (IBL-America, Immuno- Biological Laboratories, MN, USA), and Human Bcl-2 ELISA/EIA Kit Assay Designs/ Stressgen (Now Enzo Life Sciences, NY, USA) according to the manufacturer's instructions.

2.5 Analysis of apoptosis by flow cytometry

Apoptotic cell death was determined using the FITC Annexin V Apoptosis Detection Kit (BD Bioscience, NJ USA). Briefly, cells were treated for 48 h with test compounds at a concentration of 10^{-7} M. The cells were harvested, washed with PBS, stained with annexin V and propidium iodide (PI) in the binding buffer and kept for 15 min at room temperature in the dark. Then flow cytometry analysis was performed (FACS Canto II, Becton). Early and late apoptosis was visualized and quantified by constructing a dot-plot using BD FACSDiva software.

2.6 Statistical analysis

Statistical analyses were performed using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). The data were expressed as means ± SD. Differences between groups were assessed by a one-way ANOVA followed by a post-hoc multiple comparison Student-Newman-Keuls test. A probability level of 0.05 or lower was considered statistically significant.

3. Results

3.1 Effect of opioids on caspase-3, Bax and Bcl-2 gene expression

To investigate the effect of morphiceptin and analogs 1 and 2 on the induction of apoptosis in cancer cells, caspase-3, Bax and Bcl-2 mRNA expression levels were measured using quantitative real-time PCR. Concentration response (10^{-9} to 10^{-5} M) and time course experiments (12, 24, 48, 72 h) were first performed to choose the optimal conditions. The results for MCF-7 cells are given in Figure 2 (concentration-response) and in Figure 3 (time-course). In case of all three cell lines the maximal effect was observed after 48 h incubation of peptides at a concentration of 10^{-7} M. The results for the chosen concentration and incubation time are shown in Figure 4. In all three tested cell lines, mRNA levels of the pro-apoptotic genes, caspase 3 and Bax, were significantly up-regulated, while down-regulation was observed for anti-apoptotic Bcl-2 mRNA level. Only small differences were found among analogs and among tested cell lines, which could suggest an opioid receptor-independent mechanism of action. To confirm this hypothesis, the opioid anatagonist, naloxone (10^{-7} M) was included in the study. As shown in Figure 5, naloxone did not reverse opioid induced changes in caspase-3, Bax and Bcl-2 mRNA levels.

3.2 Effect of opioids on caspase-3, Bax and Bcl-2 protein level

In addition to mRNA quantification, protein levels were investigated using an ELISA assay for the genes of interest. Data showed that opioid treatment influenced caspase-3, bax, bcl-2 protein levels, however the effects were slight and not reversible by treatment with naloxone (10^{-7} M) (Figure 6).

3.3 Analysis of apoptosis by flow cytometry

Annexin V/PI analysis was applied to quantify the apoptotic profile. In apoptotic cells, the membrane phospholipide, phosphatidylserine, is translocated from the inner to the outer portion of the membrane, and thus can be detected by annexin V-FITC, a phospholipid binding protein [9]. Meanwhile, the dead cells with disintegrated membranes can be detected by the binding of PI to their cellular DNA. The results of the annexin V/PI binding analysis by flow cytometry are shown in Figure 7A. The percentage of MCF-7 cells within the population undergoing apoptosis or necrosis are given in Table 1. The total number of apoptotic cells (early and late apoptosis) was increased by 46%, 37% and 42% following treatment with morphiceptin, analog 1 and analog 2 respectively (Figure 7B). Differences between morphiceptin and its analogs were not found to be statistically significant. These results support our hypothesis that opioid induction of apoptosis is not mediated by opioid receptors.

	Control	Morphiceptin	Analog 1	Analog 2
Q_1 (necrotic cells)	5%	6%	7%	8%
Q_2 (late apoptotic cells)	14%	20%	17%	18%
Q_3 (viable cells)	71%	59%	60%	58%
Q_4 (early apoptotic cells)	10%	15%	16%	16%

Table 1. Quantitative annexin V/PI analysis of the percentage of MCF-7 cells within a population undergoing apoptosis or necrosis.

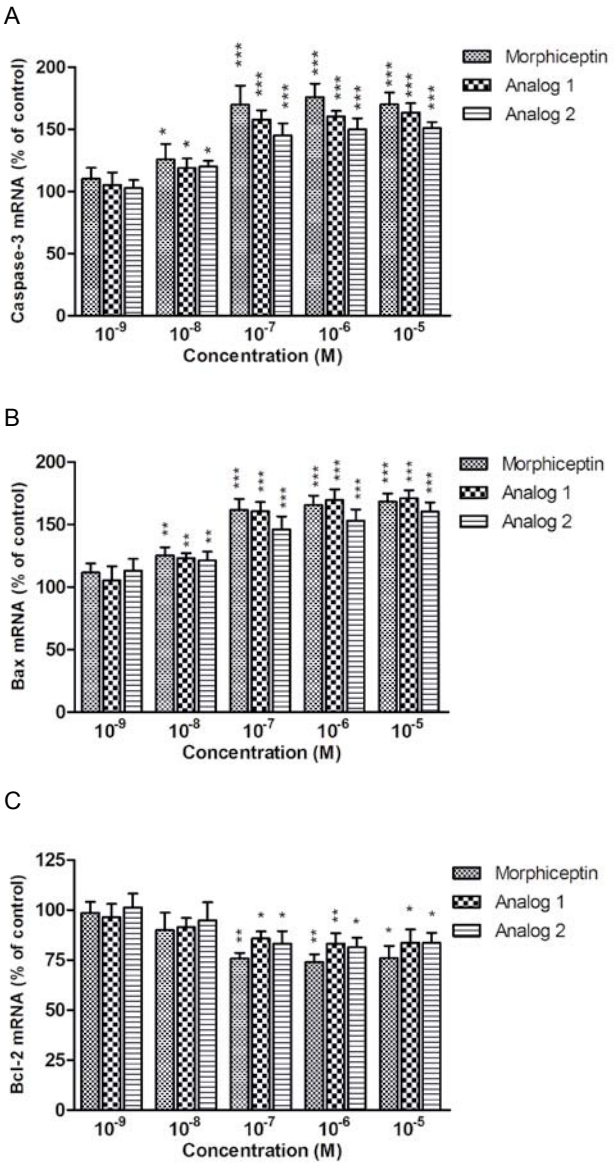

Figure 2. The concentration-response of: A) caspase-3, B) Bax and C) Bcl-2 mRNA levels in MCF-7 cells incubated for 48 h with morphiceptin or its analogs, measured by quantitative real-time RT-PCR. Data represent mean ± SD of three independent experiments performed in duplicate. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. *P<0.05, **P<0.01, ***P<0.001 was considered as significantly different from untreated cells regarded as control, 100%.

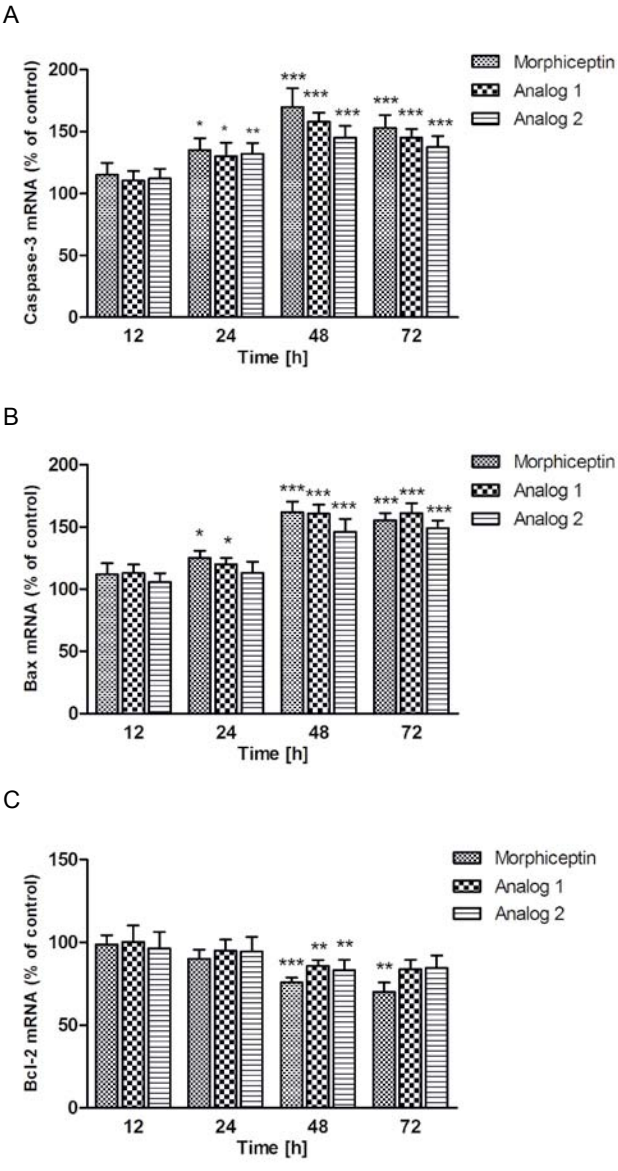

Figure 3. The time-course of: A) caspase-3, B) Bax and C) Bcl-2 mRNA levels in MCF-7 cells incubated with morphiceptin or its analogs, all at the concentration of 10^{-7} M, measured by quantitative real-time RT-PCR. Data represent mean ± SD of three independent experiments performed in duplicate. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. *P<0.05, **P<0.01, ***P<0.001 was considered as significantly different from untreated cells regarded as control, 100%.

4. Discussion

The pharmacology and function of opioids in the central nervous system have been extensively characterized, yet little is known about their effect on cancer cells.

Apoptosis is usually deregulated in cancer cells and this deregulation can contribute to uncontrollable proliferation and tumor growth [3,10,11]. Morphine has been shown to induce apoptosis in many human cancer cells lines [12-14]. However not much is known about the influence of endogenous opioid peptides on apoptosis modulation. Results are often contradictory and significant differences often occur between cell lines. For example endomorphins, endogenous MOR

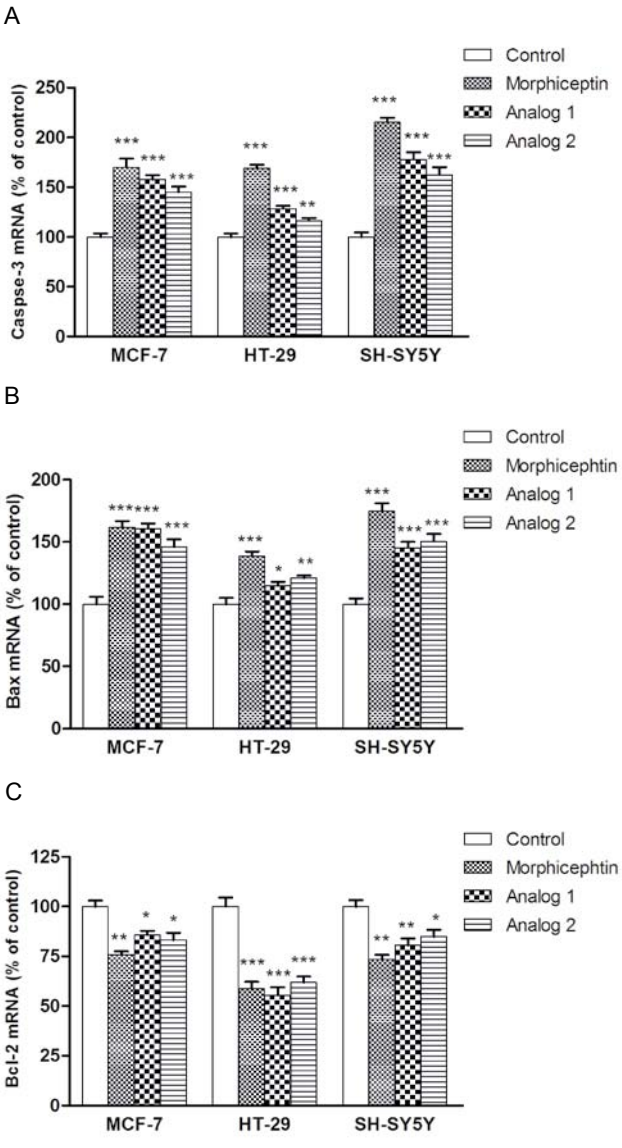

Figure 4. Quantitative real-time PCR analysis of: A) caspase-3, B) Bax and C) Bcl-2 mRNA levels in: MCF-7, HT-29 and SH-SY5Y cells incubated for 48h with morphiceptin or its analogs, all at the concentration of 10^{-7} M. Data represent mean ±SD of three independent experiments performed in duplicate. Statistical significance was assessed using one-way ANOVA and a post hoc multiple comparison Student–Newman–Keuls test. *P<0.05, **P<0.01, ***P<0.001 was considered as significantly different from untreated cells regarded as control, 100%.

agonists, were found to induce apoptosis in human leukemia HL-60 cells [15], but show no effect on cell survival in MIA PaCa-2 pancreatic adenocarcinoma, HT-29 colon adenocarcinoma, and CAL-27 oral adenosquamous carcinoma cell lines [16]. In our search for highly potent MOR-selective peptides as potential new analgesics, we have synthesized numerous analogs of MOR-selective agonists, endomorphin-2 and morphiceptin. Pharmacological studies of these analogs demonstrated that compounds with a higher affinity to MOR also had stronger antinociceptive activity.

In this study we wanted to examine whether opioid peptides (morphiceptin and its anqlogs with high MOR affinity) are able to induce apoptosis in cancer cells. One of the molecules responsible for maintaining the balance between cell proliferation and apoptosis is anti-apoptotic Bcl-2 protein [17]. This protein is localized on the outer mitochondrial membranes and antagonizes the action of pro-apoptotic Bax. The balance between these two proteins prevents translocation of cytochrome c from the mitochondria and determines apoptosis resistance. Inhibition of Bcl-2 or/and induction of Bax disrupts this balance [18,19]. Cytochrome c binds to Apaf-1 in the cytosol leading to the activation of procaspase-9, whose stimulation activates caspase-3. which leads to the execution phase of cell apoptosis [20].

We have found that morphiceptin and the two selected analogs each induced apoptosis in the three types of cancer cells tested. Apoptosis induction was associated with up-regulation of pro-apoptotic Bax protein and down-regulation of anti-apoptotic Bcl-2 and with increased activity of executioner caspase 3. Flow cytometry results confirmed that the tested opioids induced apoptosis in cancer cells but only small differences were observed between the analogs. Moreover, the effect produced by opioids was not reversed by naloxone, indicating that in tested cancer cells, opioid peptides could induce apoptosis by mechanisms that were not mediated by opiod receptors. These results are consistent with previous findings obtained for morphine. Lin *et al*. [21] demonstrated that high-dose morphine treatment led to apoptotic cell death in SH-SY5Y cells. Moreover they showed that naloxone was not able to block morphine-induced apoptosis, suggesting that a typical opioid receptor-related mechanism was not involved in opioid-induced apoptosis of tumor cells. In another study, Tegeder *et al*. [22] observed that the anti-proliferative effects of morphine were not antagonized by naloxone, suggesting that the typical opioid receptor-coupled signaling cascade involving the G_i, adenylyl cyclase, and protein kinase A was not involved. Instead, morphine treatment results in an N-terminal phosphorylation of p53 in MCF-7 cells. This p53 phosphorylation was not antagonized by naloxone and resulted in an increase of p53-dependent proteins, including Bax.

In conclusion, our results demonstrated that in cancer cells opioid peptides can induce apoptosis by mechanisms that are not mediated by the opiod receptors. Further research is necessary to provide clearer understanding of the influence of opioid peptides on apoptosis machinery.

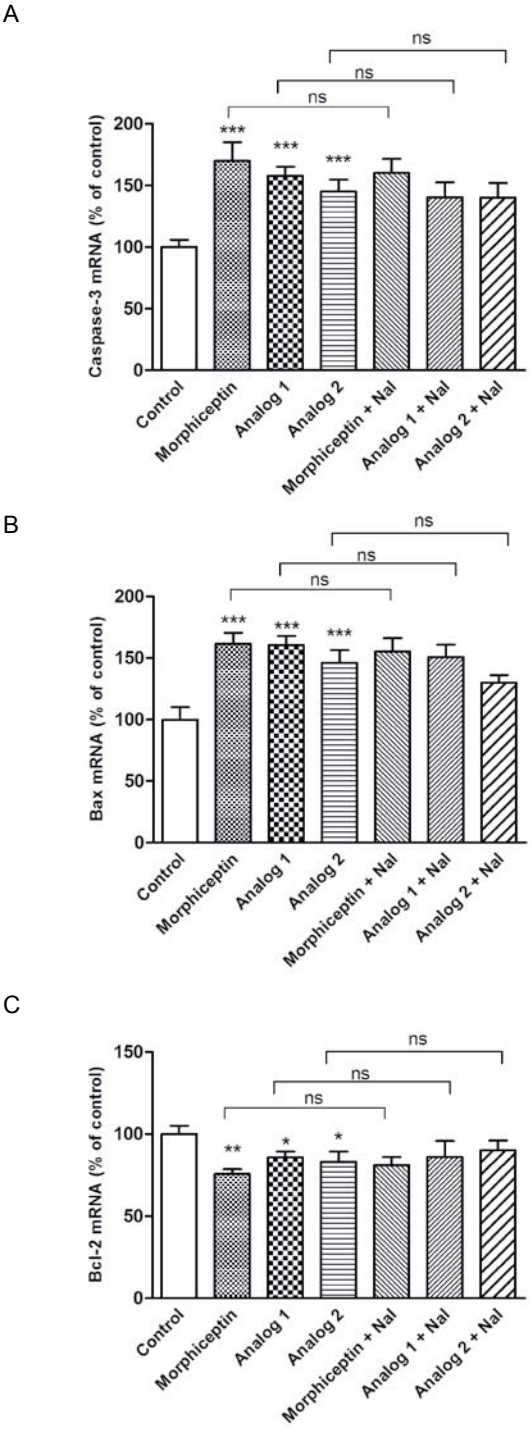

Figure 5. Quantitative real-time PCR analysis of the effect of: naloxone (Nal) (10^{-7} M) on A) caspase-3, B) Bax and C) Bcl-2 mRNA levels in MCF-7 cells incubated for 48h with morphiceptin or its analogs, all at the concentration of 10^{-7} M. Data represent mean ± SD of three independent experiments performed in duplicate. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. *P<0.05, **P<0.01, ***P<0.001 was considered as significantly different from untreated cells regarded as control, 100%; ns not statistically different from the corresponding agonist.

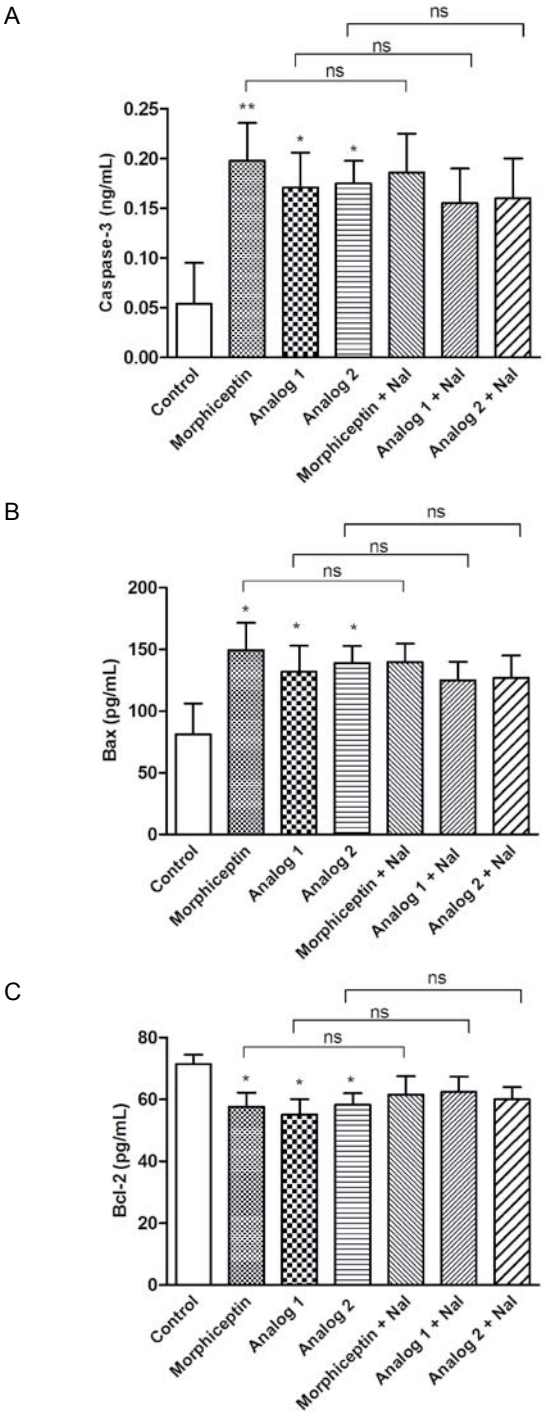

Figure 6. A) caspase-3, B) Bax and C) Bcl-2 protein concentration in MCF-7 cells incubated for 48h with morphiceptin or its analogs (10^{-7} M), alone or in combination with naloxone (10^{-7} M). Data represent mean ± SD of three independent experiments performed in duplicate. Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. *P<0.05, **P<0.01 was considered as significantly different from untreated cells regarded as control, 100%; ns not statistically different from the corresponding agonist.

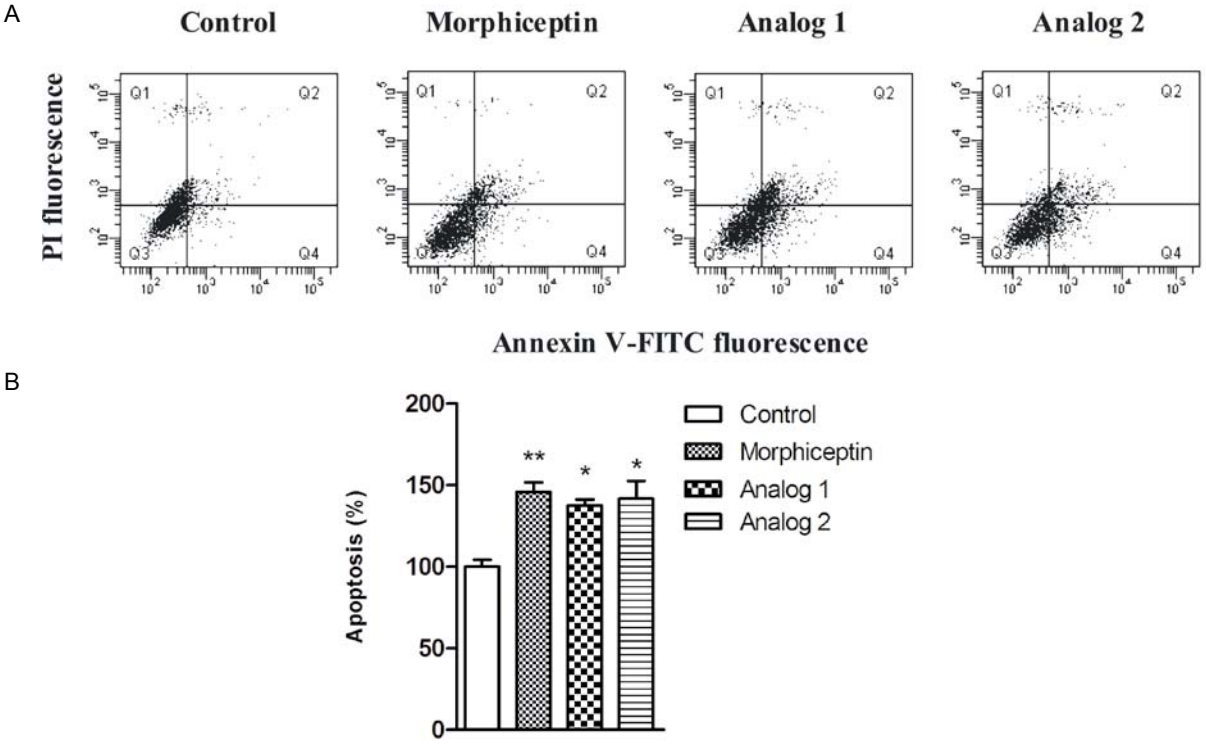

Figure 7. The results of the flow cytometry analysis of apoptosis induction in MCF-7 cells incubated for 48h with morphiceptin or its analogs, all at 10^{-7} M concentration. A) Annexin V and PI staining in MCF-7 cells (10,000 cells were counted). Q_1 quadrant indicates the percentage of necrotic cells (PI-positive), Q_2 quadrant indicates the percentage of late apoptotic cells (Annexin V + PI-stained), Q_3 quadrant indicates the percentage of viable cells and Q_4 indicates the percentage of early apoptotic cells (Annexin V-positive). B) The total percentage of apoptotic cells (Q_2 + Q_4). Statistical significance was assessed using one-way ANOVA and a post-hoc multiple comparison Student-Newman-Keuls test. *P<0.05, **P<0.01 was considered as significantly different from untreated cells regarded as control, 100%.

Acknowledgements

This work was supported by the grants from the Medical University of Lodz (No 503/1-156-02/503-01, from Polish Ministry of Science „Iuventus Plus" (No IP2010009470 for K.G.) and 'START' scholarship of the Foundation for Polish Science (for K.G.).

References

[1] Ashkenazi A., Dixit V.M., Apoptosis control by death and decoy receptors, Curr. Opin. Cell. Biol., 1999, 11, 255-260

[2] Cory S., Adams J.M., The Bcl2 family: regulators of the cellular life-or-death switch, Nat. Rev. Cancer, 2002, 2, 647-656

[3] Green D.R., Reed J.C., Mitochondria and apoptosis, Science, 1998, 281, 1309-1312

[4] Hengartner M.O., The biochemistry of apoptosis, Nature, 2000, 407, 770-776

[5] Tegeder I., Geisslinger G., Opioids as modulators of cell death and survival-unraveling mechanisms and revealing new indications, Pharmacol. Rev., 2004, 56, 351-369

[6] Gach K., Szemraj J., Stasikowska-Kanicka O., Danilewicz M., Janecka A., Opioid-receptor gene expression and localization in cancer cells, Cent Eur J Biol, 2011, 6, 10-15

[7] Fichna J., do-Rego J.C., Chung N.N., Lemieux C., Schiller P.W., Poels J., et al., Synthesis and characterization of potent and selective mu-opioid receptor antagonists, [Dmt(1), D-2-Nal(4)] endomorphin-1 (Antanal-1) and [Dmt(1), D-2-Nal(4)]endomorphin-2 (Antanal-2), J. Med. Chem., 2007, 50, 512-520

[8] Winer J., Jung C.K., Shackel I., Williams P.M., Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for

monitoring gene expression in cardiac myocytes in vitro, Anal Biochem, 1999, 270, 41-49

[9] van Engeland M., Nieland L.J., Ramaekers F.C., Schutte B., Reutelingsperger C.P., Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure, Cytometry, 1998, 31, 1-9

[10] Hanahan D., Weinberg R.A., The hallmarks of cancer, Cell, 2000, 100, 57-70

[11] Kaufmann S.H., Hengartner M.O., Programmed cell death: alive and well in the new millennium, Trends Cell. Biol., 2001, 11, 526-534

[12] Sueoka E., Sueoka N., Kai Y., Okabe S., Suganuma M., Kanematsu K., et al., Anticancer activity of morphine and its synthetic derivative, KT-90, mediated through apoptosis and inhibition of NF-kappaB activation, Biochem. Biophys. Res. Commun., 1998, 252, 566-570

[13] Hatsukari I., Hitosugi N., Matsumoto I., Nagasaka H., Sakagami H., Induction of early apoptosis marker by morphine in human lung and breast carcinoma cell lines, Anticancer. Res., 2003, 23, 2413-2417

[14] Kawase M., Sakagami H., Furuya K., Kikuchi H., Nishikawa H., Motohashi N., et al., Cell death-inducing activity of opiates in human oral tumor cell lines, Anticancer. Res., 2002, 22, 211-214

[15] Lin X., Chen Q., Xue L.Y., Ma X.J., Wang R., Endomorphins, endogenous opioid peptides, induce apoptosis in human leukemia HL-60 cells, Can. J. Physiol. Pharmacol., 2004, 82, 1018-1025

[16] Zagon I.S., McLaughlin P.J., Opioids and the apoptotic pathway in human cancer cells, Neuropeptides., 2003, 37, 79-88

[17] Sabourin J.C., Martin A., Baruch J., Truc J.B., Gompel A., Poitout P., bcl-2 expression in normal breast tissue during the menstrual cycle, Int. J. Cancer., 1994, 59, 1-6

[18] Cho M.Y., Park S.Y., Park S., Lee Y.R., Han G.D., Kim J.A., Geranyl derivative of phloroacetophenone induces cancer cell-specific apoptosis through Bax-mediated mitochondrial pathway in MCF-7 human breast cancer cells, Biol. Pharm. Bull., 2012, 35, 98-104

[19] Liu J.W., Cai M.X., Xin Y., Wu Q.S., Ma J., Yang P., et al., Parthenolide induces proliferation inhibition and apoptosis of pancreatic cancer cells in vitro, J. Exp. Clin. Cancer Res., 2010, 29, 108

[20] Yang J., Liu X., Bhalla K., Kim C.N., Ibrado A.M., Cai J., et al., Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked, Science, 1997, 275, 1129-1132

[21] Lin X., Wang Y.J., Li Q., Hou Y.Y., Hong M.H., Cao Y.L., et al., Chronic high-dose morphine treatment promotes SH-SY5Y cell apoptosis via c-Jun N-terminal kinase-mediated activation of mitochondria-dependent pathway, FEBS J., 2009, 276, 2022-2036

[22] Tegeder I., Grösch S., Schmidtko A., Häussler A., Schmidt H., Niederberger E., et al., G protein-independent G1 cell cycle block and apoptosis with morphine in adenocarcinoma cells: involvement of p53 phosphorylation, Cancer Res., 2003, 63, 1846-1852