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Evaluation of the Cytotoxic Effects of Ciprofloxacin on Human Glioblastoma A-172 Cell Line

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Abstract

Background: Glioblastoma multiforme, the most common, aggressive malignant brain tumor which affects patients of all ages, is principally resistant to treatment. Ciprofloxacin is an antibiotic that belongs to the fluoroquinolones. There are well-documented observations which indicate that ciprofloxacin has substantial anti-proliferative, apoptotic, cytotoxic and oxidative stress activities on various tumor cell lines.

Methods: We exposed the glioblastoma A-172 cell line to ciprofloxacin for 24, 48 and 72 h. Cytotoxicity was measured using MTT assay. The levels of Bax as an apoptotic and Bcl-2 as an anti-apoptotic protein were measured by ELISA and oxidative stress by the malondialdehyde assay.

Results: Ciprofloxacin induced tumor cell death in a dose-dependent manner with an IC_{50} value of 259.3 µM at 72 h. We observed an increase in Bax levels, a decrease in Bcl-2 concentrations and increased Bax/Bcl-2 ratio under the influence of ciprofloxacin. Malondialdehyde levels, as an important marker of oxidative stress, increased in the human glioblastoma A-172 cell line.

Conclusion: These results indicated that ciprofloxacin had anti-tumor, cytotoxic and apoptotic effects in the human glioblastoma A-172 cell line which might be useful as an adjuvant added to a glioblastoma multiforme chemotherapeutic protocol in the future.

Keywords: Ciprofloxacin, Glioblastoma A-172 cell line, Cytotoxicity, Apoptosis, Oxidative stress

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Introduction

Glioblastoma multiforme (GBM) is a highly lethal brain tumor (grade IV astrocytoma). Despite optimal treatment, which includes surgical resection along with radiation and chemotherapy, patients have a median survival of only 12–15 months. Among several difficulties of current standard treatment of GBM

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patients that include incomplete tumor removal, peritumoral edema, blood-brain barrier (BBB) disruption and inadequacy of maximum radiation dose for tumor destruction, the toxic adverse effects of chemo- or radiotherapy and drug resistance are the most important concerns.[1]

Ciprofloxacin (CPF) is a quinolone antibiotic used against many bacterial infections.[2] It has been suggested that the 4-fluoroquinolones (FQs) target the bacterial enzyme DNA gyrase and also stabilize DNA strand breaks created by DNA gyrase and topoisomerase IV, However the FQs have lesser affinity to the eukaryotic DNA gyrase homologue, topoisomerase II. In general, at concentrations higher than average found in blood, inhibition of topoisomerase II can lead to the formation of stabilized cleavage complexes and the ultimate production of DNA double-strand breaks.[3] In addition to the antibacterial activity of CPF, evidences have proven a substantial anti-proliferative activity among a variety of cancers such as bladder, colorectal, human prostate, osteosarcoma and leukemic cell lines.[4-8]

Apoptosis or programmed cell death, is a regulated physiological process essential for maintenance of cellular homeostasis. Apoptosis is a constitutive process that can be induced or inhibited by various stimuli. Recently, the pathogenesis of cancer, autoimmune and inflammatory diseases have been explained by the alteration of normal apoptosis regulation.[9-12] It was shown that some antibiotics could affect the life span of cells through apoptosis induction.[6,13,14] Among numerous antibiotics, the effects of quinolones have been highlighted as the major contributor in this field.[15-19] Ciprofloxacin as an anticancer agent, is used at concentrations higher than those to treat infectious diseases.[20] At these concentrations (200-300 µg/ml), CPF can effectively activate Bax, induce apoptosis of bladder carcinoma cells and yield to cell cycle arrest in the S/G2 stage.[21] Oxidative stress that results from the generation of the free radicals is known to substantially contribute to several pathological conditions, aging, cardiovascular disorders, neurodegenerative and cancer diseases.[22,23] Evidences have revealed that CPF retains oxidative stress properties.

Based on previous researches, the overall goal of this study was to examine whether CPF could effectively reduce cell proliferation and can be added to GBM treatment protocols.

Materials and Methods

Drug and reagents

3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), thiobarbituric acid (TBA) and Coomassie® brilliant blue G-250 were purchased from Merck KGaA (Darmstadt, Germany). Ciprofloxacin hydrochloride was purchased from Daroupakhsh Pharmaceutical Co., Ltd. (Tehran, Iran).

Cell culture

We cultured the human glioblastoma A-172 cell line (Avicenna Research Institute, Tehran, Iran) in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 µg/ml). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. They were harvested and used at the exponential phase of growth for cytotoxicity evaluation.

Cytotoxicity assay

The stock solution used in this study was 2.5 mM CPF in cell culture medium. A-172 cells were seeded into cell culture 96-well plates at 1×10^4 cells/well that contained 200 µl of cell culture medium and incubated at 37°C for 24 h in a 5% CO_2 atmosphere. Next, the medium was removed and replaced by fresh cell culture medium that contained different concentrations of CPF (2073.5, 1036.7, 518.3, 259.1, 129.5, 64.7, 32.3, and 16.1 µM) added in triplicate. After 24, 48 and 72 h, we added 100 µl MTT (1 mg/ml) to each well, followed by further incubation for 4 h. After the incubation time, the cell culture medium was removed and 100 µl DMSO was added to solubilize the formazan crystals formed during the

MTT assay. A solution of DMSO and cell culture medium was used for the control treatment. The absorbance was measured using a microplate reader (ELX 800 Biotek, USA) at 530 nm.

Enzyme-linked immunosorbent assay (ELISA) for apoptotic proteins

The cells were suspended in cell culture medium, seeded (1×10^6 cells) into culture plates and incubated in a humidified atmosphere of 5% CO_2 for 24 h until adherence. We added CPF (1/2 IC_{50}, IC_{50} and 2 IC_{50}) in triplicate to assess the apoptotic effects on the A-172 cell line. After 72 h, the cells were harvested. Next, we prepared the samples according to the ELISA manufacturer's information, then used the supernatant to measure the levels of Bax as an apoptotic protein and Bcl-2 as an anti-apoptotic protein with the Bax and Bcl-2 ELISA kits (Zellbio®, Germany), respectively. The absorbance was measured at 450 nm.

Malondialdehyde (MDA) assay

The malondialdehyde (MDA) levels, as an indicator of oxidative stress, were measured according to the Placer method. Briefly, samples were precipitated with a mixture of TCA and TBA and boiled. The supernatant was collected and we measured absorbance at 535 nm.[24] Protein concentration was measured according to the Bradford method. Coomassie® Brilliant Blue G-250 dye binds to proteins, the dye has both a blue and a red form. When this dye binds to a protein, the red form is converted to the blue form. The absorption was measured by a microplate reader (ELX 800 Biotek, USA) at 595 nm.[25]

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical significance was set at $P<0.05$. Graph Pad Prism 6.01 (GraphPad Software, Inc., USA) was used to analyze the drug effects. Data were analyzed using one-way analysis of variance (ANOVA) and the Tukey test, and were calculated using SPSS 21.0 (IBM, Armonk, NY).

Results

Cytotoxic effects of ciprofloxacin (CPF) treatment on the A-172 cell line

Ciprofloxacin produced significant, dose-dependent cell death after 24, 48 and 72 h exposure in the A-172 cell line. The half maximal inhibitory concentrations (IC_{50}) were found to be 388.6 µM (24 h), 308.9 µM (48 h) and 259.3 µM (72 h). We considered 259.3 µM at 72 h as the IC_{50} for more potency than the other IC_{50} values. No statistical significance existed between IC_{50} values ($P>0.05$; Figure 1).

Apoptotic protein (Bax) analysis

Protein analysis by ELISA revealed a dose-dependent increase in Bax levels in the A-172 cell line after 72 h exposure of CPF compared to the control group ($P<0.001$; Figure 2A).

Anti-apoptotic (Bcl-2) protein analysis

Protein analysis by ELISA showed a dose-dependent decrease in Bcl-2 levels in the A-172 cell line after 72 h exposure of CPF compared to the control group ($P<0.001$; Figure 2B).

Bax/Bcl-2 ratio analysis

We observed a dose-dependent increase in Bax/Bcl-2 ratio in the A-172 cell line after 72 h exposure of CPF compared to the control group ($P<0.001$; Figure 2C).

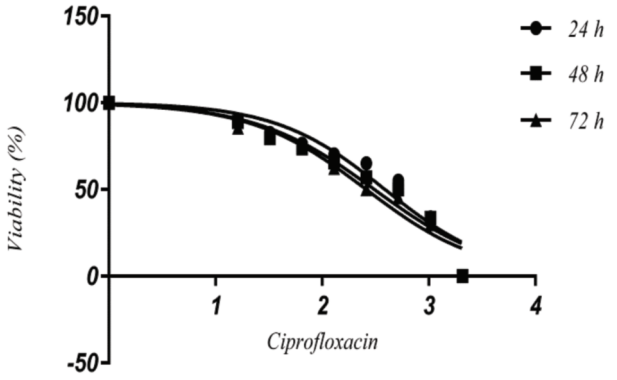

Figure 1. The effects of ciprofloxacin (CPF) treatment on proliferation of the A-172 cell line. Cells were grown in 96-well plates and treated with CPF at different concentrations for 24, 48 and 72 h. Values are represented by mean ± SD.

Analysis of the malondialdehyde (MDA) assay

Ciprofloxacin increased the MDA levels after 72 h exposure in A-172 cell line in a dose-dependent manner compared to the control group (P<0.001; Figure 3).

Discussion

In this study, we assessed the effects of CPF on human glioblastoma A-172 cancer cell line. We found that CPF had the ability to produce cytotoxicity in a dose-dependent manner. The IC_{50} value was considered to be 259.3 µM at 72 h. The drug had apoptotic effects on this cell line. Ciprofloxacin increased the levels of Bax (IC_{50} increased 8 times compared to the control group), MDA (IC_{50} increased 7.5 times compared to the control group), Bax/Bcl-2 ratio (IC_{50} increased 10 times compared to the control group) and decreased the level of Bcl-2 (IC_{50} decreased to 37% compared to the control group; P<0.001).

In vitro studies showed the cytotoxic properties of CPF on numerous cell lines such as the bladder carcinoma cell line, Jurkat T cell leukemia cell line, hormone-refractory prostate cancer (HRPC) cell lines. In addition, two transitional cell carcinoma cell lines; MBT-2 and T24, as well as sarcoma, osteosarcoma, colorectal carcinoma, GL26 murine glioma and CHOAA8 ovarian cell lines were also affected.[2,4,26-31] Ciprofloxacin inhibited tumor cell proliferation, increased population doubling time, and reduced saturation density of the cells.[32] Ciprofloxacin has been shown to inhibit mitochondrial topoisomerase II, thus affecting cellular energy metabolism. Administration of 25 µg/ml of CPF inhibited the proliferation of Jurkat cells, whereas concentrations that exceeded 80 µg/ml could induce apoptosis. The inhibition of Jurkat cell proliferation by CPF was seen in the G2/M phase of the cell cycle which compromised the formation of mitotic spindle and induced aneuploidy.[2] The current study data supported the results of the above mentioned studies on the cytotoxic effects of CPF in cancer cell lines.

However, CPF displays anti-proliferative and

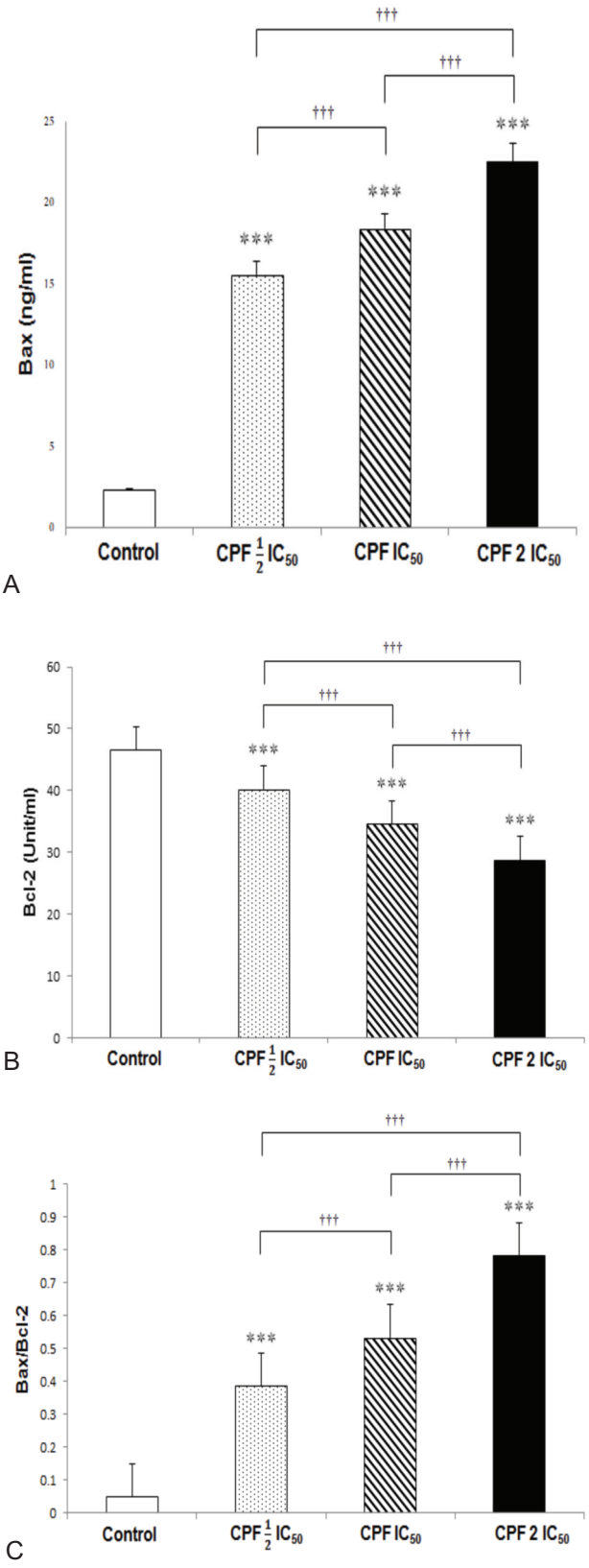

Figure 2. The effects of ciprofloxacin (CPF) treatment on level of apoptotic factors of the A-172 cell line after 72 h. (A) Bax, (B) Bcl-2 and (C) Bax/Bcl-2 ratio. Values are represented by mean ± SD. ***: P<0.001 compared to the control group; †††: P<0.001.

apoptotic effects, which is seen in malignant but not normal cells.[2] Aranha et al. have stated that CPF induces anti-proliferative and apoptotic activity on prostate cancer cells (PC-3) but not on non-tumorigenic prostate epithelial cells (MLC8891). This finding suggests that CPF could probably be used as a possible adjuvant for tumor therapy without adverse effects on normal cells.[4]

Studies have reported anti-proliferative and apoptotic activities of CPF in tumor cell lines mediated by cell cycle arrest at the S-G2/M phase. Bax translocation to mitochondrial membrane leads to an increase in the Bax/Bcl-2 ratio in some cancer cells such as PC-3 prostate cancer cells.[4] Fluoroquinolone antibiotics may also trigger the Bax-pathway of apoptosis or interfere directly with mitochondrial membrane proteins.[33,34] Researches have shown that CPF triggered apoptotic cell death. This might be attributed to the up-regulation of Bax which altered the Bax/Bcl-2 ratio in favor of apoptosis.[6] Results of this study showed that CPF has apoptotic effects on the A-172 cell line. Ciprofloxacin increased Bax levels to 339% (1/2 IC_{50}), 402% (IC_{50}) and 491% (2 IC_{50}) compared to the control group (P<0.001). It also increased the Bax/Bcl-2 ratio to 395% (1/2 IC_{50}), 543% (IC_{50}) and 798% (2 IC_{50}) compared to the control group (P<0.001). Bcl-2 levels decreased to 43% (2 IC_{50}), 37% (IC_{50}) and 30% (1/2 IC_{50}) compared to the control group (P<0.001). All increases and decreases were dose-dependent.

An imbalance between intracellular production of free radicals and the cellular defense mechanisms results in increased oxidative stress. One of the manifestations of oxidative stress is lipid peroxidation which has been known to play an important role in the toxicity of many drugs.[35] Lipid peroxidation is a significant determinant of the degree of free radical generation with MDA being one of the products, as well as an important marker of the process of the oxidative stress.[36-38] Possibly, the generation of ROS may occur during oxidative metabolism of FQs, notably CPF.[39] The previous studies have reported that the generation of ROS by FQs resulted in cellular damage.[40,41] Ciprofloxacin increased MDA concentrations in the livers of rats, which indicated increased lipid peroxidation and promotion of oxidative stress.[42] Results of this study showed that CPF increased the MDA levels to 182% (1/2 IC_{50}), 375% (IC_{50}) and 671% (2 IC_{50}) compared to the control group in a dose-dependent manner (P<0.001).

Various mechanisms are involved in the beneficial effects of CPF due to its anti-microbial activities on cancer patients who undergo chemotherapy.[29] One of these mechanisms is immune modulation which protects patients against new infections by reductions in cytokine production and improvements in immune response to infections normally found in cancer patients.[43,44] Another mechanism is based on the action of quinolones on hematopoiesis. Quinolones, particularly CPF, by means of a cyclopropyl moiety at position N1 has been shown to increase the production of colony-stimulating factors, myeloid progenitors, hemoglobin, white blood cells (lessen neutropenia) and increase the survival of cyclophosphamide-treated mice. This finding was similar to data obtained when granulocyte colony-stimulating factor were used in these mice.[29,45] Finally, the quinolone anti-tumor effects mediated by inhibition of mammalian DNA polymerase, topoisomerases I and II, were

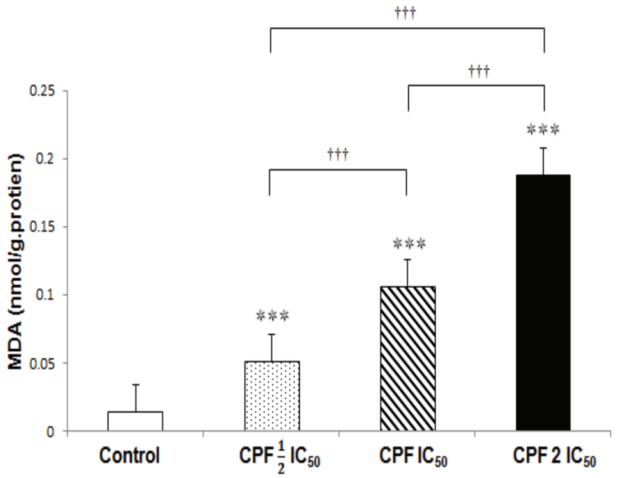

Figure 3. The effects of ciprofloxacin (CPF) treatment on level of malondialdehyde (MDA) of the A-172 cell line after 72 h. Values are represented by mean ± SD.; ***: P<0.001 compared to the control group; †††: P<0.001.

considered other CPF effects in cancer treatment.[29] Quinolones could probably reduce the mortality rate among cancer patients. Additional data have reported the use of FQs with chemotherapeutic agents in several human cancer cell lines; including head and neck, colon, prostate cancers and hepatocellular carcinoma.[46-49] In these experiments, CPF either sensitized multi-drug resistant cancer cells to chemotherapy or increased the efficacy of the chemotherapeutic agents.[47-49] In our previous study, we have reported the synergistic effects of the combination of CPF and temozolomide on the human glioblastoma A-172 cell line.[50]

We showed that CPF increased MDA levels and produced oxidative stress in the human glioblastoma A-172 cancer cell line. This drug showed cytotoxic and apoptotic effects. It increased Bax levels, increased the Bax/Bcl-2 ratio and decreased Bcl-2 levels in the cell line which supported findings from previous studies.

Conclusion

Ciprofloxacin can be used as an adjuvant to treat GBM by increasing apoptosis and ROS production in these cells.

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Conflict of interest

No conflict of interest is declared.

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