Cytotoxic Effects of Bardoxolone on HCT-116 Human Colonic Cancer Cell Line

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Abstract

Background: Nuclear factor-κB (NF-κB) transcription factors comprise a key role in many physiological processes such as innate and adaptive immune responses, proliferation of cells, cell death, and inflammations. Anti-NF-κB therapy may rescue different cases of colonic carcinoma and would be considered as a therapeutic goal. The aim of the study is to detect whether Bardoxolone is effective in treatment of colonic carcinoma by apoptosis and regression of tumor markers and activation of tumor suppressor genes as compared to other FDA approved anticancer agents as 5-FU. colonic cancer cell line (HCT 116). These cells are cultured *in vitro* according to routine cell culture protocols using specific media and reagents. **Methods**: Treatment groups are classified into 4 groups (control, Chemotherapy group, Bardoxolone and combination treatment groups).**Results:** The results revealed significant increase in inhibition concentrations IC50 of colonic cancer cells in low concentrations of bardoxolone treatment group and in combinations treatment group as compared to control group ($P< 0.05$). Also, the inhibition percent of different concentrations of 5-FU and Leucovorin on HCT-116 colonic cell line after 24h of incubation were significant ($p< 0.05$) as compared to control group(cancer cells without treatment) in all concentrations (2.5, 5, 10, 20, 40, 80 µgm/ml). **Conclusion:** Bardoxolone has a significant anticancer and cytotoxic effect in low micro-concentrations on human colonic cancer cells as compared to control groups.

Keywords: Bardoxolone, colonic cancer, HCT-116 cell line, cell cytotoxicity.

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Introduction

Colorectal cancer (CRC) is the second etiological factor of cancer death. Progress has been performed in advance of chemotherapy for disseminated colonic carcinoma. Targeted therapies against vascular growth agents are now commonly

established. However, many cases noted that tolerance arise to such management; therefore, new strategic methods are necessary to compensate the present therapies [1,2]. Some agents rise a person's risk of getting the disease comprising age, polyps of the colon, history of cancer, heredity, environmental factors, and many other agents[3-5].

Nuclear factor-κB (NF-κB) transcription factors comprise a key role in many physiological processes such as innate and adaptive immune responses, proliferation of cells, cell death, and inflammations. It has become clear that aberrant regulation of NF-κB and the signaling methods that control its activity are included in cancer progress, in addition to resistance to chemo- and radio-therapies [6]. Hence, anti-NF-κB therapy may rescue different cases of colonic carcinoma and would be considered as a therapeutic goal. Bardoxolone methyl is a pentacyclic triterpenoids. Bardoxolone-methyl (Bar-Me) (also known as "RTA 402 or CDDO-methyl ester) is an experimental and orally-bioavailable semi-synthetic triterpenoids, based on the scaffolds of the natural product oleanolic acid. Preclinical studies noticed that the agent behaves as a stimulator of the Nuclear factor erythroid-derived (Nrf2) signaling and a suppressor of the NF-κB pathway. Bardoxolone methyl is a stimulator of the KEAP1-Nrf2 pathway in mice. Bardoxolone methyl also inhibits the pro-inflammatory transcription factor NF-κB in a tissue cultured human cell line. It is in a phase 3 preclinical trial.

The aim of the study is to detect whether Bardoxolone is effective in treatment of colonic carcinoma by apoptosis and regression of tumor markers and activation of tumor suppressor genes as compared to other FDA approved anticancer agents as 5-FU.

Materials and Methods

HCT-116 colonic cell line

HCT116 cells had a mutation in the codon 13 of the K-RAS proto-oncogene, and are suitable transfection target for gene therapy researches. The cells have an epithelial morphology and can metastasize in xenograft models. When they were transducted with viral vectors carrying the p53 gene, HCT116 cells remain arrested in the G1 phase [7]. HCT116 cells are used in a different biomedical studies including colon cancer proliferation and corresponding inhibitors. The cell line has been used in tumorogenesis 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 5µl of the stock solution, completed to 1 ml of maintenance media, so the first concentration was 20 ug/ml and diluted serially (20, 10. 5, 2.5, 1.25,0.625 µg/ml).

Fluorouracil and Leucovorin serial dilutions

By using RPMI-1640 serum free media, 6 of two-fold serial working dilutions from 5FU and leucovorin solution (4 mg/ml) were prepared by taking of 5 u.ml of the stock solution, completed to 1 ml of maintenance media, so the first concentration was 80µg/ml and diluted serially(80, 40, 20, 10, 5, 2.5µgm/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-Flurouracil and Leucovorin) as a treatment for HCT-116 colonic cancer cell line; 4thgroup: Use of combination treatment of Bardoxolone, 5-flurouracil and Leucovorin for HCT-116 colonic cell line.

Crystal Violet Method

This method was used to assess the cell growth optical density in each plate well, by utilizing the plate reader. After the cytotoxicity assay end point, the test substance and the maintenance media were discarded out with the use of (200) µl of the phosphate buffer to wash the wells. Then, (200) µl of 0.5% solution of crystal violet was added for each well. After that, samples were incubated for (20) min at 37 C° with shaking. Furthermore, the plates were submersed in tap water for (15)

min. Moreover, the plates were dried in the air and dissolving the dye by using (0.2%) Triton (X-100) in water for each well and then incubation (for 30 min) with shaking. Finally, (100) ml was taken from each well into a new microplate and reading by a reader at (570 WL) absorbance length.

Iinhibition %was measured by the equation:

The inhibition % = (optical densities of control wells - optical density of test well) / the control wells optical densities] X 100.

Test Designs and the cytotoxicity measures

As noticed by Freshney (1994), the cytotoxicity tests were connected for assurance of the impact of Bardoxolone and (5FU and Leucovorin) on (HCT-116) cell lines culture. At the point when the growth in the carafes progressed toward becoming as mono-layer just prior to achieving the (exponential stage), the cell monolayer are harvested and re-suspended with a development media in a groupings of (5X 10^5) cell/ml and seeded in a (96 wells) microtiter plates. Since the cell growth achieves (90%), the very much was introduced to sequential weakening of the tests synthetic substances as in the accompanying investigations in 2 distinctive sequential weakening focuses:

Experiment No.1: The effect of Bardoxolone on HCT-116 cell 24h of duration.

Three replicates wells in a six columns of a microtiter plate were seeded with HCT-116 cells in a concentration of $5x10^5$. Three wells replicates from each columns were exposed to one of six serial dilutions starting from 20 ug/ml of Bardoxolone ending with 0.625 (20, 10, 5, 2.5, 1.25, 0.625 µgm /ml). Then the plate was covered with the plastic lid and incubated for 24h. After the end of the exposure the wells washed with 200 µl of a sterile PBS. The effect of the Bardoxolone on the HCT-116 cell line growth was assessed by C.V. cytotoxicity assay.

Experiment No.2: The effect of 5-FU and Leucovorin on HCT-116 cell line

As in experiment No.1, HCT-116 cell line microtiter plates were treated with different concentrations of 5-FU and Leucovorin starting from 80 ug/ml in six serial dilution ending with 2.5µg/ml) and incubated for 24h.

Experiment No. 3: Effect of combination of serial dilutions of Bardoxolone and (5-FU and Leucovorin) on HCT-116 cell line for 24h duration.

Cell line were treated with concomitant serial dilutions concentrations of Bardoxolone and (5-FU and Leucovorin) starting from 80 ug/mL ending with 2.5 ug/ml in triplicates wells of both reagents for 24 h of incubation. The concomitant effects were assessed by the Elisa micro-plate reader.

Results

Effects of Bardoxolone on Cell Viability %

The data in (Figure 1) showed that the cell viability percent of HCT-116 colonic cancer cell line by bardoxolone treatment after 24 hours incubation were significant ($p< 0.05$) as compared to control group(cancer cells without treatment) in all concentrations (20,10, 5, 2.5, 1.25, 0.625 µgm/ml).

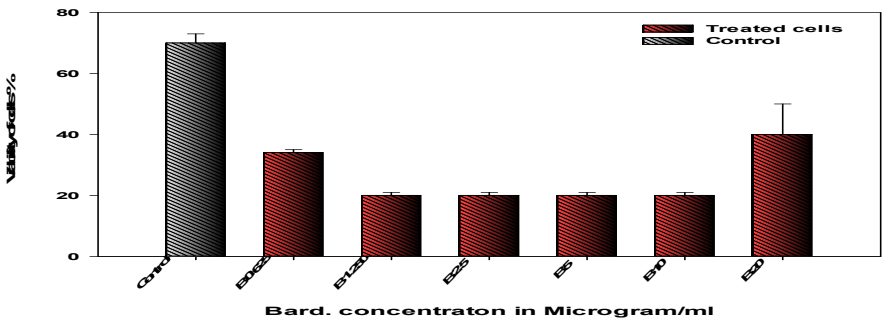

Figure 1: Effects of Bardoxolone on Percentage of viability of cells in HCT-116 colonic cancer cell line expressed as mean ± SD**.**

Effects of 5-Fluorouracil and Leucovorin on Cell viability%

The data in (Figure 2) showed that the cell viability percent of HCT-116 colonic cancer cell line by 5-FU and leucovorin treatment after 24 hours incubation were significant ($p< 0.05$) as compared to control group(cancer cells without treatment) in all concentrations (2.5, 5, 10, 20, 40, 80 µgm/ml).

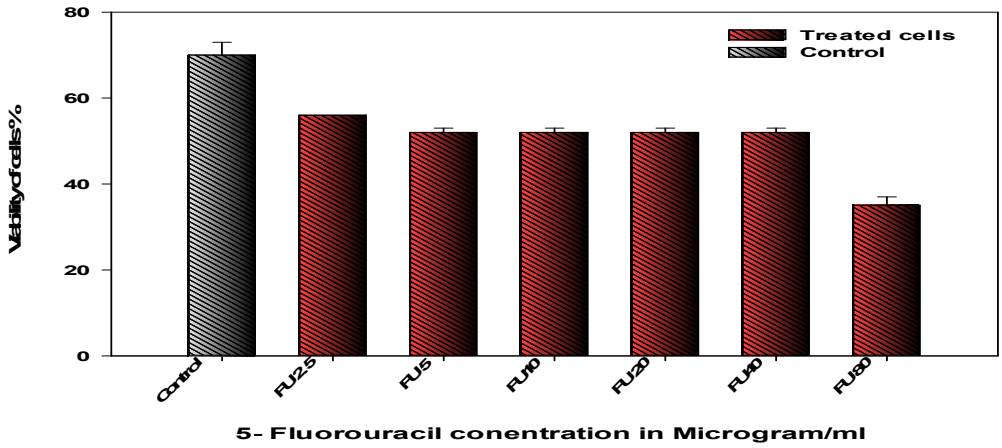

Figure 2: Effects of 5-FU and leucovorin on Percentage of viability of cells in HCT-116 colonic cancer cell line expressed as mean ± SD

Effects of combinations of Bardoxolone, 5-Fluorouracil and Leucovorin on cell viability

The data in (Figure 3) showed that the cell viability percent of HCT-116 colonic cancer cell line by a combination of Bardoxolone, 5-FU and leucovorin treatment after 24 hours incubation were significant ($p< 0.05$) as compared to control group(cancer cells without treatment) in all concentrations (5, 10, 20, 40, 80 µgm/ml) for 5FU and leucovorin and in all concentrations for bardoxolone (20,10, 5, 2.5, 1.25 µgm/ml), while there were no significant effects ($p> 0.05$) on cell viability percent in combination treatment of concentrations of bardoxolone at (0.625 µgm/ml) and 5FU concentration at (2.5 µgm/ml).

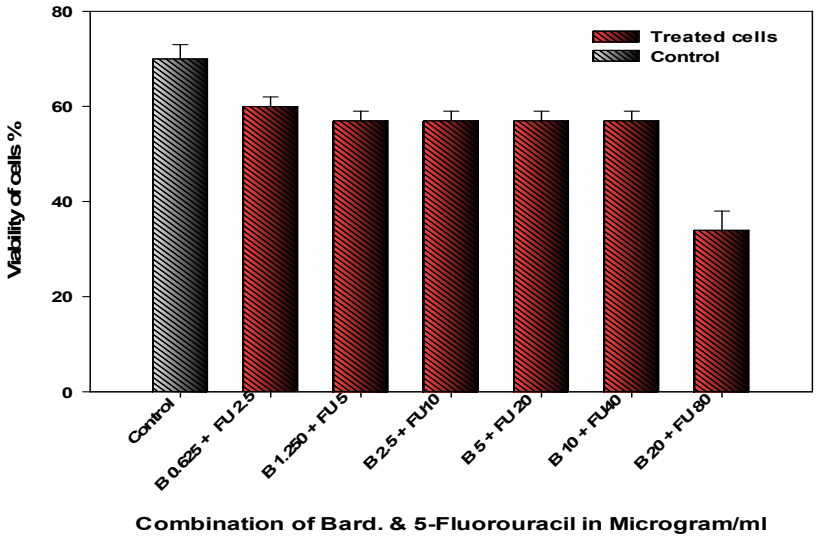

Figure 3: Effects of combinations of Bardoxolone, 5-FU and Leucovorin on Percentage of viability of cells in HCT-116 colonic cancer cell line expressed as mean ± SD.

Effects of Bardoxolone, 5-Fluorouracil and Leucovorin on cell viability in all concentrations

Figure-4 showed all the treatment drugs in different groups and different concentrations and the percentage of the viability of cells for each group.

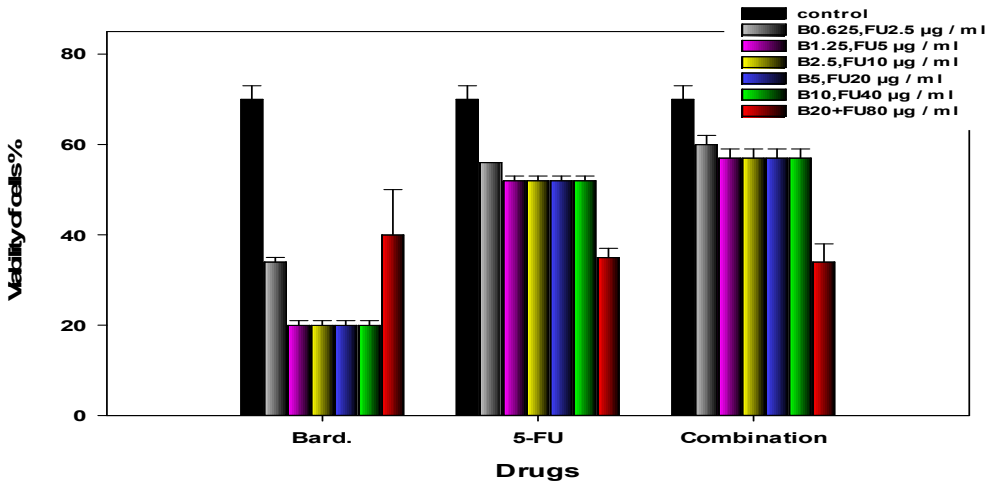

Figure 4: Effects of Bardoxolone, 5-FU and Leucovorin groups in different concentrations on Percentage of viability of cells in HCT-116 colonic cancer cell line expressed as mean ± SD

Effects of Bardoxolone on Inhibition Concentration 50% (IC50)

Figure-5 illustrates the inhibition percent of different concentrations of Bardoxolone on HCT-116 colonic cell line after 24h of incubation. There were significant ($p < 0.05$) as compared to control group (cancer cells without treatment) in all

concentrations (20, 10, 5, 2.5, 1.25, 0.625 µgm/ml) and there were significant cell cytotoxic effects in all concentrations. IC50 Baroxolone was 24.1

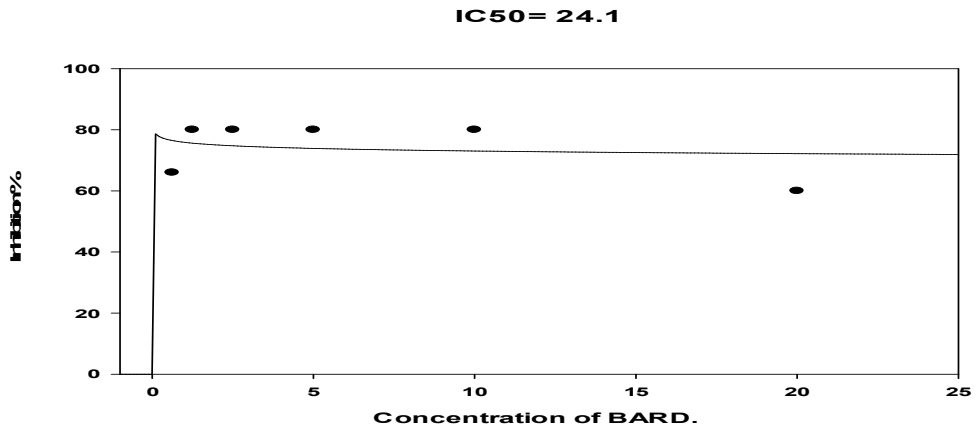

Figure 5: Inhibition-concentration curve for different concentrations of Bardoxolone on HCT-116 colonic cell line after 24 hours incubation

Effects of 5-Fu and Leucovorin on Inhibition Concentration 50% (IC50)

Figure-6 illustrates the inhibition percent of different concentrations of 5-FU and Leucovorin on HCT-116 colonic cell line after 24h of incubation. There were significant ($p< 0.05$) as compared to control group (cancer cells without treatment) in all concentrations (2.5, 5, 10, 20, 40, 80 µgm/ml). The IC50 of 5- FU and Leucovorin was 91.3.

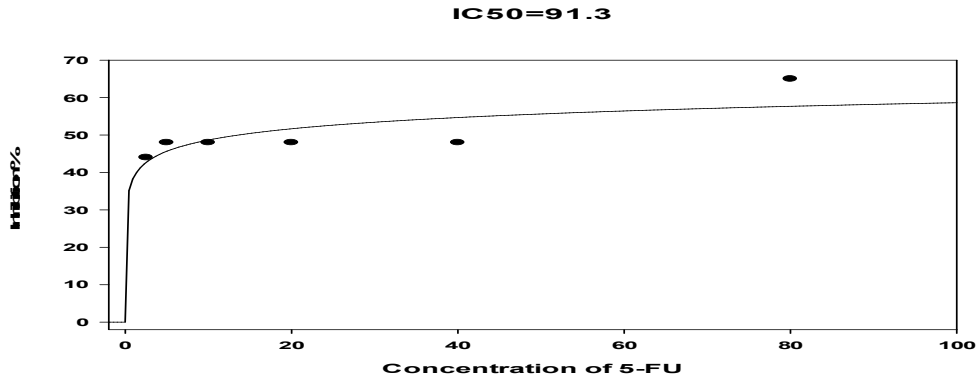

Figure 6: Inhibition-concentration curve for different concentrations of 5-FU and Leucovorin on HCT-116 colonic cell line after 24 hours incubation.

Discussion

Effects of Bardoxolone on Cell Cytotoxicity

The cell viability percent of HCT-116 colonic cancer cell line by bardoxolone treatment after 24 hours incubation were significant ($p< 0.05$) as compared to control group(cancer cells without treatment) in all concentrations (20,10, 5, 2.5, 1.25, 0.625 µgm/ml) and there were significant cell cytotoxic effects in all concentrations.

The viability percent of cells varied with different concentrations of bardoxolone. The anticancer effects of bardoxolone may be attributed to different mechanisms including the activation of Kelch-like erythroid cell-derived protein with nuclear factor (erythroid-derived 2)-like and antioxidant response element (Keap1/Nrf2/ARE) pathway as demonstrated by Yates *et al.*[8], which is involved in cyto-protection in the presence of excessive electrophiles or oxidative stress. Binding of bardoxolone to Keap1 disrupts its critical cysteine residues, leading to the release of Nrf2, which prevent its ubiquitinations and finally leads to stabilization and nuclear translocations of NF-κB. In the nucleus, Nrf2 activates the transcription of phase 2 response gene, leading to a coordinated anti-oxidant and anti-inflammatory response as explained by Dinkova [9].

Effects of 5-Fluorouracil and Leucovorin on Cell viability%

As the results shown, the cell viability percent of HCT-116 colonic cancer cell line by 5-FU and leucovorin treatment after 24 hours incubation were significant ($p< 0.05$) as compared to control group(cancer cells without treatment) in all concentrations (2.5, 5, 10, 20, 40, 80 µgm/ml). These results also demonstrated the variations in viability percent of colonic cancer cells with different concentrations of these chemotherapeutic drugs. This could be attributed to the time dependent character of 5-FU because as anticancer drug in addition to the dose of 5-FU is increased as reported by Sui *et al.*[10]. Sui *et al.* also reported that the aberrant expressions of p-53 are thought to abolish 5-FU abilities to induce p53-dependent cells growth arrest and apoptosis. These results can influence on IC50 of the cytotoxic drug. 5-FU exerts anti-proliferative effects through the inhibition of thymidylate synthase (TS), which decreases thymidylate levels and increases uracil incorporation into DNA. In the reaction catalyzed by TS, the cofactor methylene tetra-hydro-folate ($CH_2H_4PteGlu$) is the methyl and electron donor and also rate limiting in the reaction because its intracellular concentrations are lower than dUMP. The active 5-FU metabolite, FdUMP, forms a stable ternary complex with the active site (cysteine of TS and THF), thereby suicide inhibiting dTMP synthesis. Therefore, Leucovorin (folinic acid) is administered clinically in combination with 5-FU to enhance its therapeutic effects, because LV is readily converted to THF. 5-FU is directly incorporated into RNA and DNA and alter transcription and replication, respectively as reported by Berger *et al.*[11].

E**ffects of combinations of Bardoxolone, 5-Fluorouracil and Leucovorin on cell viability**

The results demonstrated that the cell viability percent of HCT-116 colonic cancer cell line by a combination of Bardoxolone, 5-FU and leucovorin treatment after 24 hours incubation were significant ($p< 0.05$) as compared to control group(cancer cells without treatment) in all concentrations (5, 10, 20, 40, 80 µgm/ml) for 5FU and leucovorin and in all concentrations for bardoxolone (20,10, 5, 2.5, 1.25 µgm/ml), while there were no significant effects ($p> 0.05$) on cell viability percent in combination treatment of concentrations of bardoxolone at (0.625 µgm/ml) and 5FU concentration at (2.5 µgm/ml). Bardoxolone can implement its synergistic anticancer effects with other chemotherapeutic agents via different ways such as induction of ROS in cancer cell lines, and that ROS plays a vital role in drug-mediated growth inhibitions, induction of apoptosis and differentiation, and down-regulation of cMyc. These results were in agreement with the reported data by Jutooru *et al.*[12].

Conclusion

Bardoxolone has significant anticancer and cytotoxic effects in low micro-concentrations on human colonic cancer cells as compared to control groups.

Conflicts of interest: None of the authors have any conflicts of interest relevant to this research subject.

Ethical Approval

Ethical Committee at the Al-Furat Al-Awsat University, Iraq, approved the study.

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