Effects of celecoxib and L-NAME on apoptosis and cell cycle of MCF-7 CD44+/CD24−/low subpopulation

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Abstract: Recent studies have reported that cancer stem cells (CSCs) play a pivotal role in treatment failure, causing cancer recurrence. Here, we investigated the effects of L-NAME (an iNOS inhibitor) and celecoxib (a selective COX-2 inhibitor) on CSC-like cells (CSC-LCs) and their parental cells. Breast CSC-LCs derived from the MCF-7 cell line were sorted and characterized with the CD44+/CD24−/low phenotype. After isolation, the percentage of the subpopulation expressing CD44+/CD24−/low biomarkers increased considerably from 0.96% to 28.6%. Use of L-NAME and celecoxib showed antiproliferative activity towards both MCF-7 and CSC-LCs. Although celecoxib enhanced apoptotic cell death, the CSC-LC population was more resistant than parental cells. Moreover, L-NAME was less effective at inducing apoptosis, suggesting an involvement of different mechanisms of cell death. L-NAME caused cell cycle arrest in the S-phase in CSC-LCs, while celecoxib induced G0/G1 arrest in CSC-LCs and their parental cells. Immunocytochemistry results demonstrated that L-NAME had a similar potency to attenuate iNOS expression in MCF-7 and CSC-LCs; however, celecoxib reduced COX-2 expression in MCF-7 cells. The results show the crucial role of NOS and COX-2 in the maintenance of CD44+/CD24−/low breast CSC-LCs and suggest that L-NAME and celecoxib could have clinical implication in combination therapy.

Key words: Celecoxib, L-NAME, cancer stem cell-like cells, CD44+CD24−/low, cytotoxicity

1. Introduction
Breast cancer is the primary cause of death in malignancies among females (Regulski et al., 2016). It is suggested that a major population of breast tumor was incapable of further growth but only a minute fraction was able to seed new cancers, called cancer stem cells (CSCs) (Al-Hajj et al., 2003). During recent years, studies have identified CSCs as the subpopulation of tumor cells with a unique capacity for self-renewal and the ability to give rise to a heterogeneous population of cancer cells to form tumors. Genomic instability via several gene mutations has been reported to form CSCs from normal stem cells, progenitor cells, or differentiated cells. Cancer development could be due to the fact that CSCs are associated with tumor initiation, progression, and metastasis as well as treatment resistance. Thus, CSCs have been discovered to be a suitable therapeutic target for prevention and treatment of cancer (Soltanian and Matin, 2011; Sotiropoulou et al., 2014). Breast CSCs are multiple, distinct, and nonoverlapping populations coexisting within the tumor mass (Wright et al., 2008). In 2003, breast CSCs with CD44+/CD24−/low/ESA+ cell surface markers were isolated for the first time by Al-Hajj et al. (2003). Additionally, ALDH1, which belongs to the aldehyde dehydrogenase family, is a putative CSC marker, including breast cancer. To date, several breast cancer stem cell markers have been proposed (CD133, CD29, and CD49f), of which CD44+/CD24−/low and ALDH1 are used exclusively to identify these highly tumorigenic cells (Carrasco et al., 2014).

The cyclooxygenase enzyme, which mediates prostaglandin production, consists of three isoforms, COX-1, COX-2, and COX-3. COX-1, a house-keeping enzyme, has a crucial role for internal homeostasis. Conversely, COX-2 is undetectable in normal tissues while it is inducible in the setting of neoplasia and inflammation (Regulski et al., 2016). COX-2 upregulation by modulating various signaling pathways can enhance production of prostaglandins, which promote tumor growth, invasion, angiogenesis, and apoptosis resistance. Recent studies have also attributed the overexpression of COX-2 to breast cancer stem-like cell (CSC-LCs) properties and cancer development (Jeong et al., 2010; Singh et al., 2011).
Moreover, the beneficial effect of celecoxib administration as an adjunct to chemotherapy in advanced-stage cancer patients has been confirmed (Gee et al., 2009).

NO, a commonly seen radical reactive oxygen species, is synthesized from L-arginine by the catalytic action of NO synthases (NOS) in a wide variety of tissues, e.g., vascular endothelium or neurons. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are calcium- and calmodulin-dependent NOS isoforms that synthesize a small amount of NO. In contrast, inducible NOS (iNOS), the calcium-independent isoform, produces larger amounts of NO (Amale et al., 2011). L-NAME is an L-arginine analog that inhibits the synthesis of NO by competitive antagonism of NOS. Due to the contribution of COX-2 and NOS to carcinogenesis, our objective is to study the effects of celecoxib and L-NAME on growth inhibition, apoptosis, and cell cycle arrest in CD44+/CD24−/low human breast CSC-LCs and their parental cells.

2. Materials and methods

2.1. Chemicals, reagents, and drugs

RPMI 1640 (LM-R1638), fetal bovine serum (FBS; FB-1001), and penicillin–streptomycin (XC-A4122) were purchased from Biosera (East Sussex, UK). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; M2003) and dimethyl sulfoxide (DMSO; 472301) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC and propidium iodide (PI) were purchased from eBioscience (88-8008-72; San Diego, CA, USA). 4',6-Diamidine-2-phenylindole dihydrochloride (DAPI; 236 276) was obtained from Roche (Mannheim, Germany). Celecoxib (PZ0008) and L-NAME (N-5751) were provided by Sigma-Aldrich. CD44-PE-Cy7 (560533), CD24-APC (658331), and ESA-FITC (347197) antibodies were purchased from BD Pharmingen (San Diego, CA, USA). For magnetic cell sorting, all reagents were obtained from Miltenyi Biotec (Auburn, CA, USA). All other chemicals were of analytical grade and used as received.

2.2. Cell culture

The human breast cancer MCF-7 cell line was obtained from the Pasteur Institute Cell Bank of Iran (Tehran, Iran). Cells were maintained in 89% RPMI-1640 culture medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin and incubated at 37 °C in a humidified 5% CO2 atmosphere.

2.3. Fluorescence-activated cell sorting analysis (FACS)

To evaluate the putative breast CD44+/CD24− cells, flow cytometry analysis was employed. Briefly, MCF-7 cells were cultured in complete growth medium in 75-cm² culture flasks. Adherent cells, which were detached using trypsin, were collected by centrifugation and washed twice with phosphate-buffered saline (PBS). Then 1 × 10⁶ cells were labeled with CD44-PE-Cy7, CD24-APC, and ESA-FITC antibodies for 30 min on ice. After washing to remove unbounded antibodies, cells were analyzed on a FACSCalibur flow cytometer (BD Pharmingen).

2.4. Magnetic-activated cell sorting (MACS)

A human breast CD44+/CD24− subpopulation was isolated using MACS from the MCF-7 cell culture. Cell pellets were resuspended in 80 µL of Miltenyi running buffer (130-091-221; PBS without Ca²⁺ or Mg²⁺ and supplemented with 2.5 mM EDTA and 0.5% BSA) at the density of 1 × 10⁷ cells/mL. Single-cell suspensions were incubated with 20 µL of MACS anti-CD44-conjugated microbeads (130-095-194) at 4 °C for 15 min. After washing with PBS, cells were resuspended in 500 µL of Miltenyi buffer and applied to the LD positive selection column. CD44-negative cells passed through the column, while CD44-positive cells were separated by washing the column with 2 × 1 mL of Miltenyi buffer. CD44+ cells were cultured for 3 weeks. Afterward, CD44+ cells (1 × 10⁷) were resuspended in 40 µL of Miltenyi running buffer and labeled with 10 µL of biotin-conjugated anti-CD24 antibodies (130-095-951) at 4 °C for 15 min. After washing with 500 µL of Miltenyi buffer, cells were resuspended in 80 µL of Miltenyi running buffer and incubated with 20 µL of antibiotin microbeads at 4 °C for 15 min. The cells were washed with PBS to remove unbound microbeads, resuspended in Miltenyi buffer (500 µL), and applied to the LD/deletion column to obtain CD24-negative cells (Sajadian et al., 2015). According to the instructions provided by the manufacturer, there is no need to remove the microbeads from the cells. Hence, collected cells were CD44+/CD24− CSC-LCs. In order to preserve the cellular properties of the CSC-LCs, not only were the cells used in this study kept up to 5 passages, but their characteristics were also reexamined at the end of the experiments.

2.5. Viability assay

For viability assay, cells were cultured in a 96-well plate at 7 × 10³ cells/well and treated with various doses of celecoxib and L-NAME. Following 48 h of treatment, the medium was removed and 20 µL of 5 mg/mL MTT solution in PBS was added and incubated for additional 4 h at 37 °C. Subsequently, 100 µL of DMSO was added to each well. The formazan salts were quantified by reading the absorbance at 570 nm with a reference wavelength of 690 nm using a microplate reader (Anthos, London, UK).

2.6. Analysis of drug-induced apoptosis

2.6.1. Annexin V/PI

The percentage of both apoptotic and necrotic cells labeled with annexin V-FITC and PI were determined by flow cytometry. MCF-7 cells (3 × 10⁶ cells/well) were seeded in 6-well plates. The next day, cells were treated either with 25 µM and 50 µM celecoxib (Bocca et al., 2011) or 0.1 mM, 1 mM, and 10 mM of L-NAME (Sawyer, 1998) for
48 h. Afterwards, cells were trypsinized and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) following by incubation with annexin V-FITC (5 µL) and PI (10 µL) for 10 min at room temperature. Finally, for each assay, 10^6 cells diluted in 500 µL of binding buffer were analyzed for induction of apoptosis.

2.6.2. DAPI staining
Cells were treated with 50 µM celecoxib or 10 mM L-NAME for 48 h. After washing with PBS, cells were incubated with permeabilization buffer (PBS containing 0.01 M glycine and 0.1% Triton X-100) for 10 min. Then cells were washed with PBS and fixed with 2% paraformaldehyde and 5 µg/mL DAPI solution for 10 min. Following washing, apoptotic cells were monitored under fluorescence microscopy.

2.7. Cell cycle assay
To identify the effects of treatment on cell cycle distribution, flow cytometry analysis was used. Treated cells were centrifuged at 200 × g for 5 min and the pellet was fixed in cold 70% ethanol on ice for 60 min. Following washing with PBS, cells were centrifuged and resuspended in 1 mL of PI staining solution (0.1% (v/v) Triton X-100, 10 μg/mL PI, and 100 μg/mL DNase-free RNase in PBS) and kept in the dark at 37 °C for 10 min. Quantification of cell cycle progression was then performed using FACS analysis. ModFit software was used to analyze the obtained data.

2.8. Immunocytochemistry
Cells were seeded at a density of 5 × 10^4 cells in chamber slides (Nunc, Roskilde, Denmark) and treated with celecoxib and L-NAME for 48 h. Subsequently, cells were fixed with methanol-acetone (9:1) and incubated overnight with primary antibodies against iNOS (1:50, ab15323-500; Abcam, Cambridge, UK) or COX-2 (1:100, sc-7951; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. Following incubation with biotinylated secondary antibodies (1:200) for 30 min at room temperature, DAB chromogen (K0673; Dako, Glostrup, Denmark) was used as the HRP substrate. Cells were then washed and slightly counterstained with Mayer's hematoxylin (Hx948000; Merck, Darmstadt, Germany), and slides were mounted with Faramount (Dako). Microscopic evaluation of three randomly selected fields in each experiment was accomplished using ImageJ software on an Olympus microscope (Olympus, Tokyo, Japan).

2.9. Statistical analysis
To compare differences between various treatment groups, one-way analysis of variance followed by Tukey's post hoc test was used with GraphPad Prism 5.01 (San Diego, CA, USA). A statistical probability of P < 0.05 was considered significant.

3. Results
3.1. Isolation and characterization of CD44+/CD24−/low subpopulation
To assess the expression of CSC markers in the MCF-7 breast cancer cell line, flow cytometry analysis was applied. Three markers, CD44, CD24, and ESA, were chosen among the putative breast CSC markers. In the next step, cancer cells were purified using MACS according to the dual expression of CD44/CD24 and characterized as epithelial specific antigen+ (ESA), ABCG2+, CD44+, and CD24−/low.

The percentage of cells with CD44+/CD24−/low phenotype increased from 0.96% to 28.6% (Figures 1A and 1B).

Figure 1. Flow cytometry analysis of the CD44+/CD24− cell population in (A) MCF-7 cell line; part D: 0.96% and (B) CD44+/CD24− CSC-LCs; part D: 28.6%. CD44+/CD24−/low breast CSC-LCs were isolated from the main population of MCF-7 cell line using MACS and evaluated the CD44/CD24 expressions in parent cells and CSC-LCs by flow cytometry.
3.2. Cytotoxic activity of celecoxib and L-NAME on MCF-7 CD44+/CD24−/low cells
MCF-7 parent and stem-like cells were treated with different doses of celecoxib and L-NAME for 48 h. According to Figure 2, proliferation of both MCF-7 parent and stem-like cells was inhibited by celecoxib and L-NAME in a dose-dependent manner. In the case of L-NAME, the concentration causing 50% inhibition of cell viability (IC50) was 18.1 ± 2.03 mM for MCF-7 and 16.27 ± 0.96 mM for their stem-like cells, although the results did not reach statistical significance (Figure 2A). Moreover, celecoxib abolished cell growth of MCF-7 and their CSC-LCs with an IC50 value of 74.03 ± 5.68 μM and 92.52 ± 6.8 μM (P < 0.05), respectively, implying that it had a less cytotoxic effect on CD44+/CD24− CSC-LCs than on their parental cells (Figure 2B).

3.3. Effect of celecoxib on apoptosis of MCF-7 CD44+/CD24−/low cells
Cells were treated with 25 μM and 50 μM celecoxib to determine whether celecoxib could induce apoptosis in MCF-7 CSC-LCs. Treated cells were incubated with annexin V-FITC and PI as described in Section 2. As shown in Figure 3, more total apoptotic cells mediated by 25 and 50 μM celecoxib treatment were detected in the main population (30.71%, 44.48%) than in CD44+/CD24− CSC-LCs (13.83%, 25.38%). Celecoxib treatment could increase the percentage of early apoptotic cells in MCF-7 parental cells from 14.28% to 28.37% at 50 μM (Figure 3A), whereas this proportion was less for their stem-like cells, though no significant difference was observed. Furthermore, MCF-7 CSC-LCs exhibited more resistance to celecoxib exposure than parental cells (Figure 3B).

Additionally, apoptotic cells were characterized by nuclear fragmentation and condensed chromosomes. DAPI staining was used to investigate nuclei morphological changes in MCF-7 and CSC-LCs following treatment with 50 μM of celecoxib. At this concentration, MCF-7 cells underwent higher levels of DNA fragmentation relative to the CD44+/CD24− subpopulation. However, round nuclei with clear margins were observed in the control cells.

3.4. L-NAME effects on apoptosis of MCF-7 CD44+/CD24−/low cells
To determine whether L-NAME could induce apoptosis in MCF-7 CSC-LCs, cells were treated with L-NAME (0.1, 1, and 10 mM) for 48 h and incubated with annexin V-FITC and PI, as described in Section 2. Figures 4A and 4B indicate that 10 mM L-NAME augmented total apoptosis in 32.79% and 20.97% of MCF-7 and their CSC-LCs, respectively, but this was not statistically significant. Importantly, the percentage of apoptotic cells was predominant in parental cells compared to MCF-7 stem-like cells. However, in nuclear staining with DAPI solution, the cell nuclei of most cells were intact at 10 mM of L-NAME. This phenomenon was significant in MCF-7 cells (Figure 4A).

3.5. Celecoxib effects on cell cycle of MCF-7 CD44+/CD24−/low cells
Cell cycle progression in cells treated with 25 μM and 50 μM celecoxib was evaluated using flow cytometry. After exposure to celecoxib, a significant increase in the G0/G1 phase in MCF-7 parental cells, as well as parent cells, in comparison to untreated control cells was observed. With regard to MCF-7 parent cells, celecoxib could dramatically reduce the percentage of cells in the S-phase compared with the control group (45.71% versus 17.41%; Figure 5A). As shown in Figure 5B, celecoxib induced a noticeable dose-dependent reduction in the percentage of stem-like cells in the G2/M phase (38.11% vs. 23.15%), while the

![Figure 2](image_url)

**Figure 2.** The cytotoxic effect of L-NAME and celecoxib on MCF-7 cell line and CD44+/CD24− CSC-LCs isolated from it. (A) L-NAME; (B) celecoxib. Results are presented as mean ± standard deviation. ***P < 0.001, **P < 0.01 relative to corresponding concentration.
3.6. L-NAME effects on cell cycle of MCF-7 CD44+/CD24–/low cells

Cell cycle progression in cells treated with L-NAME (0.1, 1, and 10 mM) was evaluated using flow cytometry. In MCF-7 cells, L-NAME induced a reduction in the percentage of cells in the S-phase, with a weak accumulation in the percentage of cells in G2/M, in comparison to the control (26.94% vs. 21.16%; Figure 6A). Moreover, a marked increase in the S-phase in MCF-7 stem-like cells compared with untreated control cells (Figure 6B) was observed.

3.7. Immunocytochemical analysis of iNOS and COX-2 expression

Immunocytochemical (ICC) analysis of COX-2 expression showed that celecoxib (74 µM) diminished COX-2 expression in MCF-7 cells. However, administration of celecoxib caused no change in COX-2 expression of CD44+/CD24–/low breast CSC-LCs relative to the control (Figures 7A and 7B). Moreover, iNOS expression was reduced due to L-NAME treatment at the IC50 dose in both MCF-7 and CSC-LCs compared to control cells (Figures 7C and 7D).

4. Discussion

The presence of cancer stem cells is associated with tumorigenic potential, resistance to current therapies, and cancer relapse. Several mechanisms, including the activity of multiple drug resistance transporters (MDR-1), hyperactivation of DNA damage checkpoint response, and free radical scavengers, are believed to facilitate their resistance to conventional cancer therapies like chemotherapy and radiotherapy (Carrasco et al., 2014). Purification and identification of cancer stem cells in human tumors or cancer cell lines are often mediated through cell surface markers. In this regard, key biomarkers such as CD44, CD24, CD133, CD166, and EpCAM have been used extensively in different tumors (Soltysova et al., 2005; Jaggiupilli and Elkord, 2012). In this study, CD44+/CD24–/low breast CSC-LCs were isolated from the main population of the MCF-7 cell line using MACS and CD44/CD24 expressions were evaluated by flow cytometry.
After enrichment, the percentage of the subpopulation expressing CD44+/CD24−/low increased significantly, from 0.96% to 28.6%. Extensive studies have proposed CD44+/CD24−/low as putative CSC markers with undifferentiated basal/mesenchymal features in MCF-7 and MDA-MB-231. However, noticeable variation in expression levels was observed, even between various lines of one cancer type (Jaggupilli and Elkord, 2012).

Figure 4. L-NAME treatment resulted in apoptosis-mediated cell death in MCF-7 cells and their CSC-LCs. (A) MCF-7 cells (upper panel: annexin/PI staining; lower panel: DAPI staining); (B) MCF-7 CSC-LCs (upper panel: annexin/PI staining; lower panel: DAPI staining). MCF-7 stem-like cells were more resistant to apoptosis induced by L-NAME treatment. Results are presented as mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 relative to vehicle-treated controls. #P < 0.01 relative to the corresponding concentration.

Figure 5. Celecoxib treatment inhibited cell cycle progression in a dose-dependent manner, inducing G0/G1 arrest in MCF-7 and CSC-LCs derived from it. (A) MCF-7 cells; (B) MCF-7 CSC-LCs.
Previous studies have reported enhanced COX-2 regulation in multiple neoplasms, such as breast, colorectal, pancreatic, and bladder cancer (Dubois et al., 1998; Mohammed et al., 1999). Over the last decades, selective COX-2 inhibitors have been introduced and used in clinical trials to reduce the risk of malignancies, especially in breast cancer, via reduction of prostaglandin synthesis, causing apoptosis induction and angiogenesis inhibition (Mazhar et al., 2006; Ashok et al., 2011). Here, we found that celecoxib displayed dose-dependent cytotoxicity against MCF-7 and MCF-7 CSC-LCs. However, CSC-LCs were less sensitive than their parental cells. In accordance with cytotoxicity results, MCF-7 CSC-LCs were more resistant to celecoxib treatment and had fewer apoptotic cells than the parental cells. Nevertheless, earlier studies revealed that celecoxib did not modulate the expression of Bak, procaspase, and Bax in MCF-7 cells and thereby had no effect on apoptosis induction (Bocca et al., 2011).

Figure 6. L-NAME treatment inhibited cell cycle progression and induced S-phase arrest in MCF-7 CSC-LCs. (A) MCF-7 cells; (B) MCF-7 CSC-LCs.

Figure 7. Immunocytochemical analysis of COX-2 and iNOS localization. The expression of COX-2 was investigated in MCF-7 cells (A), MCF-7 CSC-LCs treated with celecoxib at an IC₅₀ dose for 48 h (B), and iNOS expression in MCF-7 cells (C) and MCF-7 CSC-LCs treated with L-NAME at an IC₅₀ dose for 48 h (D).
In CD44+/CD24− breast CSC-LCs, celecoxib induced G0/G1 cell cycle arrest in a dose-dependent manner, similar to their parental cells. These findings are in line with previous studies on MCF-7 cells (Dai et al., 2012). According to the ICC assay, COX-2 expression was reduced significantly in MCF-7 cells, with no reduction in their CSC-LCs. It has also been observed that celecoxib, at 10 μM, dramatically suppressed the tumorsphere and colony-forming capacity of MCF-7 cells, which is a hallmark of CSCs in vitro (Singh et al., 2011). It was recently demonstrated that celecoxib can effectively harness MDR-1 expression in the doxorubicin-resistant MCF-7 cell line, which is mediated by inhibiting AP-1 and NF-κB transcription factors, suggesting the use of a selective COX-2 inhibitor in combination therapy as a promising avenue for successful breast cancer treatment (Chen et al., 2011).

In the CSC model, investigations have clarified that developmental pathways such as Hedgehog, Wnt, Notch, PTEN, and Bmi-1 seem dispensable in CSC regulation (Soltanian and Matin, 2011). Intriguingly, Charles et al. (2010) observed that the activation of Notch signaling by eNOS led to the acquisition of stem-like properties in glioma cells. NO produced by iNOS has multiple physiologic and pathologic effects (Ohtsu et al., 2010). A positive correlation between iNOS and poor outcomes in patients with breast cancer and melanoma has been established (Ekmeckicoglu et al., 2006; Prueitt et al., 2007). As a result, the antitumor effect of L-NAME as an iNOS inhibitor on breast CSC-LCs was investigated in this study. L-NAME treatment suppressed breast CSC-LC proliferation with the same potency as their parental cells. L-NAME could increase the percentage of apoptotic cells in parental cells, though it was less active in terms of the apoptotic effect on CSC-LCs. Exposure to L-NAME arrested MCF-7 stem-like cells predominantly in the S-phase at a higher concentration, whereas a minimal increase in the G2/M phase was found in parental cells. Finally, a reduced expression of iNOS was observed in MCF-7 and their stem-like cells.

In conclusion, L-NAME and celecoxib possess antiproliferative effects on both the main population and stem-like cells of the MCF-7 breast cancer cell line. L-NAME and celecoxib led to a concentration-dependent S- and G0/G1-phase arrest, respectively, and increased total apoptosis in breast CSC-LCs. Additionally, L-NAME and celecoxib attenuated iNOS and COX-2 expression in the main population and subpopulation of MCF-7 cells, respectively. Although combination therapy was not examined in this study, considering the results of this experiment, a combination of COX-2 and iNOS inhibitors with standard therapeutic agents may improve breast cancer treatment. This hypothesis needs further investigation.

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References


