Original Article

Effects of Methadone on the Toll-like Receptor 4 Expression in Human Non-Small Cell Lung Carcinoma A549 Cell Line Using *In-silico* and *In vitro* Techniques

Golnaz Vaseghi[1], Nastaran Rashidi[2], Nasrin Zare[3], Fahimeh Ghasemi[4], Marjan Pourhadi[2], Laleh Rafiee[2], Shaghayegh Haghjooy Javanmard[2,3]

[1]Isfahan Cardiovascular Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran, [2]Department of Physiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran, [3]Applied Physiology Research Center, Cardiovascular Research Institute, Isfahan University of Medical Sciences, Isfahan, Iran, [4]Department of Bioinformatics and Systems Biology, School Advanced Technologies in Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: In this study, the effects of methadone and naloxone on the expression of toll-like receptor 4 (*TLR4*) gene have been evaluated in human non-small cell lung carcinoma A549 cell line migration using *in-silico* and *in vitro* techniques.

Materials and Methods: Lung cancer A549 cell cultures were stimulated for 24 h with methadone (5, 10, and 20 μM) and naloxone (20 and 40 μM) concentrations. The level of *TLR4* expression was determined by the quantitative real-time polymerase chain reaction. Migration of the A549 cells was investigated after a 4-h incubation period with methadone using the Boyden Chamber assay.

Results: Migration rate of the A549 cells treated with 5 ($P < 0.05$) and 20 ($P < 0.01$) μM methadone was, respectively, increased and decreased with 20 μM naloxone ($P < 0.05$). Furthermore, the *TLR4* expression was enhanced with 5 ($P < 0.05$) and 20 ($P < 0.01$) μM methadone and decreased with 20 ($P < 0.05$) and 40 μM naloxone ($P < 0.01$). In addition, *in silico* docking analysis revealed docking of methadone to MD-2 and *TLR4*.

Conclusion: According to the present DATA, methadone affects the *TLR4* expression. It may however cause adverse consequences by increasing the *TLR4* expression. Therefore, the useful analgesic properties of methadone should be separated from the unwanted *TLR4*-mediated side effects.

Keywords: A549 cells, lung cancer, methadone, naloxone, toll-like receptor 4

Address for correspondence: Dr. Shaghayegh Haghjooy Javanmard, Department of Physiology, Applied Physiology Research Center, Faculty of Medicine, Isfahan University of Medical Sciences, Hezar Jerib Avenue, Isfahan, Iran.
E-mail: shaghayegh.haghjoo@gmail.com
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Introduction

Lung cancer is the second fatal illness, causing cancer-related mortality in both genders nowadays. As such, a critical component of tumorigenesis and tumor progression is inflammation.[1] However, the gold standard for the clinical pain management is administering different types of opiates.[2] Notably, opioid receptors are present in the central (central nervous system) and peripheral nervous system and they are expressed on the immune cells; therefore, opioids could modulate immune functions.[3] Opioids seem to interact with the innate immune receptors, such as toll-like receptors (*TLRs*) and in particular TLR 4 (*TLR4*) and affect their signaling pathways.[4] The important role of *TLRs* in innate immune function and inflammatory responses is related to recognizing pathogen-associated molecular patterns, such

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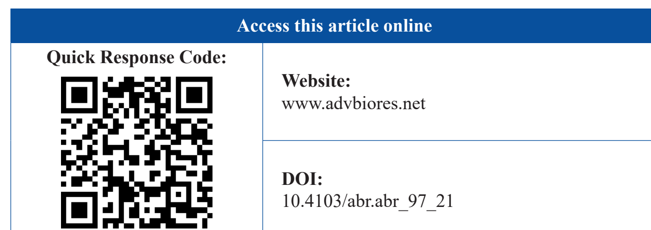

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as lipopolysaccharide (LPS), lipopeptide, double-strand RNA, and bacterial DNA.[5] They are expressed on immune, endothelial, and various types of cancer cells.[6] Furthermore, the stimulation and activation of *TLR4* could invoke the production of pro-inflammatory cytokines and mediators against pathogens.[7] The *TLR4*-induced anti-apoptotic and pro-inflammatory signaling through chronic stimulation of the *TLR4* pathway contributes to tumor-promoting escape.[8] Morphine, an opioid medication, may reduce the beneficial effect of radiotherapy on breast and cervical cancer patients.[9] Treatment of breast cancer cells by morphine might change the *TLR4* gene expression. While moderate morphine dose decreased *TLR4* gene expression, it had a reverse effect in higher concentrations.[10] Previous findings suggested that *TLR4* signaling in cancer cells could not only promote the expression of angiogenic and inflammatory factors but also increase cancer invasiveness and metastasis.[11] The TLR4 expression correlated with the differentiation degree, stage, and metastasis of lung cancer cells.[12] However, methadone, a synthetic opioid agonist with a high affinity for both μ-receptors (key mediators in supra-spinal analgesia) and δ-receptors (probably important in spinal analgesia), has been recommended as an effective analgesic to relieve chronic and complex pain in cancer patients.[13] In previous studies, incubation of isolated natural-killer (NK) cells with methadone has decrease NK cell activity.[14] Similarly, prenatal methadone exposure enhanced the level of inflammatory cytokines and upregulated the *TLR4* and Myeloid differentiation primary response 88 (MyD88) mRNA in the neonatal peripheral circulation brain.[15] Therefore, present series of *in-silico* and *in vitro* studies was firstly designed to investigate whether methadone influences the TLR4 expression in human non-small cell lung carcinoma (NSCLC) A549 cell line. Furthermore, the TLR4-induced effects of methadone on tumor cell migration were evaluated.

Materials and Methods

Cell culture

The human lung cancer cells (cell line A549) were obtained from the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured in Roswell Park Memorial Institute medium, which was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin streptomycin. The cells were incubated in a humidified atmosphere containing 5% CO2 (at 37°C), and harvested at 70%–80% confluency with a culture medium renewal every 3 days.

Drug exposure

Naloxone hydrochloride (Caspian Tamin Co., Iran), morphine sulfate (Darou Pakhsh Co., Iran), and methadone (Exir Co., Iran) were used for the A549 cell line experiments, *in vitro*. Cell cultures were stimulated with 5, 10, and 20 μM methadone[16] and 20 and 40 μM naloxone[17] for 24 h. The level of *TLR4* expression was determined by quantitative real-time polymerase chain reaction (RT-qPCR). Furthermore, transwell migration assay (or, Boyden Chamber assay) was used to measure the migration rate in treated cells with 5 μM and 20 μM methadone, 20 μM naloxone, 5 μM methadone plus 10 μM naloxone, and 20 μM methadone plus 20 μM naloxone.

RNA extraction and cDNA synthesis

The A549 cells were stimulated with 5 and 20 μM methadone as well as 20 and 40 μM naloxone for 24 h. The total RNA was extracted from the treated cells using RNA mini Kit (Total RNA Prep Kit, Bio FACT, South Korea) according to manufacturer instructions. After the DNase I treatment, the RNA quantity and quality were evaluated by a spectrophotometer and gel electrophoresis. The RNA was reverse-transcribed into cDNA using a 2XRT-PCR Pre-mix cDNA synthesis kit (Bio FACT, South Korea) in compliance with manufacturer protocols. Finally, the total RNA was reverse-transcribed by this kit in a 20 μL reaction volume (respectively, for 5, 30, and 5 min at the room temperature, 5, and 95°C, then immediately cooled to 4°C).

Real-time polymerase chain reaction for toll-like receptor 4: Gene expression assays

To measure the level of *TLR4* and *GAPDH* mRNA expression, an RT-PCR system was implemented. The forward (F) and reverse® primer sequences were used for *TLR4* and *GAPDH* genes:

(*TLR4* forward primer: 5-CGCTTTCA CCTCTGCCTTCAC-3; *TLR4* reverse primer: 5-TTGCCGTTCTTG TTCTTCTTC-3; *GADPH* forward primer: 5-GAAGGTGGTGAAGCAGG CATC; and *GADPH* reverse primer: 5-GTGGGAGTTGCTGTT GAAGTC).

An RT-PCR assay was developed based on SYBR Green I and in a total reaction volume of 10 μL using a Corbett Rotor-Gene 6000 system (Life Science, Australia). The following thermal cycling protocol was applied: Incubation at 95°C for 15 min, followed by 45 cycles at 95, 60, and 72°C, each for 20 s. The relative quantitative expression of *TLR4* mRNA was calculated using the 2-ΔΔCt method. The ΔΔCt value was calculated by obtaining the difference between the ΔCt values of the treated and untreated cells (ΔΔCt = ΔCt (treated cell)-ΔCt (untreated cell). Also, the ΔCt values were calculated by subtracting the *GAPDH* Ct from the *TLR4* Ct of for each sample (ΔCt = *TLR4* Ct-*GAPDH* Ct).

Migration assay

An *in vitro* trans well migration assay was performed by uncoated 8 μm pore size membranes (SPL Life Sciences, South Korea). The untreated control A549 cells and the ones treated with methadone and naloxone were seeded in the upper compartment, adding 500 μl of 10% FBS media to the lower chamber. After a 4-h incubation period, the nonmigrated cells were removed from the top membrane and the migrated ones were fixed and stained using 0.05% (w/v) crystal violet solution.[10,16] Subsequently, the captured images by ImageJ software were used to analyze the migration inside the chamber.

Docking simulations in-silico

A molecular docking study was performed by Auto Dock (v. 4.2). The crystallographic structure of the human *TLR4*/human MD-2 complex (PDB ID: 3FXI, X-ray resolution: 3.1 Å) was obtained from RCSB Protein Data Bank (PDB) archive.[18] After retrieving the appropriate PDB file (ID: 3FXI), the methadone ligand structure, the (S)-methadone and ®-methadone otherwise shown as-(-), was downloaded from the PubChem (https://pubchem.ncbi.nlm.nih.gov). Subsequently, it was converted to a PDB file using BioVia Discovery Studio v. 16 (2016). Water molecules, crystallized with targets, were removed to generate an original crystalline structure. Hydrogens were then added to the PDB protein structure. In the protonation step, ionization and tautomeric states of residues were done. Merz-Kollman charges were calculated and nonpolar hydrogens were merged with the adjacent carbon atoms. The protein structures were first re-saved as PDBQ format and then as PDBQT one. After adding hydrogens of-(-) methadone molecules, Gasteiger-Marsili charges were calculated and nonpolar hydrogens were merged. Finally, the rotatable bonds in the ligand were detected and saved as the PDBQT format. The modified PDB files for receptors and ligands were inputted in Auto Dock v. 4.2.[19] Biological targets were applied as an Auto Grid input so that the atomic affinity grid maps for each atom type in the ligand, as well as the additional electrostatic and desolvation maps, could be calculated. All maps were calculated inside a grid box with 60 × 60 × 60 points in the X, Y, Z directions and 0.375 Å grid spacing (centered at 27.991, 0.851, and 19.625).[20] All-important active sites of amino acids were involved in the grid box. Notably, the grid parameter files were built by the Auto Grid program. While running an Auto Dock search using a Lamarckian genetic algorithm (Lamarckian GA or LGA), 150 GA runs were conducted; several parameters were set as the default LGA settings (2.5 × 106 energy evaluations, 2.7 × 104 generations, as well as mutation and crossover rates: 0.02 and 0.8). Furthermore, the conformation with the lowest estimated free energy was visualized by AutoDockTools v. 1.5.6 and BioVia Discovery Studio v. 16.[21]

Statistical analysis

All data were presented as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) test. Furthermore, comparisons between multiple data from different treatment groups were conducted using the least significant difference (*post hoc*) test. Besides, SPSS 20. Inc., Chicago, Ill., USA was used for the statistical analyses. $P < 0.05$ ($P < 0.05$) was considered statistically significant. The error bars on graphs represent the standard error of the mean.

Results

Effects of methadone and naloxone on the toll-like receptor 4 gene expression in the A549 cells

TLR4 gene expression was assessed by the RT-PCR technique. As shown in Figure 1, the level of *TLR4* expression in the A549 cells were increased significantly (ANOVA with $P = 0.0001$) after a 24-h treatment period with methadone concentration at doses of 5 μM (1.274 ± 0.0108) and 20 μM (3.387 ± 0.0976) compared to the control group (1.061 ± 0.0182). However, the level of *TLR4* expression was decreased significantly (ANOVA with $P = 0.0001$) after a 24-h treatment with naloxone concentrations at doses of 20 μM (0.927 ± 0.0033) and 40 μM (0.837 ± 0.0731) compared to the control group [Figure 1] (both ANOVA with $P = 0.0001$).

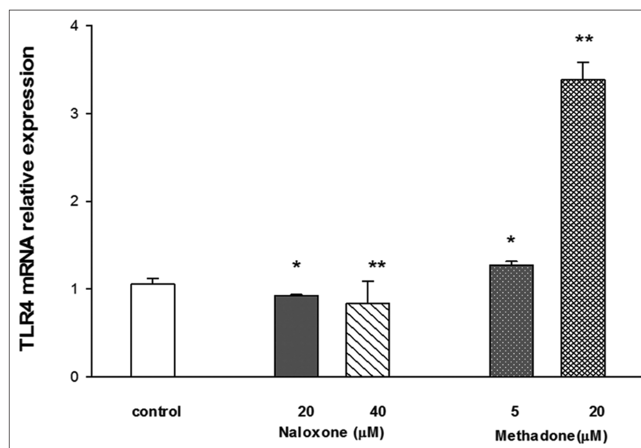

Figure 1: Fold changes of toll-like receptor 4 expression after incubation with various concentrations of naloxone and methadone for 24 h. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the control

Effects of methadone and naloxone on the migration of the A549 cells

The A549 cells were incubated for 24 h with 5 μM (234.637 ± 1.168) and 20 μM (239.322 ± 2.426) methadone concentration. This treatment increased the migration of A549 cells compared to the control group (227.378 ± 3.223). However, there were no significant correlations in the 20 μM naloxone (224.150 ± 3.535) compared to the control group regarding the cell migration. Finally, no significant relationship was observed in the 5 μM methadone plus 20 μM naloxone (231.039 ± 0.736) and 20 μM methadone plus 20 μM naloxone (237.289 ± 1.127) groups compared to the control group [Figure 2].

In-silico studies on human toll-like receptor 4: Human MD-2

Docking simulations are suitable tools in medicinal chemistry for exploring most possible ligand-target interactions with currently known three-dimensional structures. Therefore, AutoDockTools (v. 4) was utilized to analyze the interactions of methadone with human *TLR4*/human MD-2 complex to explore the conformation of ligand binding sites. The results are summarized in Table 1.

According to Table 1, molecular conformers were clustered into 19 categories. The best group, having 35 out of 150 conformers, presented −5.63 mean energy. The highest conformation was selected based on the lowest binding energy and highest populations. The lowest binding energy in docking

Table 1: Data of molecular conformers

Cluster rank	Number of conformers in cluster	Lowest binding energy	Mean binding energy	Mean RMSD
1	3	−7.57	−7.33	0.7
2	25	−5.99	−5.58	1.5
3	15	−5.86	−5.62	1.2
4	21	−5.86	−5.72	0.7
5	35	−5.75	−5.63	0.3
6	2	−5.72	−5.61	0.75
7	17	−5.57	−5.46	1.5
8	4	−5.48	−5.40	0.7
9	3	−5.48	−5.38	0.5
10	10	−5.47	−5.37	1.9
11	2	−5.43	5.39	0.5
12	2	−5.39	5.36	0.2
13	2	−5.37	5.27	0.4
14	3	−5.29	5.23	0.4
15	2	−5.25	5.23	0.2
16	1	−5.23	−5.23	0
17	1	−5.16	5.16	0
18	1	−5.12	−5.12	0
19	1	−4.97	−4.97	0

RMSD: Root mean square deviation

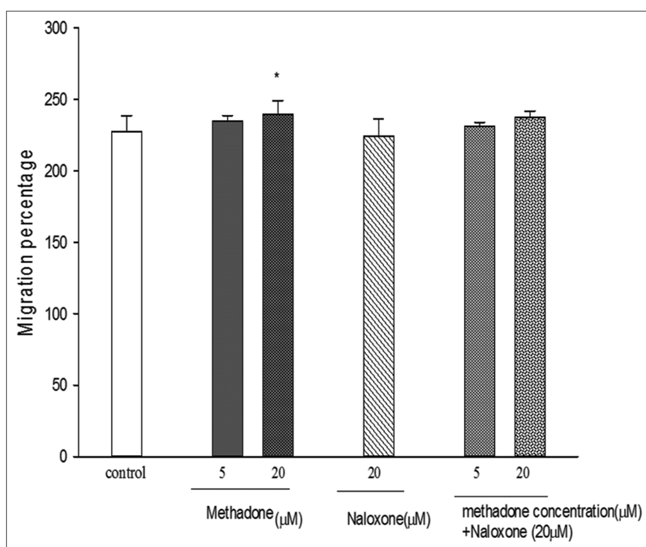

Figure 2: Migration of the A549 cells after 4 h incubation with naloxone and methadone or DMSO (control). The mean of cell counts is shown. **P* < 0.05 compared with the control

studies was calculated as −7.57 in cluster 1 with 3 conformers from all runs. Approximately, 75% of runs had a binding energy value higher than −5.5. This compound's placement in the active site of human *TLR4*-human MD-2 docking is demonstrated in Figure 3.

The ligand-binding pocket was stabilized through hydrophobic interactions with Phe-76. As illustrated in this figure, methadone binds to the LPS-binding pocket on human *TLR4*-human MD-2 in an *in-silico* model. Also, the methadone benzene ring may form pi-sulfur hydrophobic interactions with CYS-133. In addition, most conformers indicated hydrophobic interaction

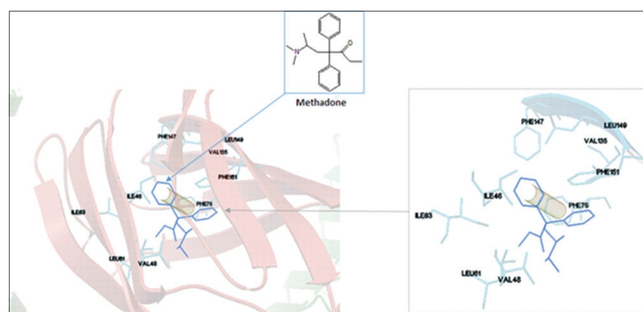

Figure 3: Interaction between methadone and the active site of human toll-like receptor 4-human MD-2 visualized by auto dock tools 1.5.6. PHE 76 shows Pi-Pi interaction with methadone

with Phe-76. Other ligand-target interactions are presented via BioVia Discovery Studio v. 16 [Figure 4].

Discussion

In the current study, a significant increase in the level of *TLR4* expression was observed in the methadone-treated A549 cells. The effects of methadone on *TLR4* might be dose-dependent as treatment with higher methadone concentrations (5, 20 μM) for 24 h increased the *TLR4* expression. Some studies have indicated a correlation between low serum soluble *TLR4* (*TLR4s*) and poor survival of early-stage NSCLC patients who received surgical resection.[22] It is also reported that high expression of *TLR4* in lung cancer tissues had a meaningful relationship with the malignancy of lung cancer.[12]

Our findings demonstrated a significant reduction in the *TLR4* expression in the A549 cells treated with naloxone. Similarly, previous findings had revealed that clinical use of opioid antagonists, such as naltrexone and naloxone inhibited

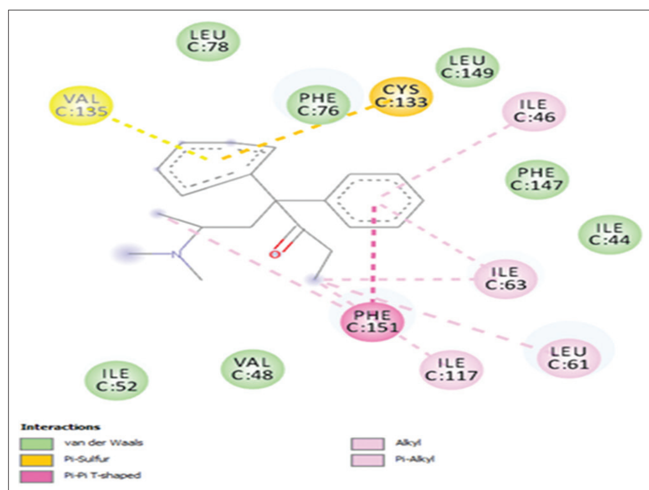

Figure 4: The interaction between methadone and active site of toll-like receptor 4 and MD-2 visualized by Discovery Studio 2016

opioid-induced *TLR4* signaling.[23] Therefore, the effects of morphine on gene expression might be time-dependent.[11] For instance, increasing morphine concentrations for 24 h increased the *TLR4* expression but exposure to higher morphine concentrations (5 μM) for 48 h decreased the *TLR4* expression.[16]

In this study, some common residues in the *TLR4*, MD, and methadone binding sites exhibited chemical interactions. Moreover, the π–π bond with Phe-76 and ligand ring seemed to be effective in *TLR4* potency. Methadone's chemical structure was proper for making conformation to these important chemical interactions in the *TLR4*-binding sites. In other studies, insignificant interactions between these molecules were indicated as well.[20,24]

As such, in the cross-talk between peroxisome proliferator-activated receptor (PPARs) and *TLRs*, they are reported to have mutually affected each other. In other words, while PPARs might have altered *TLRs'* behavior, in a reverse mechanism, *TLRs* might also alter PPARs' effects. Therefore, an adjuster feedback loop existed between their signaling pathways.[25]

In a former study, pioglitazone (a PPAR γ agonist) suppressed melanoma cancer in mice by inhibiting the *TLR4* signaling. Furthermore, pioglitazone presented no beneficial protective effects against melanoma by interfering with the *TLR4* expression and PPARπ activation. The PPAR γ agonist reduced tumor volume, expression of *TLR4*, Myd-88, nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-kb1) mRNA, and development of tumor necrosis factor α in the melanoma tumors, and particularly in the LPS-stimulated cells.[26]

Moreover, the growth of human lung cancer cells is reported to have been inhibited by methadone, in both *in vitro* and *in vivo* experiments. The inhibitory effects of methadone occurred at low concentrations and were related to alterations in cell morphology and viability.[27] In the present study, methadone increased the migration rate in A549 cells after 4 h. Therefore, the effects of methadone may depend on its dosage and time.

Recent findings consider methadone as a potential antineoplastic compound. In addition, methadone, similar to other opioids, shows pro-apoptotic effects and proliferation-stimulating properties. However, it is still unclear whether or not different types of receptors (such as μ-opioid, cell death, *TLR4*, N-Methyl-d-aspartate, and opioid growth factor receptors are responsible for these effects of methadone.[28]

Conclusion

In summary, it was observed that methadone enhanced the expression of *TLR4* in the A549 cell line. Comparing this result with the findings of other studies, there is incomplete knowledge regarding the application of methadone as a cancer inducer or inhibitor. Moreover, data from *in-silico* studies suggest an opioid agonist activity, including methadone, for *TLR4*. The *TLR4* signaling seems to be associated with opioid-induced pro-inflammatory activations. Therefore, it is necessary to determine and separate the beneficial opioid-analgesic neuronal actions from the unwanted and detrimental *TLR4*-mediated side effects.

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Conflicts of interest

There are no conflicts of interest.

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