



Research Article

The Combination of Berberine and Methotrexate Enhances Anti-Cancer Effects in Hela Cancer Cell Line: A Morphological Study

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Abstract

Background: Co-administration of two or several either chemotherapeutic agents or conventional drugs as a combination treatment is the most effective method to increase therapeutic efficiency. Additive or synergistic influence are two mechanisms by which combination therapy causes a rise in optimal cancer therapy compared to a single treatment.

Methods: Colorimetric assay was carried out to estimate the cytotoxicity of the combined system, followed by apoptosis assay to calculate the number of apoptotic cells. Both 4',6-diamidino-2-phenylindole (DAPI) staining and DNA ladder were complemented tests to illuminate morphological changes and DNA fracturing on HeLa cancer cells.

Results: We revealed that the combination of berberine (BER) and methotrexate (MTX) could inhibit the growth of HeLa cancer cells noticeably. Nevertheless, single BER and MTX were not as effective as a combined system to reduce cell viability at the same dose. Regarding the apoptosis induction and change in morphology of cancer cells' nucleus, co-treatment of BER and MTX was more effective. The result was complemented with flow cytometry, DAPI staining and DNA ladder, which showed that BER+MTX depicted more anti-cancer effects.

Conclusion: The combination therapy of HeLa cancer cells with BER and MTX showed high inhibition effect compared to other treated groups.

Introduction

Published cancer statistics in GLOBOCAN by the International Agency for Research on Cancer (IARC) in 2012 draw the attention towards 14.1 million new cancer cases, with 8.2 million deaths at the same year resulted from an unregulated transformation, unbalanced apoptosis, unlimited rate of proliferation, invasion, excessive angiogenesis, and micrometastasis.¹⁻³ According to the latest updates, cervical cancer ranked as the fourth affecting cancer incidence and mortality rates.¹ Single chemotherapy is widely used to treat cancer worldwide, although fast-growing healthy cells might be attacked by a high dose of chemotherapeutic agents resulted in failed conventional chemotherapy.⁴ Hence, optimization of concentration and pharmacological action of anti-cancer drugs in combination system minimize mentioned side effect. The combination of multiple anti-cancer drugs

has a synergistic effect using similar molecular pathways and reduces chemoresistance via a diverse mechanism of action. Hence, herbal-based components with a defined mechanism of action are under the focus of scientists to reduce or even eliminate side effects while increasing the therapeutic index of conventional anti-cancer drugs.⁵ According to the classifications, berberine (BER) is a natural isoquinoline alkaloid displaying various biological effects, including anti-microbial, anti-viral, anti-inflammatory, anti-diarrhea, free radical scavenger, antioxidant and anti-tumor effect. In more detailed consideration, BER imposing its anti-cancer effects by introducing apoptosis and cell viability inhibition in different human cancers such as breast, lung, and liver.⁶ Besides, methotrexate (MTX) as a folate antagonist and the first antineoplastic agent is used for more than a half-

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century to control childhood acute lymphoblastic leukemia. Take into account that MTX is administered either as a first-line monotherapy or anchor drug combined with other anti-cancer drugs or natural components for patients with insufficient responses.⁷ As a folate antagonist, MTX is considered an inhibitor of folate-dependent enzymes that eradicate purine and pyrimidine nucleic acids synthesizing, essential for DNA and RNA production in fast-growing and dividing cancer cells.⁸ Hair loss, nausea, headaches, fatigue, MTX related liver disease, and cirrhosis are several side effects of MTX.⁹ In this study, the combination of BER and MTX was used to assess both medical plants' anti-cancer potential and conventional chemotherapeutic drugs together. Hence, different BER and MTX concentration was mixed to reaches optimum concentration of both BER and MTX. In order to illustrating the effect of synergistic or additive consequence of combination therapy compared to the single-state use of each drug, i.e. BER and MTX, the anti-cancer effect at the same level of concentration has been investigated via MTT, flow cytometry, DAPI staining and DNA ladder.

Materials and Methods

Materials

HeLa cancer cells were obtained from the National Cell Bank of Iran (NCBI). The fetal bovine serum (FBS), penicillin-streptomycin (catalog number: 10566016), and RPMI-1640 (Roswell Park Memorial Institute) media were purchased from Gibco BRL Life Technologies (catalog number: 11995065). MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide, MTX and trypsin were supplied from Sigma-Aldrich. Culture plates were gained from SPL Life Sciences Co., Ltd (Gyeonggi-do, Korea). AnnexinV-FITC/PI apoptosis detection kit was provided from Oncogene Research Products (San Diego, USA). BER hydrochloride with purity>95% was prepared from Santa Cruz Biotechnology, Dallas, USA (CAS number: 633-65-8).

MTT assay

HeLa cells were cultured in RPMI-1640 culture media containing antibiotics (100 units/mL penicillin and 50 units/mL streptomycin) and 10% fetal bovine serum in a relative humidity of 95% and 5% CO₂ concentration. The effect of BER, MTX, and combination form (BER+MTX) was evaluated on the proliferation of HeLa cancer cells using MTT assay. The cells were seeded in 96-well plates at the density of 8×10³ cells/well containing 200 μL fresh complete medium and incubated overnight. Different BER, MTX, and BER+MTX concentrations were prepared and added into each well and then incubated for 24 h and 48 h. Next, 50 μL of MTT solution was prepared in PBS buffer (2 mg/mL) and added to each well, and incubated for another 4 h. 200 μL of DMSO was added to dissolve the formazan crystals formed by the viable cells. In the end, the spectrophotometric plate reader (BioTek Instruments Inc, Vermont, USA) was used for UV absorbance measurements

at 570 nm. All experiments were repeated three times.¹⁰

Flow cytometry

The flow cytometry method was accomplished for the quantitative evaluation of apoptosis inductions. The HeLa cells were seeded in 6-well plates at a density of 3×10⁵ cells/well in complete culture media and incubated overnight to reach 70% confluence. Then, obtained IC₅₀ concentration of BER, MTX, and combined BER and MTX was used to treat HeLa cells for 48h. Next, the medium was aspirated, the cells rinsed with PBS, and detached via trypsin. After centrifuging harvested cells at 1200 rpm, the cell pellet was resuspended and washed in cold PBS three times. 200μ of Annexin V Binding Buffer was used for dissolving cell pellet followed by exposing collected cells to 5μl of Annexin V/propidium iodide, respectively and incubating for 15 minutes in a dark place. The stained cells were analyzed by fluorescence-activated cell sorting (FACS).¹¹⁻¹³

DAPI staining

DAPI staining assay was carried out to show the quality of apoptosis via condensed and fragmented DNA in the nucleus of HeLa cancer cells. First, 1×10⁴ cells were distributed in 96 well plates and incubated overnight. The cells were then treated with IC₅₀ concentration of BER, MTX, and combined MTA and BER for 48h. After discarding the culture media, the cells were fixed with 4% paraformaldehyde for 30 min, followed by permeabilization with 0.1% (w/v) Triton X-100 for 5 min. Finally, the washed cells with PBS were exposed to 4, 6-diamidino-2-phenylindole (DAPI) for 10 min and then washed with PBS. DNA fragmentation was visualized with Cell Imaging Multi-Mode Reader (BioTekCytation™ 5, USA).¹²

DNA ladder

DNA ladder was accomplished as a complementary assay for confirming DNA fragmentation as a sign of apoptosis in HeLa cancer cells. The mentioned cells were plated into a 6-well plate and, after incubation, an overnight treated with a defined IC₅₀ concentration of BER, MTX, and BER+MTX and incubated for 48h. As for DNA extraction, the cells were harvested and suspended in 1 ml lysis buffer and proteinase K at 56 °C for 1h. Denaturation of total protein was performed using 400 μL chloroform followed by separation of total DNA into the upper phase of suspension. After transferring of speared DNA, absolute ethanol (800 μL) was supplemented and incubated for 20 min in -20°C and then removed by centrifugation at 12,000 rpm for another 20 minutes at 4°C. In the final stage, the obtained DNA pellet was electrophoresed in 2% agarose gel containing 2 mL/100mL SYBER green (1 h at 70 V/30 mA). An ultraviolet gel documentation system was used to image fractures in DNA.^{14,15}

Statistical analysis

Statistical data measurements were performed using Graph pad Prism 6.0 software. One-way ANOVA was used

to determine the significance. A P-value of less than 0.05 was considered to be statistically significant.

Results

Cell viability

To investigate the anti-cancer effect, various BER and MTX concentrations, including 5, 10, 50, 100, 200, 400, 600 µg/mL, solely and in a combination of each other, are used to treat HeLa cancer cells. The obtained results in Figure 1 showed that simultaneous administration of MTX and BER could reduce the proliferation of cancer cells up to 50% at 80 and 40 µg/mL in 24 and 48h, respectively. However, BER and MTX were not as effective as a combination therapy in decreasing the growth of HeLa cancer cells, and their IC50 was just 95 and 100 µg/mL in 48h, respectively.¹⁶

Flow cytometry

Annexin V/PI staining assay was carried out to distinguish apoptotic cells from necrotic cells. The percentage of apoptotic cells was demonstrated with flow cytometry. Annexin V and PI double-negative cells were categorized as live cells, and cells colored with annexin V and PI were classified as late apoptotic cells. Annexin V positive and PI-negative cells were early apoptotic cells, while necrotic cells were positive to PI and negative to annexin V. As can be seen in Figure 2, combined BER and MTX had the highest number of apoptotic cells at 52.9%, followed by BER and MTX with 22.5% and 7.03%, respectively in comparison with unstained cells.

DAPI staining

The presence of condensed, fragmented, and degraded

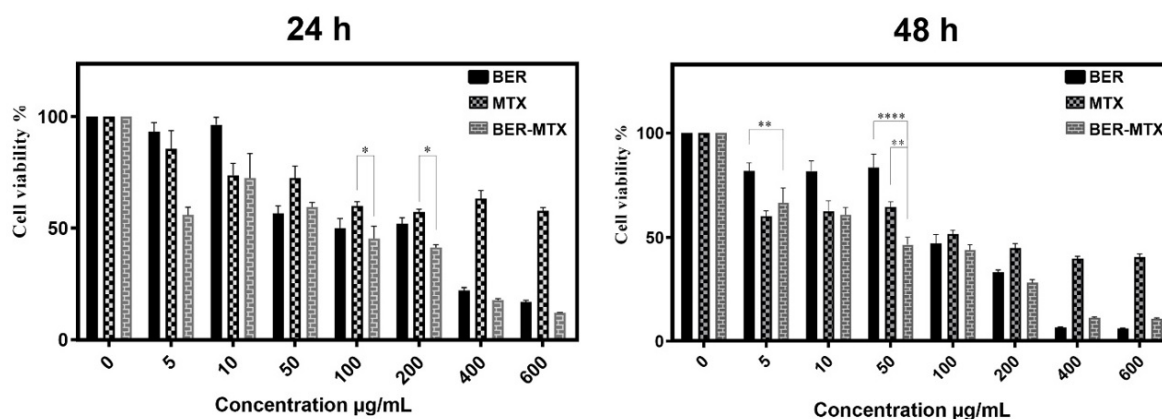


Figure 1. MTT assay diagrams for investigating the viability of HeLa cancer cells exposed to different BER, MTX, and BER-MTX concentrations (MTX concentration was IC50 value and constant in combination therapy), ranging from 25 to 100 µg/ml, for A) 24 and B) 48h time intervals. The results are shown based on cell numbers related to the control group. *P < 0.05, ** P < 0.01 and *** p < 0.001. Untreated cells served as a negative control (n = 3).

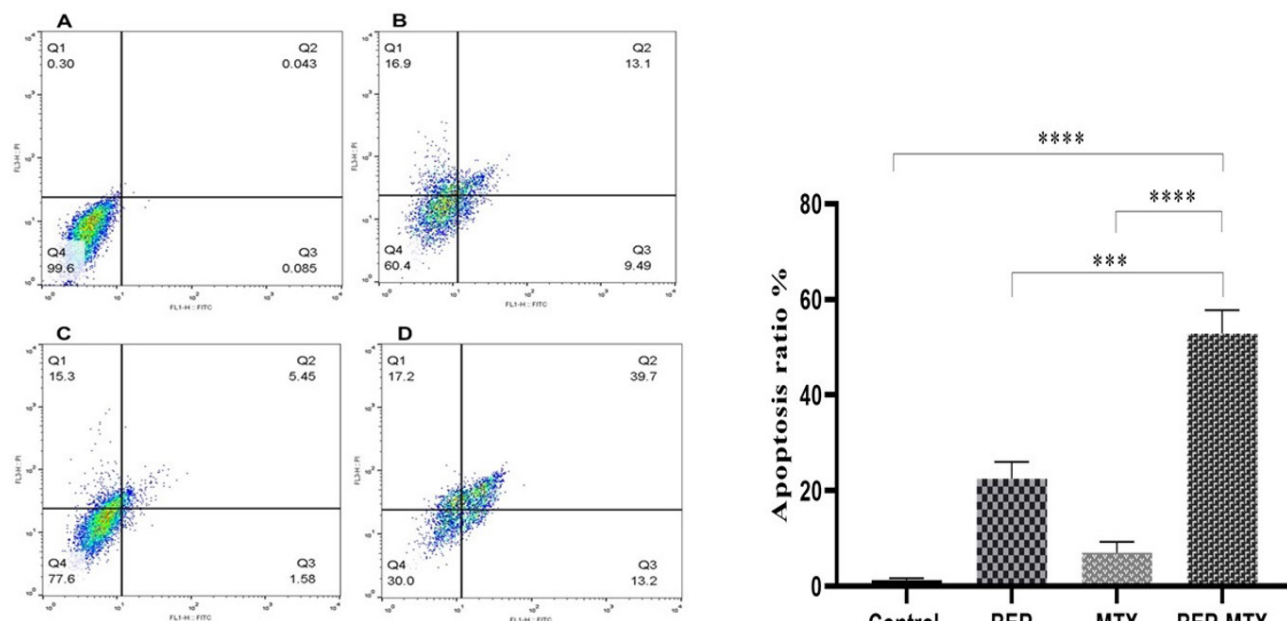


Figure 2. Apoptosis detection after treatment of HeLa cancer cells with IC50 concentration of A) Untreated groups are considered as a control group. B) BER, C) MTX and D) BER-MTX after 48h carried out by FITC-labeled Annexin V/PI flow cytometry assay. 2) *P < 0.05, ** P < 0.01 and *** p < 0.001 (n = 3).

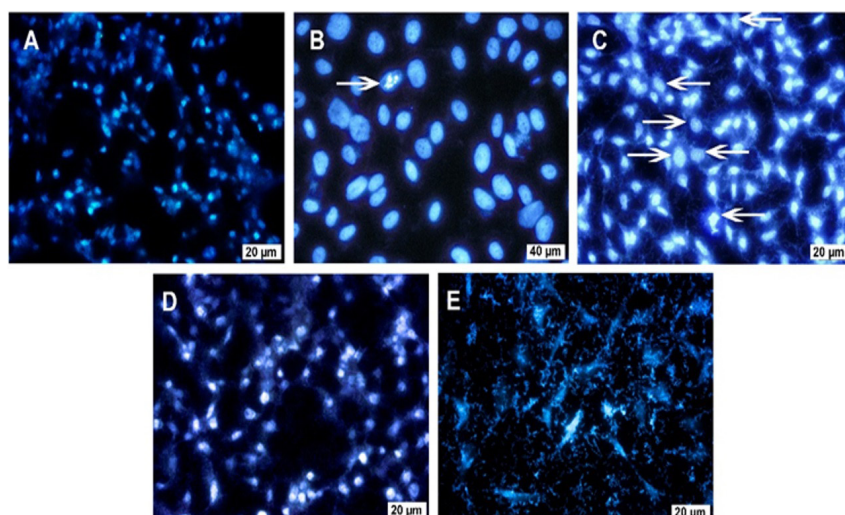


Figure 3. Representing fluorescent images of DAPI stained HeLa cancer cells treated with IC₅₀ concentration of A) BER, B) MTX, C) BER combined with MTX, D) untreated control group and E) positive control treated with 5% DMSO. Arrows showed chromatin and DNA fragmentation.

nuclei recognize apoptotic cells qualitatively visualizing by fluorescence microscopic analysis of DAPI staining in HeLa cells. As shown in Figure 3, in combination treatment upon MTX and BER after 48h, the nuclei of HeLa cancer cells showed various apoptotic cells, including chromatin fragmentation and nuclear shrinkage like positive control. The untreated cells did not exhibit any change in the morphology of the cells. Besides, either BER or MTX alone depicted a gentle sign of apoptotic cell features compared to negative control and simultaneous administration of MTX and BER.

DNA ladder

DNA fragmentation is a qualitative method to distinguish the adverse effect of the studied component using DNA ladder pattern widening from 180 to 200 bp on agarose gels electrophoresis. The obtained results in Figure 4 showed that co-administration of BER and MTX was more effective in fracturing DNA structure when exposed to HeLa cancer cells during 48h while BER and MTX showed gentle fragmentation in proportion to the negative control. Besides, since necrosis is known by random DNA fragmentation and formation of “smear” on agarose gels, the attained results showed that the mechanism of cell death via combination therapy with BER and MTX was both apoptotic and necrotic.

Discussion

BER is a natural bioactive component inducing apoptosis and inhibiting the viability of a wide range of cancer cell lines from colon, lung, and breast despite its low pharmaceutical profile.¹⁷ Furthermore, BER is known to be a suitable modulating agent curbing drug efflux, and consequently, drug resistance in highly expressed cancer cells resulted in an apoptotic reaction.¹⁸ MTX is a clinical chemotrophic drug that is active based on the high-affinity binding. However, MTX application is limited due to several dose-dependent toxicity and resistance of cancer cells.¹⁹

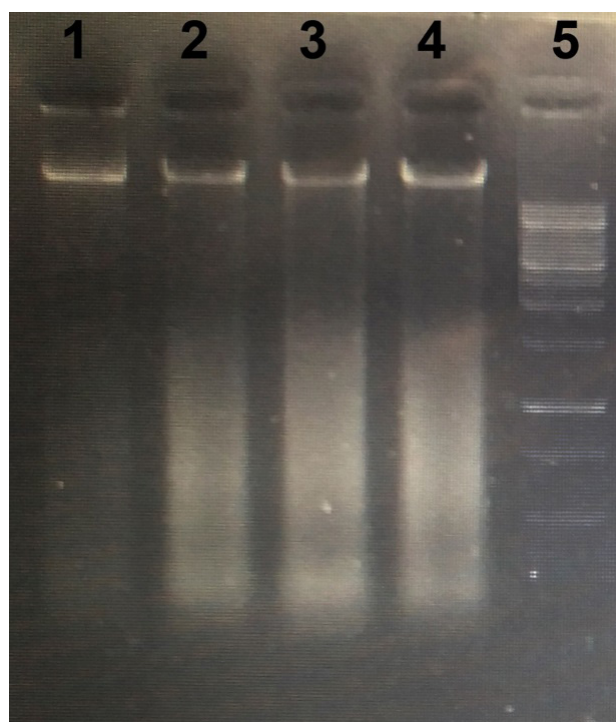


Figure 4. DNA ladder assay illustrated DNA fragmentation of HeLa cancer cells treated with MTX (Lane 2), BER-MTX (Lane 3), BER (Lane 4). Lane 1 is a Negative control group, and Lane 5 is a DNA marker.

Hence, the combination is considered a promising method to mix the rational amount of either herbal-based plants or conventional cancer therapy drugs to maximize therapeutic impact using additive or synergistic mechanisms and reduce the unwanted effects.^{20,21} In this study, we used MTX and BER to investigate their simultaneous anti-cancer effect and compare the cytotoxicity of the combined system with bare BER and MTX. Firstly, we examined the anti-proliferative efficacy of three different treated groups, including single BER administration, MTX, and BER+MTX on HeLa cancer cells. The cell cytotoxicity

results revealed that MTX, combined with BER, could significantly decrease cervical cancer cells' growth in a dose and time-dependent manner. Besides, there was a reduction in the number of cancer cells treated with bare BER and MTX, which was not as higher as a combined group. In line with our results, Liu *et al.*²² assessed the anti-cancer effect of BER mixed with cisplatin (DDP) on OVCAR3 and POCCLs cells. They found that co-treatment of BER with DDP reduced proliferation of cancer cells time and dose-dependently. Apoptosis is being studied as a genetically programmed cell death, playing a significant role in biological processes and abnormalities in extrinsic and intrinsic pathways resulted in several malignancies such as resistance to chemotherapy.²³ Thus, apoptosis activation prevents tumor formation via cytotoxic drugs and also is a leading approach to evaluate the potential of anti-cancer agents in cancer therapy.²⁴

When it comes to the anti-apoptotic effect of BER+MTX, the results showed that the combination of BER and MTX had the highest potential in inducing apoptosis among treated groups. Single therapy of BER and MTX could include apoptosis compared to untreated cells in HeLa cancer cells. We observed incidence of early/late stages of apoptosis and even necrosis within HeLa cancer cells treated with BER and MTX and mix of BER and MTX using FITC-labeled annexin V flow cytometry and the most late apoptosis percentage was observed for cells treated with mix BER and MTX. In line with this study, Ren and his colleagues investigated the co-treatment effect of BER and Galanin in apoptosis induction. The results depicted that co-administration of BER and Galanin led to dramatic apoptosis induction in human oesophageal carcinoma cells. However, BER and Galanin alone induced apoptosis moderately in comparison with control groups.²⁵ DNA fragmentation is a noticeable biochemical sign of apoptosis forming from DNA breakdown into nucleosome-sized fragments.^{26,27} DNA fragmentation factor (DFF) or caspase-activated DNase (CAD) is responsible for promoting apoptotic DNA fragmentation. Activated caspase 3, 7 cleave DFF resulted in chromosomal DNA fragmentation by generating double-stranded breaks.²⁸

Given the DAPI staining and DNA ladder results, we indicated that simultaneous treatment of BER and MTX broke, condensed, and remodeled DNA structure and revealed other morphological changes in the nucleus of HeLa cancer cells such as nuclear shrinkage, body formation, and plasma membrane blebbing, which was imaged by fluorescence microscope. However, a single treatment of BER and MTX caused gentle DNA fraction compared to the intact nucleus of the control group and mixed BER and MTX. Furthermore, BER incorporated with MTX had the highest amount of fragmentation in double-strand DNA followed by BER and MTX with the moderate DNA breakage ratio. According to attained the results, it can be concluded that combination therapy with BER and MTX showed synergistic effect and can be considered as an ideal candidate for the induction of

apoptosis. However, further experiments are required to approve our observation.

Conclusion

To sum up, the results of this study revealed that the combination of MTX and BER could inhibit the growth of HeLa cancer cells noticeably. Nevertheless, single BER and MTX were not as effective as a combined system to reduce cell viability at the same dose. Regarding the apoptosis induction and change in morphology of cancer cells' nucleus, co-treatment of BER and MTX was more effective. The result was complemented with flow cytometry, DAPI staining and DNA ladder, which showed that BER+MTX depicted more anti-cancer effects than other treated groups and control groups.

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Author Contributions

HM: Formal analysis, Investigation and Writing original draft; MA: Conceptualization; Maryam Ghaffari: Formal analysis and Investigation; AJ: Formal analysis and Investigation; DS: Investigation; MT: Conceptualization; JE: Conceptualization; Formal analysis; Supervision and Writing - review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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