EJMO

DOI: 10.14744/ejmo.2024.61923
EJMO 2024;8(2):135–140

Research Article

Effect of Losartan on Cell Proliferation and Reactive Oxygen Species Scavenging in Gastric Cancer Cell Lines

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Abstract

Objectives: The study aims to investigate the mechanisms underlying the anti-cancer effects of losartan in gastric cancer cell line.

Methods: In this experimental investigation, MKN-45 cells were cultivated in RPMI-1640 medium supplemented with 10% fetal bovin serum and 100 µg/ml streptomycinin, and 100 IU/ml penicillin, and maintained under controlled conditions of temperature and CO2. Following washing with PBS, all cells were detached using trypsin, centrifuged and then 8×103 cells re-plated onto 96- well plates. Then various concentrations of Losartan (1000, 2000 and 3000 µM) and 5-fluorouracil (12.5 µM) were added to each well in triple therapy. Anti-proliferative effects of this treatment were evaluated through MTT assay and ROS detection by ROS-sensitive fluorescence indicator after 24 hours.

Results: Losartan greatly enhanced the ant-proliferative effect at all tested doses, especially with an IC50 of about 3000 µM in contrast to other groups (P<0.01). Also, cell ROS content due to losartan treatment was significantly reduced compared to untreated group (p<0.05), and the cells treated with Losartan (3000 µM) had considerably lower fluorescence than other groups (p=0.000).

Conclusion: In conclusion, this study demonstrated that the various concentration of losartan treatment reduced the viability of MKN-45 gastric cancer cell proliferation, concomitant with a notable decrease in ROS production.

Keywords: Gastric cancer, Losartan, Renin-angiotensin system, MTT, MKN-45 cell line.

Cite This Article: *Raei M, Ahmadi M, Abrotan S, Razavi A, Hedayatizadeh-Omran A, Shamshirian A, et al. Effect of Losartan on Cell Proliferation and Reactive Oxygen Species Scavenging in Gastric Cancer Cell Lines. EJMO 2024;8(2):135–140.*

Gastric cancer is a leading cause of cancer-related mortality worldwide, exhibiting both high incidence and mortality rates.[1,2] Although advancements in early detection and treatment strategies have been made, long-term outcomes for patients diagnosed with gastric cancer remain poor.[3] Consequently, there is a critical need to iden-

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Submitted Date: May 23, 2024 **Accepted Date:** July 28, 2024 **Available Online Date:** July 10, 2024

tify novel therapeutic agents that can effectively target the molecular mechanisms underlying gastric cancer development and progression.

The renin-angiotensin system (RAS) is comprises multiple components, including angiotensin-converting enzyme (ACE), angiotensinogen, renin and angiotensin (Ang).[4,5] Within the RAS, ACE plays a critical role as a key enzyme, facilitating the conversion of angiotensin I to angiotensin II.[6] Angiotensin II, a bioactive octapeptide, is believed to induce tumorigenic effects primarily by binding to Angiotensin II receptor type I (AT1R) and Angiotensin II receptor type II (AT2R). Notably, ACE inhibitors (ACEIs) or Angiotensin II Receptor Blockers (ARBs) have been shown to reduce tumor progression in various cancer types.[7,8]

Numerous studies examining local AT1 receptor expression in gastric cancer have implicated angiotensin II as a potential critical factor involved in tumor growth and metastasis mediated by the AT1 receptor.[9-11] Losartan, a widely used angiotensin II type 1 receptor blocker (ARB) for hypertension, is a potential therapeutic candidate in this context. Recent epidemiological studies suggest anticancer effects of ARBs in various cancers, including gastric cancer.[12-14] Losartan has been shown to inhibit migration, invasion, and proliferation of gastric cancer cell lines, possibly through RAS regulation.[15,16]

Furthermore, elevated reactive oxygen species (ROS) levels are a hallmark of gastric cancer progression.[17] Analyzing ROS production in cancer cells can offer valuable insights for potential therapeutic interventions.[18] Regarding previous studies, we extend our investigation to the gastric cell lines by quantifying ROS levels, building upon our earlier findings. This approach will allow us to discern the nuanced variations in ROS dynamics within the gastric cancer population.

The present investigation aimed to evaluate the therapeutic efficacy of losartan on cell proliferation in MKN-45 cell lines by utilizing MTT assay and ROS detection. The study intends to provide insights into the mechanisms underlying the anticancer effects of losartan and potentially recognize novel molecular targets for the management of gastric cancer.

Methods

Material

Fluorouracil (5-FU) was purchased from Darupaksh Pharmaceutical Company (Iran), while Thiazolyl blue tetrazoliumbromide (MTT) was provided by Sigma (USA). Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI-1640), Phosphate-buffered saline (PBS), Trypsin/EDTA and penicillin were obtained from Gibco (Paisley, UK). The gastric cancer cell lines of MKN-45 were purchased in frozen vials from the Pasteur Institute of Iran. Losartan was kindly gifted by Razak Co. (Iran), 5-fluorouracil and dimethyl sulfoxide (DMSO) were obtained from Sigma aldrich (Germany), the drug was dissolved in PBS and utilized in all experiments at varying concentrations.

Cell Culture

The cells were cultured in flask (Nunc, Denmark) in RPMI-1640 supplied with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 µg/ml streptomycin (PAA, Pasching, Austria). The cells were grown in a humid environment with 5% CO_2 at 37°C. While the culture reached 80-90% confluency, the growth medium was removed and washed with Phosphate-buffered saline (PBS) (pH=7.4), separated with 0.25% trypsin, centrifuged at 485 RFC for 5 minutes, and replated onto 96- well plates.[19] Cells were treated with various concentrations of losartan (1000, 2000 and 3000 µM) and 5-fluorouracil (12.5 µM).[20,7] Then, plate returned to the incubator for either 24 h. A haemocytometer was used to count the cells, and 8×10^3 cells/well were utilized for all experiments.

Cell Viability Assay

The MTT assay was carried out to evaluate the Losartan's cytotoxicity on the MKN-45 cell line. First, various concentrations of losartan (1000, 2000 and 3000 µM) and 5-fluorouracil (12.5 µM) were applied to the cells for 24 hours. Then, MTT solution (5 mg/mL in PBS) was added to each well and cells were incubated for an additional 4 h at 37°C. After extracting the superficial solution, 200 µL of dimethyl sulfoxide (DMSO) was applied to each well to dissolve the cell membrane and release formazans, and shacked for 10 minutes. Finally, the samples read by spectrophotometric plate reader (Bio Tech-USA) at 570 nm and optical density (OD) was calculated for each well.[21] The study was performed in triplicate to improve the efficiency and accuracy. Also, the zero medicine concentration group (untreated group) served as the control.

Intracellular Reaction Oxygen Species Scavenging Activity

A ROS-sensitive fluorescence indicator referred to as DCFH-DA (Abcam, US. Cat No.: AB113851) was used to determine the cellular reactive oxygen species (ROS) content. Their scavenging activities were assessed through various concentrations of losartan and 5-fluorouracil. In this regard, in a 96-well plate, MKN-45 cells were cultivated in each well.

After 24 hours, they were incubated for 20 hours at 37° C with 5% CO^2 in the presence of 1000 µM, 2000 µM and 3000 µM concentrations of Losartan and 12.5-µM concentrations of 5-fluorouracil without changing the culture condition. By the next day, Cells were exposed to DCFH-DA (25 µM) for 45 minutes at 37°C in a red phenol free environment, and ROS were quantified according to the manufacturer's instruction. At 485 wavelengths, the fluorescent intensity was quantified using a microplate reader manufactured by Bio Tech-USA.[22]

Statistical Analysis

The SPSS software (version 20) was used to analyze the data. All findings were presented as mean±SD. ANOVA and Tukey test were used to assess the significance of difference. The p-value of 0.05 was considered statistically significant.

Results

Effect of Losartan on Cell Viability in MKN-45

We used a modified MTT assay (in vitro), to determine growth inhibition of different concentrations of Losartan (1000-3000 µM) on MKN-45 cell lines. The rates of cell proliferation in the losartan-treated gastric cancer cells are presented in Figure 1. Treatments induced a reduction of 38%, 48%, 90% and 50% in cell proliferation in MKN-45 cells when cells treated with 1000, 2000, 3000 µM of losartan and 12.5 µM of 5FU, respectively. After a 24-hour incubation, losartan dramatically reduced the proliferation of MKN-45 cells at all tested doses, especially in a concentration-dependent manner with an IC50 of approximately 3000 µM compared to other groups ($p<0.01$) (Fig. 1). However, non-significant difference was observed between comparison of Losartan 1000 & 2000 µM individually, with other groups ($p>0.05$).

The effects of Losartan on Antioxidant Balance

To evaluate the antioxidant effects of Losartan in various doses, the intracellular ROS was measured. In the 24-hour treatment of MKN-45 cells with various concentrations of Losartan (1000-3000 µM), the ROS production was significantly reduced compared to untreated group ($p<0.05$) (Fig. 2). From the evidence for the attraction of ROS-sensitive fluorescence indicators referred to as DCFDH, a decrease in peroxide ions was discovered. Furthermore, the cells treated with Losartan (3000 µM) had significantly lower fluorescence than other groups ($p=0.000$). However, the difference between the Losartan (2000 µM)-treated group and Losartan (1000 µM)-treated group was not significant ($p=0.989$).

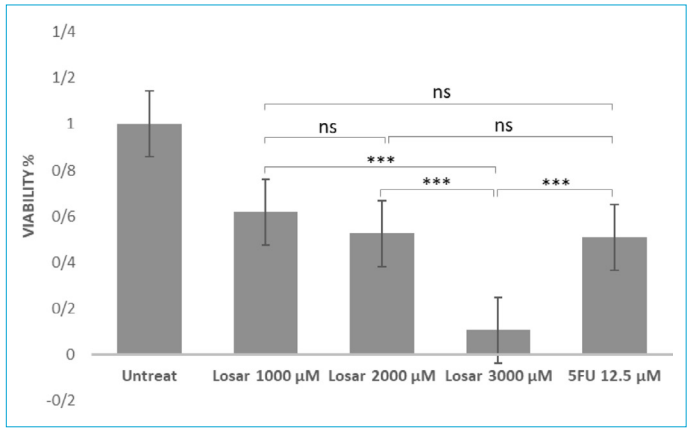

Figure 1. The Effect of Losartan at different concentrations (1000, 2000 and 3000 µM) and 5-fluorouracil (12.5 µM) on Viability of gastric cancer cells MKN-45. *** represents $p<0.001$; ns: represents statically non-significant.

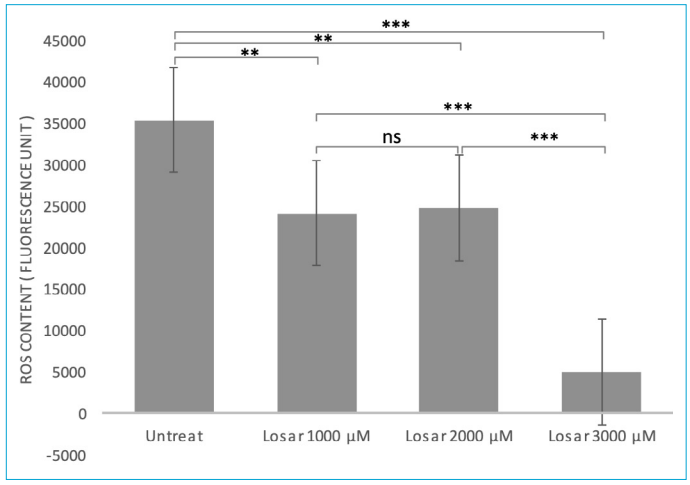

Figure 2. Measurement of Losartan's antioxidant activity in gastric cancer MKN-45 cells. ** represents $p<0.01$; *** represents $p<0.001$; ns: represents statically non-significant.

Discussion

The overall goal of our study was to gain insights into losartan's therapeutic effect on cell proliferation in gastric cancer cell lines and ROS levels. We provide here preliminary evidence supporting the role of anticancer effects of losartan against cell proliferation in gastric cancer. One of the more surprising findings in our study is the significant reduction in MKN-45 gastric cancer cell viability observed with losartan treatment. Losartan significantly inhibited the proliferation of MKN-45 cells after 24 hours of incubation, particularly in a concentration-dependent manner with an IC50 of around 3000 µM compared to other groups. Also, Losartan at various concentrations (1000 to 3000 µM) significantly reduced the production of ROS in MKN-45 cells compared to the untreated group.

The proliferation, migration, and angiogenesis of different

cancer cells are mediated by angiotensin II (ATII), a biologically active peptide from the renin-angiotensin system (RAS).[23-25] The ATII/ Angiotensin II receptor type 1 (AT1R) pathway promotes vascular development. Following the activation of mitogen-activated protein kinase (MAPK) by ATII, vascular endothelial growth factor (VEGF) expression increased.[26] There is evidence that RAS inhibitors decrease tumor angiogenesis by lowering VEGF expression and changing the tumor microenvironment.[7] By blocking downstream ATII effectors in endothelial cells within the tumor stroma and cancer cells, ARBs may also exert anticancer effects.[27,28] As well, Losartan decreased angiogenesis in azoxymethane-induced colorectal cancer (CRC) by lowering VEGF protein levels and expression.[29] The study by Neo et al shows that renin-angiotensin system blockers, captopril, and irbesartan, reduced tumor growth in CRC liver metastases.[30] We found losartan decreased the proliferation of MKN-45 cells dramatically after 24-hour incubation at all tested concentrations compared to other groups.

Previous research has shown that RAS is related to gastric cancer.[31-33] Furthermore, gastric cancer growth has been suppressed by both ACE inhibitors and AT1R antagonists.[34,35] Gastric cancer tissues exhibit increased protein levels of ATII, AT1R, and AT2R, alongside elevated ACE enzyme activity. These studies demonstrated that ATII increases the size and weight of gastric cancer tumors in mice, as well as the migration and proliferation of MKN45 human gastric cancer cells. At the other end of the spectrum, losartan significantly decreased the size and weight of tumors in mice with gastric cancer, as well as the migration and proliferation of human gastric cancer cells MKN45.[36]

A 2017 study, human prostate cancer cell lines PC3, DU145, and LNCap-Ln3 were evaluated for growth, viability, proliferation, and migration in the presence of ARBs (Fimasartan, losartan, eprosartan, and valsartan) at 100, 200, and 400 mM concentrations.[37] According to the results, ARBs reduced cell viability compared to the control group, and, at a concentration of 400 mM, all ARBs inhibited prostate cancer cell proliferation. Comparatively to other ARBs, Fimasartan showed the greatest cytotoxic effect and valsartan had the lowest antiproliferative effect.[37] In a xenograft model of colon cancer, the therapeutic potential of targeting RAS using losartan was investigated. It was shown that losartan can inhibit cell growth and cell cycle progression and cause an increase in CRC cells in the G1 phase. Losartan significantly reduced tumor growth, metastasis, and angiogenesis and increased tumor cell necrosis. Losartan's antiproliferative effects may be explained by impacts on the inflammatory response, particularly the upregulation of proinflammatory cytokines and chemokines in colon cancer cells.[7] Similarly, Valuckaite et al. demonstrated that Losartan inhibited angiogenesis in azoxymethane-induced CRC by lowering VEGF protein levels and expression.[29] We confirmed that losartan at different concentrations (1000 to 3000 μM) significantly reduced both ROS production in MKN-45 cells and proliferation of MKN-45 cells compared to the untreated group. By modulating tumor-associated fibroblasts, ARBs can alter tumor desmoplasia. Desmoplasia constricts the vascular and prevents immune cells from infiltrating. In this way, remission of tumor desmoplasia allows for T-cell infiltration, which increases drug delivery.[38]

Cell viability is decreased in correlation with ROS production. Furthermore, it causes cell apoptosis.[39,40] Cancer cell metastasis was inhibited by oxidative stress.[7] According to these findings, Ahmadian et al. demonstrated that Azilsartan, a new AT1R blocker, increased ROS before triggering the apoptotic pathway.[41] Our work interestingly revealed a decrease in peroxide ions based on the attraction of ROS-sensitive fluorescence indicators (DCFDH). Similarly, the cells treated with Losartan (3000 μM) had significantly lower fluorescence than other groups. Our findings imply that one of the elemental mechanisms driving Losartan's antitumor effects against gastric cancer cells may be changes in the oxidant-antioxidant state.

Typically, cancer cells have higher levels of ROS due to an imbalance between oxidants and antioxidants. ROS serves a dual function in cell metabolism. At low to moderate levels, it acts as a signal transducer to activate cell proliferation, migration, invasion, and angiogenesis.[42] Some evidence showed a decrease in ROS levels in myeloid-derived suppressor cells enhances the activity of CD8+ and CD4+ T lymphocytes, leading to efficient suppression of tumor cell proliferation.[43] Our work suggests that reducing ROS levels may inhibit the proliferation of MKN-45 gastric cancer cells.

There are also some limitations to our study. First, the intracellular pathways such as ERBB, epidermal growth factor receptor (EGFR), and platelet-derived growth factor receptor (PDGFR) were not explicitly investigated. We also did not have access to normal cell lines for comparison, limiting our ability to demonstrate the specificity of the observed activities to cancer cells. Moreover, while we utilized MTT and ROS assays, these are general experiments and may not provide the depth of information that more specific assays involving the measurement of protein expression or antioxidant enzymes could offer. Also, evaluating the expression of genes related to the control of cell cycle progression and DNA damage response in MKN-45 gastric cancer cells was not on our agenda. Furthermore, we did not employ Western blot analysis, flow cytometry, or immunohistochemistry for confirmation of our findings.

Conclusion

We showed the significant role of losartan in the acquisition of inhibition of MKN-45 gastric cancer cell proliferation. Also, we provided evidence of a significant reduction in the production of ROS in MKN-45 cells after receiving different doses of losartan compared to the untreated group. More in vitro studies are needed to validate the use of ARBs in treating gastric cancer.

Disclosures

Ethics Committee Approval: This study was approved by the Biomedical Research Ethics Committee in Imam Hospital in Mazandaran University of Medical Sciences with code IR.MAZUMS.IMAMHOSPITAL.REC.1400.077.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – S.A., A.H., M.S.; Design – R.A-N.; Supervision –R.A-N., M.A- ; Materials – M.R., A.R-; Data collection &/or processing – A.R-S.A; Analysis and/or interpretation – K.H-R.A-N; Literature search – M.S; Writing – M.R-A.R; Critical review – All authors.

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