

Effects of Bardoxolone on Nuclear Factor-Erythroid 2-Related Factor 2 Signaling Pathway in HCT-116 Human Colonic Cancer Cell Line: *In vitro* Study

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Abstract

Background: Bardoxolone inhibited proliferation and induced apoptosis *in vitro* in wide types of human cancer cells. It has also been shown to inhibit the growth of tumor in the mice model. Several studies demonstrated that bardoxolone may modulate multiple molecular targets that have a fundamental role in both the development and progression of cancer. **Objectives:** The aim of this study is to assess the effects of Bardoxolone (CDDO-Me) in the treatment of colonic carcinoma by induction of the nuclear factor erythroid 2-related factor 2 signaling pathway (Nrf2) with regression of tumor markers as compared to other chemotherapeutic agents like 5-fluorouracil. **Materials and Methods:** Treatment groups are classified into four groups (control, chemotherapy group, bardoxolone, and combination treatment groups). **Results:** The results revealed that there were significant effects of bardoxolone treatment on cytoplasmic Nrf2 level ($P < 0.05$) as compared to control group (cancer cells without treatment). **Conclusion:** Bardoxolone has significant anticancer effects ($P < 0.05$) in low micrograms concentrations on human colonic cancer cells as compared to control groups.

Keywords: Apoptosis, bardoxolone, colon cancer, HCT-116 cell line, nuclear factor erythroid 2-related factor 2

INTRODUCTION

The colorectal cancer is considered as the third-most frequent cancer universally, but more than 50% of all cases of deaths happen in the more developed countries.^[1] The manifestations of colonic carcinoma mainly depends on tumor locations in the intestine and if it has spread or not. Clinical features can be divided into local, constitutional, and metastatic. Different etiological causes increase the person's risk of getting the cancer involving age, presence of polyps, hereditary factors, smoking, dietary factors, physical inactivity, some viruses, diminished selenium, inflammatory bowel diseases, environmental impacts, exogenous hormones, and alcohol.^[2]

Bardoxolone inhibited proliferation and induced apoptosis *in vitro* in wide types of human cancer cells.^[3,4] It has also been shown to inhibit the growth of tumor in the mice model.^[5,6] The studies demonstrated that bardoxolone may modulate multiple molecular targets that have an important role in

both the development and progression of cancer. Nuclear factor- κ B (NF- κ B), phosphoinositide 3-kinase/protein kinase, and janus-activated kinase/STAT are the bardoxolone main targets where it makes anticancer impacts.

The aim of this study is to assess the effects of Bardoxolone (CDDO-Me) in the treatment of colonic carcinoma by induction of nuclear factor erythroid 2-related factor 2 signaling pathway (Nrf2), regression of tumor markers as compared to other chemotherapeutic agents like 5-fluorouracil (5-FU), colonic cancer cell line (HCT 116).

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Submission: 18-11-2019 **Accepted:** 30-11-2019 **Published Online:** 17-03-2020

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How to cite this article: Al-Khafaji NA, Al-Rikabi SH, Kammona TH, Obied HN. Effects of bardoxolone on nuclear factor-erythroid 2-related factor 2 signaling pathway in HCT-116 human colonic cancer cell line: *In vitro* study. Med J Babylon 2020;17:25-9.

Access this article online

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DOI:
10.4103/MJBL.MJBL_90_19

MATERIALS AND METHODS

HCT-116 colonic cell line

HCT-116 cells (from National bank of Iran) had a mutation in the codon 13 of the K-RAS proto-oncogene, and are suitable transfection targets for gene therapy researches. The cells have an epithelial morphology and can metastasize in xenograft model. When they were transduced with viral vectors carrying the p53 gene, HCT116 cells stay arrested in the G1 phase.^[7]

HCT116 cells are used in different biomedical studies, including colonic cancer proliferation and corresponding inhibitors. The cell line has been used in tumorigenesis studies.

Bardoxolone serial dilution

By using RPMI-1640 serum-free media, 6 of two-fold serial working dilutions from Bardoxolone stock solution (4 mg/ml) were prepared by taking of 5 µl of the stock solution, completed to 1 ml of maintenance media, and hence, the first concentration was 20 µg/ml and diluted serially (20, 10, 5, 2.5, 1.25, and 0.625 µg/ml).

Fluorouracil and Leucovorin serial dilutions

By using RPMI-1640 serum-free media, 6 of two-fold serial working dilutions from 5-FU and leucovorin (LV) solution (4 mg/ml) were prepared by taking of 5 µl of the stock solution, completed to 1 ml of maintenance media, so the first concentration was 80 µg/ml and diluted serially (80, 40, 20, 10, 5, and 2.5 µg/ml).

Study groups

Study groups included the following: 1st group: Control group (cancer cells without treatment); 2nd Group: Use of Bardoxolone alone as a treatment for HCT-116 colonic cell line; 3rd Group: Use of chemotherapeutic agents (5-fluorouracil and LV) as a treatment for HCT-116 colonic cancer cell line; and 4th Group: Use of combination treatment of Bardoxolone, 5-fluorouracil, and LV for HCT-116 colonic cell line.

Measurement of nuclear factor erythroid 2-related factor 2

The Nrf2 is a fundamental leucine zipper transcription factor that belongs to the family (the Cap'n'Coller) (CNC) families. On stimulation by various stimuli (like oxidative substances, electrophile, or toxic stresses like (natural) molecules). It is one of the principle players to keep and restore cell hemostasis. Diminished clearance of oxidative pressure or misfolded proteins, marker for a lacking (Nrf2) activities, is frequently associated with the cause for different diseases, for example, malignant growth, and other.

Principle of nuclear factor-erythroid 2-related factor 2

The plates of the kit were precoated with antibodies specific to Nrf2. Standards or tests are then added to the right microplate wells with an antibody which is conjugated with biotin specific to Nrf2. At that point, Avidin conjugated to Horseradish Peroxidase is appended to each plate wells and incubated. After T. M. B substrate was inserted, just those wells that contain Nrf2, biotin-conjugated antibodies, and

enzyme-conjugated Avidin will demonstrate color change. The enzyme substrate is finished by adding of H₂SO₄ solution, and the color change is assessed by spectrophotometry at a wavelength of (450) nm ± 10 nm. The amount of Nrf2 in the samples is then evaluated by evaluating the OD of the samples to the standard curve.

Cell lysates

Cells are required to be lysed before examining as follows:

1. First, the adherent cells would be washed gently by phosphate solution, and after that, they were disaggregated with trypsin and collected by centrifugation at (1000×g) for 5 min
2. Wash cells three times in the phosphate solution
3. Resuspend cells in fresh lyses buffer 4 with the concentration of 10⁷ cells/ml. If it is required, the cells would be subjected to ultrasonication until it is clarified
4. Centrifugation at (1500×g) for (10 min) at (2C°–8C°) to clean any cellular debris. Test soon or keep them at ≤–20C°.

Testing procedures

- a. Assess wells for diluted standard, blank, and tests. Preparation of (7 wells) for standard, 1 well for the blank. Then, add (100 µl) every one of dilutions of standard, clear and tests to the right (wells). Spread with the sealer. Incubate for (1 h) at (37C°)
- b. Discard the liquid of each well, without washing
- c. After that, adding (100 µLs) of Reagent (A) working agent for each well, covering the well with the sealer and incubate for (1) hour at (37C°)
- d. Then, Aspiration of the solution and washing with (350) µl of (×1 Wash Solution) to each well by multi-channel pipettes, manifold dispenser or auto-washing, and let it stable for (1–2) min. Expel the remaining fluid from all wells completely by absorbent paper. Absolutely wash (multiple times). After that, evacuate any residual Wash Buffer by suctioning. Invert the plates and blotting it against absorbent paper
- e. Furthermore, adding (100 µls) of Reagent (B) working agent for each well, covering the well with the sealer and incubate for (30) min
- f. Recur the aspiration/wash method done in step d
- g. Moreover, the addition of (90 µl) of Substrate Solutions to each well. Covering with another plate seal. Incubation for (10–20) min at (37C°). The fluid will transform to blue by the addition of substrate agent
- h. Furthermore, the addition of (50 µl) of Stop Solution to each well. The fluid may transform to yellow by the insertion of Stop agents. Mixing the fluid with taping the sides of the plates. In the event that color changing does not sound uniform, tapping the plate softly to guarantee the whole mixing
- i. Finally, discard the drops of H₂O on the bottom of the plate and be sure that there was no fluid surface bubbles. Next, run the plate reading and do measurement at (450 nms) soon.

Statistical analysis

These data were implemented as a mean $\pm$ standard deviation. Furthermore, paired *t*-test was utilized to compare differences between groups. $P < 0.05$ was considered as statistically significant consider as. Analyses were performed using the SPSS software version 21 (SPSS, IBM Company, Chicago, USA), and Sigma plot update version.

RESULTS

Effects of Bardoxolone on nuclear factor-erythroid 2-related factor 2 level (ng/ml)

The data in [Figure 1] showed that the cytoplasmic Nrf2 level of HCT-116 colonic cancer cell line by bardoxolone treatment after 24 h incubation were significant increase in Nrf2 level ($P < 0.05$) as compared to control group (cancer cells without treatment) in the concentrations (20, 10, 5, 2.5, and 1.25) $\mu\text{g/ml}$.

Effects of 5-fluorouracil on nuclear factor-erythroid 2-related factor 2 (ng/ml)

The data in Figure 2 showed that the cytoplasmic Nrf2 level of HCT-116 colonic cancer cell line by 5-FU and leucovorin treatment after 24 h incubation were significant increase in Nrf2 level ($P < 0.05$) as compared to control group (cancer cells without treatment) only in the concentrations (5, 20) $\mu\text{g/ml}$ While there were no significant effects of 5-FU and LV on the Nrf2 level in the concentrations (2.5, 10, 40, 80) $\mu\text{g/ml}$ ($P > 0.05$).

Effects of combinations of bardoxolone, 5-fluorouracil and leucovorin on nuclear factor-erythroid 2-related factor 2 level (ng/ml)

The data in [Figure 3a] showed that the cytoplasmic Nrf2 level of HCT-116 colonic cancer cell line by combination treatment of bardoxolone, 5-FU and LV after 24 h incubation were significant increase in Nrf2 level ($P < 0.05$) as compared to control group (cancer cells without treatment)

in the concentrations (20, 10, 5, 2.5, 1.25) ($\mu\text{g/ml}$) of bardoxolone. Furthermore, 5-F with LV in the concentrations (5, 10, 20, 40, 80) $\mu\text{g/ml}$; while there were no significant effects on Nrf2 in combination of bardoxolone concentration at (0.625 $\mu\text{g/ml}$) and 5-FU and LV at (2.5 $\mu\text{g/ml}$) ($P > 0.05$).

Effect of bardoxolone, 5-fluorouracil and leucovorin on nuclear factor-erythroid 2-related factor 2 level in all concentrations

Figure 3b shows all the treatment drugs in different groups and different concentrations and the Nrf2 concentration for each group.

DISCUSSION

Effects of bardoxolone on nuclear factor-erythroid 2-related factor 2 levels

The results had shown the cytoplasmic Nrf2 level of HCT-116 colonic malignant cell line in the bardoxolone treatment group was increased in a significant level ($P < 0.05$) as compared to a control group (without treatment) in the concentrations (20, 10, 5, 2.5, 1.25 $\mu\text{g/ml}$).

These results could be attributed to the main role of bardoxolone as a strong activator to Nrf2 and hence as a suppressor to NF- κ B and to cyclin D1 which have important roles in carcinogenesis and inflammation. This effect was in consistent with as reported by Chun *et al.*^[8]

Bardoxolone has α , β -unsaturated carbonyl groups on its ring (A and C) which can give a reversible adduct with the cysteine residues (thiol group) in target proteins such as Kelsh-like ECH-associated protein 1 (KEAP1) and inhibitor of KB kinase.

It was noticed that bardoxolone at low nanomolar concentrations protects the cells from oxidative stress through the inhibition of reactive oxygen species (ROS) production, while badoxolone at low micro-molar concentrations induces

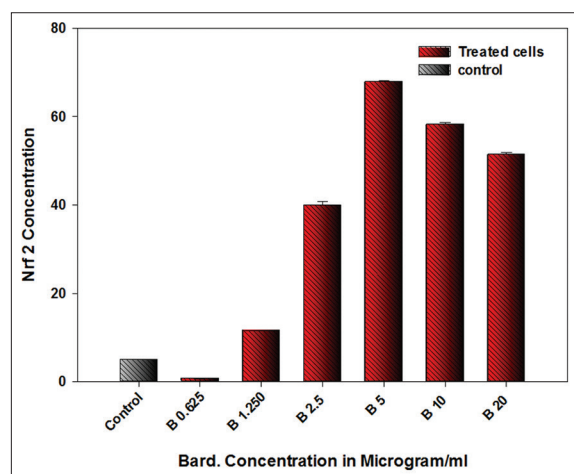


Figure 1: Effect of bardoxolone on nuclear factor erythroid 2-related factor 2 level (ng/ml) in HCT-116 colonic cancer cell line expressed as mean $\pm$ standard deviation

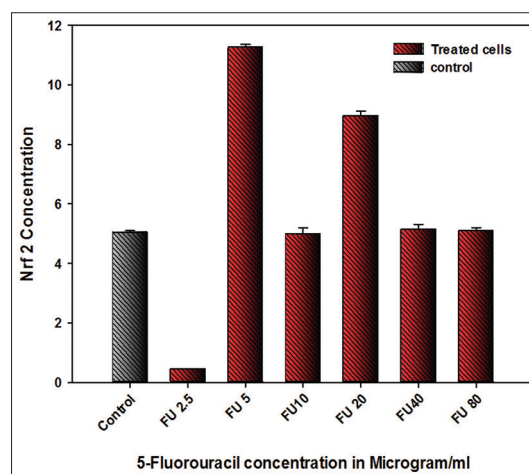


Figure 2: Effect of 5-fluorouracil and leucovorin on nuclear factor erythroid 2-related factor 2 level (ng/ml) in HCT-116 colonic cancer cell line expressed as mean $\pm$ standard deviation

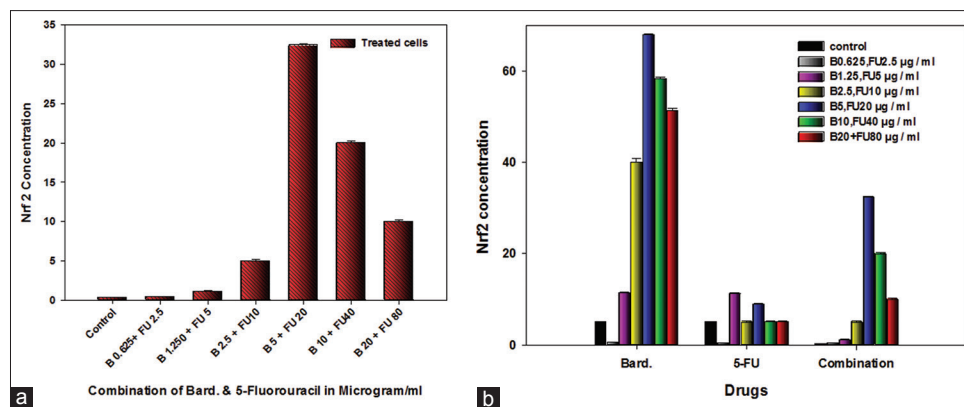


Figure 3: (a) Effect of bardoxolone, 5-fluorouracil and leucovorin on nuclear factor erythroid 2-related factor 2 level (ng/ml) in HCT-116 colonic cancer cell line expressed as mean $\pm$ standard deviation. (b) Effects of bardoxolone, 5-fluorouracil and leucovorin groups in different concentrations on nuclear factor erythroid 2-related factor 2 level (ng/ml) in HCT-116 colonic cancer cell line expressed as mean $\pm$ standard deviation

the apoptosis by elevating ROS and diminishing intra-cellular glutathione.

Nrf2 is considered as an active transcription factor for mRNA expressions that coded for production of cyto-protective substances.

These molecules or substances comprise the enzymes that directly damage ROS. In addition, enzymes that form the small molecules like glutathione have a role in scavenging the potentially damaging electrophiles. These mechanisms were consistent with as reported by Liby *et al.*^[9]

Effects of 5-fluorouracil and leucovorin on nuclear factor-erythroid 2-related factor 2 level

The results demonstrated the cytoplasmic level of Nrf2 in HCT-116 colonic malignant cell line after treatment with 5-FU and LV and 24 h incubation. The results showed a significant increase in Nrf2 level only in the concentrations (5, 20 µg/ml) ($P < 0.05$) while there were no significant effects of 5-FU and LV in the concentrations (2.5, 10, 40, 80 µg/ml) ($P > 0.05$) as compared to control group (cancer cells without treatment).

It is noticed that 5-FU is associated with the inhibition of thymidylate synthase and also incorporation of 5-FU into DNA and RNA as reported by Longley *et al.*^[10]

The nuclear Nrf2 is considered as a redox-sensitive transcription agent which regulates the phase II antioxidant genes expression including hem oxygenase-1 (HO-1) which gives a cyto-protective mechanism in oxidative stress. Regarding the unstressed cells, Nrf2 has been sequestered by KEAP1 which facilitates its fast proteasome-mediated degradations as reported by Zhang.^[11]

In spite of a good understanding of the 5-FU mechanism of action in the last years, the drug-resistant phenotype have been appeared which remains as a part of its clinical use limitation. Therefore, a new management strategies are needed to overcome any resistance can happen. Nrf2 modulations might be considered as a key system in 5-FU treatment regimen and decrease drug resistance.

Effects of combinations of bardoxolone, 5-fluorouracil, and leucovorin on nuclear factor erythroid 2-related factor 2 levels

As had been shown, the results of a combination treatment of bardoxolone and the two chemotherapeutic agents (5-FU+LV) on HCT-116 colonic malignant cells line demonstrated as significant increase ($P < 0.05$) in Nrf2 level in all concentrations except in one concentration (0.625 µg/ml) and in all concentrations of 5-FU and LV except in (2.5 µg/ml) concentration.

It was noticed that bardoxolone possess a synergistic effects to other chemotherapeutic drugs. Nrf2 transcription pathway can promote the detoxification process of ROS. In addition, Nrf2 is considered as a transcriptional modulator and a possible target to overcome the resistance of colonic malignant cells to 5-FU therapy.

HO-1 is shown to play a vital role in the maintenance of cellular homeostasis and prevention of transformations from normal to premalignant conditions by counteracting ROS that mediate the development of malignancies. It is regulated by Nrf2.

However, abnormal increase in HO-1 leads to adverse effects and behaves as precancer molecule and can be present in different colonic malignancies because of its anti-apoptotic role as reported by Tanaka *et al.*^[12]

Furthermore, when there is abnormal over-expressions of HO-1, this leads to active survival process which results in a poor clinical consequences.

Finally, it was shown that HO-1 expression (in malignant cells) is increased after treatment with chemotherapeutic drugs and in radiotherapy. Hence, HO-1 and its regulatory Nrf2 have been implicated in modifications of drug resistance by malignant cells as reported by Berberat *et al.*^[13]

CONCLUSION

Bardoxolone has significant anticancer effects in low microconcentrations on human colonic cancer cells as compared to control groups ($P < 0.05$).

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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