Original Article
Middle East Journal of Cancer; January 2017; 8(1): 31-38

The Synergistic Effects of the Combination of Ciprofloxacin and Temozolomide on Human Glioblastoma A-172 Cell Line

Ashkan Zandi*, Taraneh Moini Zanjani*, Seyed Ali Ziai*♦, Yalda Khazaei Poul*, Mostafa Haji Molla Hoseini**

*Department of Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
**Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Background: Combination therapy has generated remarkable motivation in the clinical setting since it boosts the therapeutic potential of anticancer drugs. Glioblastoma multiforme is the most common, aggressive malignant brain tumor which affects patients of all ages. This brain tumor is principally resistant to treatment.

Methods: In this study, we combined temozolomide as a standard chemotherapeutic drug for human glioblastoma multiforme, with ciprofloxacin in an attempt to determine whether ciprofloxacin could potentiate the cytotoxic effects of temozolomide. The glioblastoma A-172 cell line was exposed to ciprofloxacin and temozolomide either alone or in combination for 24, 48 and 72 h. Cytotoxicity was measured by the MTT assay.

Results: Ciprofloxacin and temozolomide induced tumor cell death in a dose-dependent manner with an IC_{50} value of 259.3 µM for ciprofloxacin and 62.5 µM for temozolomide at 72 h. These values shifted to 22.8 µM for ciprofloxacin and 8.6 µM for temozolomide in the presence of the IC_{50} of the other drug. The combination index values were <1.

Conclusion: These results showed synergism across a broad range of concentrations of ciprofloxacin and temozolomide in a glioblastoma tumor cell line. In this study, ciprofloxacin increased the anti-tumor cytotoxic effects of temozolomide in the glioblastoma A-172 cell line.

Keywords: Temozolomide, Ciprofloxacin, Glioblastoma A-172 cell line, Synergism, Cytotoxicity

♦Corresponding Author:
Seyed Ali Ziai, PharmD, PhD
Department of Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Koodakyar St., Daneshjoo Blvd., Velenjak, Tehran, Iran
Tel: +98-2122439969
Fax: +98-2122439969
Email: saziai@gmail.com, aliziai@sbmu.ac.ir

Introduction

Glioblastoma multiforme (GBM) is a highly lethal brain tumor (grade IV astrocytoma). The median survival

Received: July 15, 2016; Accepted: September 14, 2016

of GBM patients is only 12–15 months despite optimal treatment, which includes surgical resection along with radiation and temozolomide (TMZ)-based chemotherapy.[1] Among several difficulties of current standard treatment of GBM patients with incomplete tumor removal, the most important concerns include: peritumoral edema, blood-brain barrier (BBB) disruption, inadequacy of maximum radiation dose to destruct the tumor, the toxic side effects of chemo- or radiotherapy, and drug resistance. Temozolomide is the current standard of care for the treatment of GBM. According to reports, patients' survival can be prolonged for approximately 2 months when combined with surgery and radiation.[1,2] The mechanism of action of TMZ as an alkylating agent is based on its capacity to methylate DNA, which causes cellular cytotoxicity by forming O6-methylguanine adducts.[2] Unfortunately, GBM cells show resistance to TMZ which is mediated by a DNA repair protein, O6-methylguanine-DNA methyltransferase (MGMT) that removes the TMZ-generated DNA adduct.[3] Resistance to TMZ is a major barrier to cure in GBM patients. It has been reported that GBM patients with a methylated MGMT promoter have an increased total survival and better response to combined TMZ and radiation therapy in comparison with radiation itself.[4] Lack of MGMT expression is considered a good prognostic factor in TMZ-treated GBM patients.[5]

Ciprofloxacin (CPF) is a quinolone antibiotic used against many bacterial infections.[6] 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 they 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.[7] In addition to the antibacterial activity of CPF, evidences have proven a substantial anti-proliferative activity among a variety of cancer cells, such as prostate, bladder, colorectal, osteosarcoma and leukemic cancer cell lines.[8-12] Ciprofloxacin, as an anticancer agent, is used at concentrations higher than for treatment of infectious diseases.[13] At these concentrations (200-300 µg/ml), CPF can effectively induce apoptosis of bladder carcinoma cells and yield to cell cycle arrest in the S/G2 stage.[14] Based on previous researches, this study intends to examine whether the addition of CPF can improve the potency and efficacy of TMZ in the human glioblastoma A-172 cell line.

Materials and Methods

Drugs and reagents

Temozolomide, 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). Ciprofloxacin hydrochloride was purchased from (Daroupakhsh Pharmaceutical Co., Ltd., Tehran, Iran).

Cell culture

The human glioblastoma A-172 cell line (Avicenna Research Institute, Tehran, Iran) was cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 µg/ml). Temozolomide was freshly dissolved in DMSO. The final DMSO concentration was kept below 0.25% in the cell culture. At this concentration DMSO did not demonstrate any toxic effects on cell growth or viability. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were harvested and used at their exponential phase of growth in order to evaluate the cytotoxicity.

Cytotoxicity assay

The stock solutions used in this study consisted of 400 mM TMZ in DMSO and 2.5 mM CPF in cell culture medium. A-172 cells were washed three times with phosphate-buffered saline (PBS), after which 0.25% trypsin was applied to detach the cells, followed by centrifugation at 1500 rpm

for 10 min. The cells were re-suspended in cell culture medium, seeded (1×10^4cells/well) into 96-well plates and incubated in a humidified atmosphere of 5% CO_2 for 24 h to allow for cell adherence. In order to obtain the half maximal inhibitory concentrations (IC_{50}) we added CPF at concentrations of 2073.5, 1036.7, 518.3, 259.1, 129.5, 64.7, 32.3, and 16.1 µM and TMZ at final concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µM. The drugs were added in triplicate. After 24, 48 and 72 h we added 100 µl MTT (1 mg/ml) to each well, followed by an additional incubation period of 4 h. After the incubation time, we removed the cell culture medium and added 100 µl DMSO to solubilize the formazan crystals that formed during the MTT assay. The absorbance was measured by a microplate reader (ELX 800 Biotek, USA) at 530 nm.

Analysis of combined drug effects

Drug synergism was recognized by the isobologram and combination index (CI) methods, produced from the median effect basic principle of Chou-Talalay using CalcuSyn software (Biosoft, Ferguson, MO).[15] Data obtained from the growth inhibitory experiments were used to perform these analyses. The isobologram method is a graphical representation of the pharmacologic interaction to be shaped by choosing the desired fractional cell kill (Fa) and plotting the individual drug doses required to create the Fa on their respective x- and y-axes. A straight line is then drawn to connect the points. The observed dose combination of the two agents that achieve the particular Fa is then plotted on the isobologram. Combination data points which fall on the line represent additive drug-drug interactions, whereas data points that fall below or above the line show synergism or antagonism, respectively. The CI method is a mathematical and quantitative representation of a two-drug pharmacologic interaction. Using data from the growth inhibitory experiments and computerized software, we have generated CI values over a range of Fa levels from 0.25-0.97 (25%-97% growth inhibition). A CI of 1 indicates an additive effect between two agents, whereas a CI <1 or CI >1 shows synergism or antagonism, respectively.

Statistical analysis

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

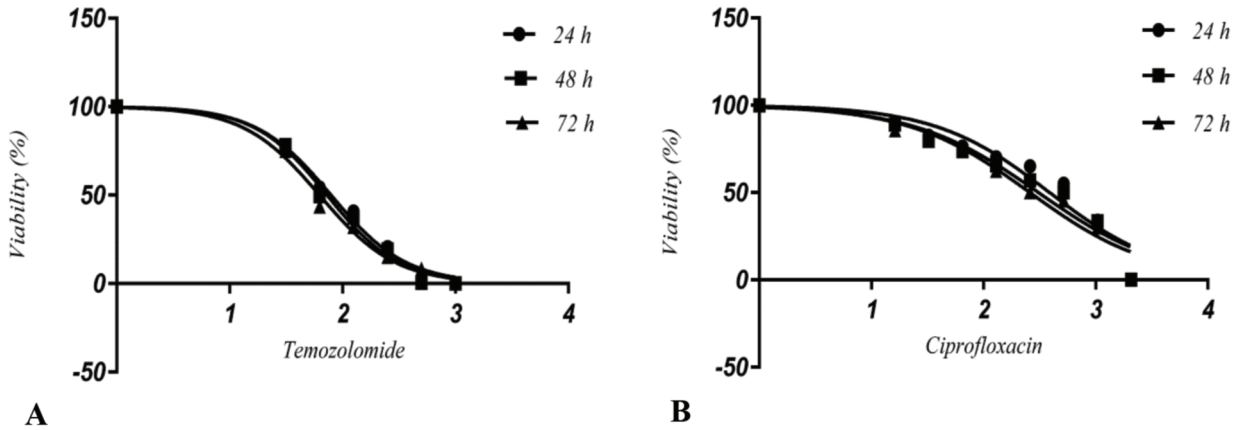

Figure 1. The effects of temozolomide (TMZ) and ciprofloxacin (CPF) treatment on proliferation of the A-172 cell line. Cells were grown in 96-well plates and treated with TMZ and CPF at different concentrations for 24, 48 and 72 h. "A" shows growth inhibition of different concentrations of TMZ. "B" indicates growth inhibition of distinct concentrations of CPF. Values represent mean±SD.

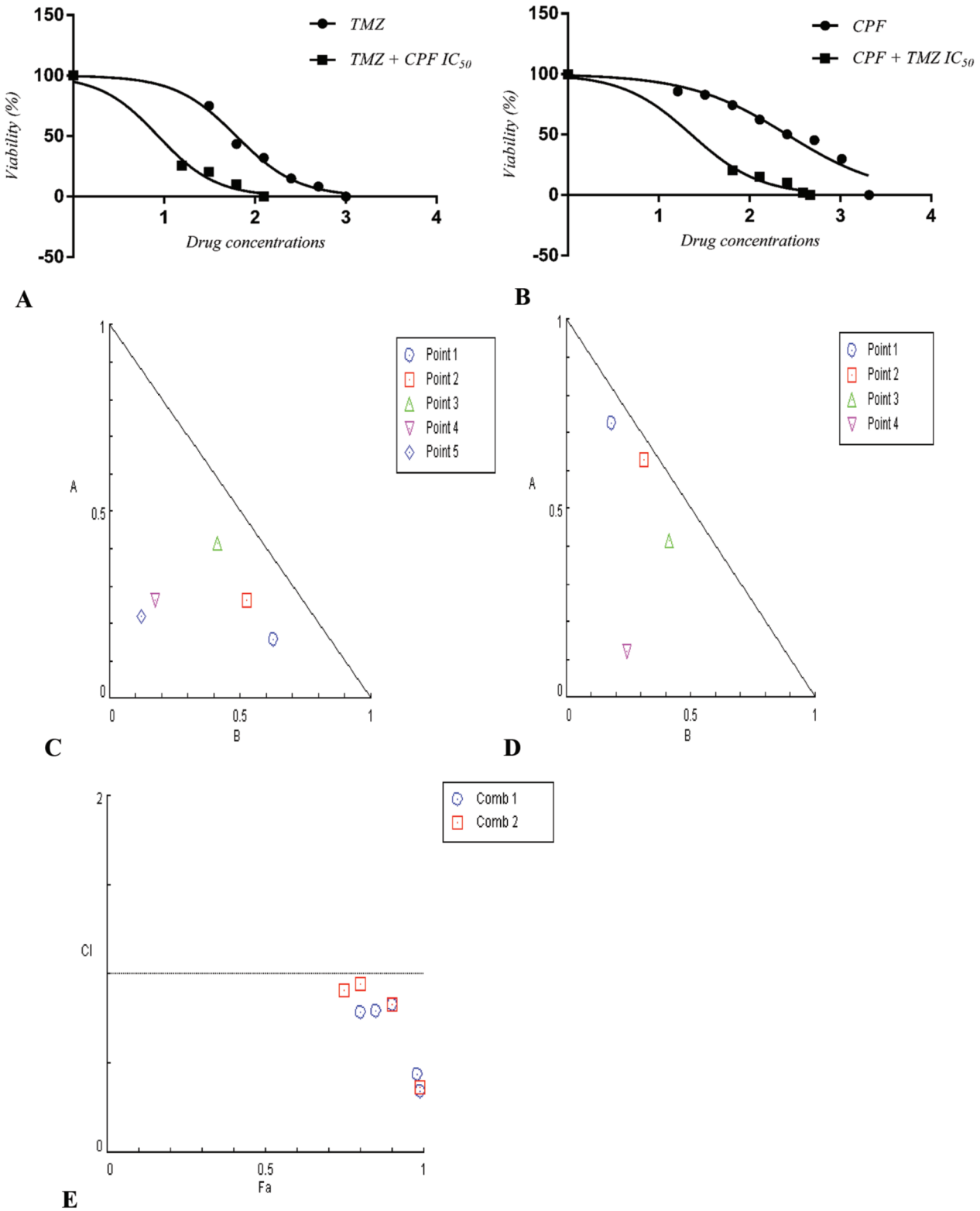

Figure 2. A comparison of the combined temozolomide (TMZ) and ciprofloxacin (CPF) treatment on proliferation of the A-172 cell line. Half maximal inhibitory concentration (IC_{50}) of TMZ concentrations-viability curve in the presence of CPF IC_{50} decreased from 62.5 µM to 8.6 µM (A), and the IC_{50} of CPF concentrations-viability curve in the presence of TMZ IC_{50} decreased from 259.3 µM to 22.8 µM (B). Isobolographic analysis of combined TMZ and CPF treatment on the A-172 cell line. Combination index (CI) plots obtained from median-effect analysis of Chou–Talalay,[15] respectively. CI <1 indicates synergistic effects (C-E). Points 1, 2, 3, 4, 5 are the drug combination effects.; Comb1 = CPF + TMZ IC_{50}; Comb2 = TMZ + CPF IC_{50}

Results

Cytotoxic effects of temozolomide (TMZ) treatment on the A-172 cell line

Temozolomide showed a significant dose-dependent cytotoxic effect on the viability of A-172 cells. We determined the IC_{50} value to be 62.5 µM at 72 h. Temozolomide at the highest dose (1000 µM) showed maximum cell death compared to the control following 72 h of exposure (P<0.05; Figure 1A).

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

Ciprofloxacin produced a dose-dependent, significant cell death after 72 h of exposure in the A-172 cell line. The IC_{50} value was found to be 259.3 µM at 72 h. We observed maximum cell death at the 2073.5 µM concentration compared to the control following 72 h of exposure (P<0.05; Figure 1B).

Cytotoxic effects of combined temozolomide (TMZ) and ciprofloxacin (CPF) on the A-172 cell line

The isobologram generated for Fa values of 0.25, 0.50, 0.75, 0.90 and 0.97 represented growth inhibition of CPF + TMZ IC_{50} and TMZ + CPF IC_{50} (Figure 2C,D).

The effects of different concentrations of TMZ combined with the CPF IC_{50} at 72 h significantly shifted the TMZ IC_{50} from 62.5 µM to 8.6 µM (P<0.001; Figure 2A) and resulted in CI values of 0.910, 0.943, 0.832 and 0.367 (Figure 2E).

Graded concentrations of CPF combined with the TMZ IC_{50} at 72 h significantly changed the CPF IC_{50} from 259.3 µM to 22.8 µM (P<0.001; Figure 2B) and resulted in CI values of 0.786, 0.789, 0.832, 0.438 and 0.343 (Figure 2E). These values indicated a strong synergistic effect on growth inhibition (Figure 2E).

Discussion

In this study, for the first time, we assessed the effects of CPF individually and in combination with TMZ on the human glioblastoma A-172 cancer cell line. We found cytotoxic and synergistic effects of CPF on growth inhibition.

Glioblastoma multiforme is the most common cancer of the central nervous system that affects patients of all ages. Being essentially resistant to treatment, the clinical outcome seems disappointing regardless of age at the time of diagnosis. The median survival of a patient with glioblastoma is 15 months, which has improved in the past four decades.[16] Common treatment measures include surgical resection and radiotherapy, most often with the use of nitrosourea-based chemotherapy. Recently, although the use of TMZ has emerged as a new standard of care in patients, unfortunately a modest improvement in median survival has been seen.[1] *In vitro* studies showed cytotoxic properties of CPF on other cancer cell lines such as a bladder carcinoma cell line, Jurkat T cell leukemia cell line, hormone-refractory prostate cancer cell lines (HRPC); including the PC-3 and LNCaP cell lines, two transitional cell carcinoma cell lines; MBT-2 and T24, sarcoma, osteosarcoma, colorectal carcinoma, and CHOAA8 ovarian cell lines.[6,17-19,8,20,21] Ciprofloxacin inhibited proliferation of cells, increased population doubling time and reduced saturation density of the cells.[22]

Ciprofloxacin inhibits mitochondrial topoisomerase II thus affecting cellular energy metabolism. Administration of 25 µg/ml of CPF inhibits the proliferation of Jurkat cells, while concentrations exceeding 80 µg/ml induce apoptosis. The inhibition of Jurkat cell proliferation by CPF has been seen in the G2/M-phase of the cell cycle which compromised the formation of mitotic spindle and induced aneuploidy.[6]

Ciprofloxacin displays anti-proliferative and apoptotic effects observed in malignant cells, but not normal cells. Fortunately, normal and non-tumorigenic cells are not targeted by CPF. In a study, PC-3 cells as well as normal prostate epithelial cells (MLC8891) were treated with 25 to 400 µg/ml CPF, and cell counting was performed 3 days after treatment. This study showed that CPF, as an FQ, induced anti-

proliferative and apoptosis activity on prostate cancer cells (PC-3) but not on non-tumorigenic prostate epithelial cells (MLC8891). This finding suggested that CPF could probably be used as a possible adjuvant for tumor therapy without any side effects on normal cells.[8]

Considering the previous relevant study, the data showed that CPF induced morphological changes in the GL26 murine glioma cell line. It was cytotoxic against GL26 murine glioma cells which decreased their viability and had dose and time-dependent cytotoxicity effects. They showed that GL26 cells lost their regular shape, size, and cell-cell contact after incubation with CPF. Many cells also lost their adherence. Most cells showed a rounded phenotype and were aggregated.[23]

Studies revealed that CPF has anti-proliferative and apoptotic activities in tumor cell lines which are mediated by cell cycle arrest at the S-G2/M phase. Bax translocation to mitochondrial membrane leads to the increase in the Bax/Bcl-2 ratio in some cancer cells such as PC-3 prostate cells.[8]

There are a number of different mechanisms involved in the beneficial effects of CPF according to its anti-microbial activity on cancer patients who undergo chemotherapy.[20] One of these mechanisms is immune modulation which protects patients against new infections by reducing cytokine production and improving their immune response to infections normally found in cancer patients.[24, 25] Another mechanism is based on the action of quinolones on hematopoiesis. In terms of 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 (shorten neutropenia), and increase survival of cyclophosphamide-treated mice which was similar to data obtained when granulocyte colony-stimulating factor was used in these mice.[20,26] Finally, the quinolone anti-tumor effect mediated by inhibition of mammalian DNA polymerase, topoisomerases I and II, are considered to be another CPF action in cancer treatment. Quinolones appear to probably reduce the mortality rate among cancer patients.[20]

Additional data reported the use of FQs with some chemotherapeutic agents in several human cancer cell lines, including head and neck cancers, colon cancer, prostate cancer and hepatocellular carcinoma.[27-30] In these experiments, CPF sensitized multi-drug resistant cancer cells to chemotherapy or increased the efficacy of the chemotherapeutic agents.[28-30]

In our research, we obtained synergistic cytotoxic effects when CPF was used in combination with TMZ in the glioblastoma A-172 cancer cell line, which correlated with previous studies.

Conclusion

Ciprofloxacin exhibited cytotoxic properties and enhanced the inhibitory effects of TMZ on the cell viability of human glioblastoma A-172 cells. Our data indicated a strong inhibitory effect of CPF individually and in combination with TMZ on the proliferation of A-172 cells.

We suggest that, in relation to the anti-proliferative features of CPF, this drug can be a novel adjuvant administered in a therapeutic regimen in clinical trials relevant to the curative strategy in human GBM and other cancers. The mechanisms underlying these effects are not well understood and further investigations are needed to assess the exact mechanism of CPF in tumor cells.

Acknowledgement

The authors would like to express their appreciation to Mr. Hadi Kalantar and Ms. Shiva Ghafghazi for their kind collaboration. This article was extracted from a thesis written by Mr. Ashkan Zandi, PhD candidate of Pharmacology, Shahid Beheshti University of Medical Sciences (Registration No.165).

The present article is financially supported by the Research Department of the School of Medicine, Shahid Beheshti University of Medical Sciences (Grant No. 6132).

Conflict of Interest

No conflict of interest is declared.

References

1. Stupp R, Mason WP, Van Den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med.* 2005;352(10):987-96.
2. Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, et al. A randomized trial of bevacizumab for newly diagnosed glioblastoma. *N Engl J Med.* 2014;370(8):699-708.
3. Ochs K, Kaina B. Apoptosis induced by DNA damage O6-methylguanine is Bcl-2 and caspase-9/3 regulated and Fas/caspase-8 independent. *Cancer Res.* 2000;60(20):5815-24.
4. Hegi ME, Diserens AC, Gorlia T, Hamou MF, De Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* 2005;352(10):997-1003.
5. Hegi ME, Liu L, Herman JG, Stupp R, Wick W, Weller M, et al. Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. *J Clin Oncol.* 2008;26(25):4189-99.
6. Koziel R, Szczepanowska J, Magalska A, Piwocka K, Duszynski J, Zablocki K. Ciprofloxacin inhibits proliferation and promotes generation of aneuploidy in Jurkat cells. *J Physiol Pharmcol.* 2010;61(2):233-9.
7. Burden DA, Osheroff N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. *Biochem Biophys Acta*. 1998;1400(1-3):139-54.
8. Aranha O, Grignon R, Fernandes N, McDonnell TJ, Wood DP Jr, Sarkar FH. Suppression of human prostate cancer cell growth by ciprofloxacin is associated with cell cycle arrest and apoptosis. *Int J Oncol.* 2003;22(4):787-94.
9. Aranha O, Zhu L, Alhasan S, Wood DP Jr, Kuo TH, Sarkar FH. Role of mitochondria in ciprofloxacin induced apoptosis in bladder cancer cells. *J Urol.* 2002;167(3):1288-94.
10. Herold C, Ocker M, Ganslmayer M, Gerauer H, Hahn EG, Schuppan D. Ciprofloxacin induces apoptosis and inhibits proliferation of human colorectal carcinoma cells. *Br J Cancer.* 2002;86(3):443-8.
11. Miclau T, Edin ML, Lester GE, Lindsey RW, Dahners LE. Effect of ciprofloxacin on the proliferation of osteoblast-like MG-63 human osteosarcoma cells *in vitro*. *J Orthop Res*. 1998;16(4):509-12.
12. Somekh E, Douer D, Shaked N, Rubinstein E. *In vitro* effects of ciprofloxacin and pefloxacin on growth of normal human hematopoietic progenitor cells and on leukemic cell lines. *J Pharmacol Exp Ther.* 1989; 248(1):415-8.
13. Smart DJ, Halicka HD, Traganos F, Darzynkiewicz Z, Williams GM. Ciprofloxacin-induced G2 arrest and apoptosis in TH6 lymphoblastoid cells is not dependent on DNA double-strand break formation. *Cancer Biol Ther.* 2008;7(1):113-9.
14. Aranha O, Wood DP Jr, Sarkar FH. Ciprofloxacin mediated cell growth inhibition, S/G2-M cell cycle arrest and apoptosis in a human transitional cell carcinoma of the bladder cell line. *Clin Cancer Res.* 2000;6(3):891-900.
15. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul.* 1984;22:27-55.
16. Mrugala MM, Chamberlain MC. Mechanisms of disease: temozolomide and glioblastoma-look to the future. *Nat Clin Pract Oncol.* 2008;5(8):476-86.
17. Zehavi-Willner T, Shalit I. The inhibitory effect of ciprofloxacin on proliferation of a murine bladder carcinoma cell line. *J Antimicrobial Chemother.* 1991;29(3):323-8.
18. Pinto AC, Moreira JN, Simões S. Ciprofloxacin sensitizes hormone-refractory prostate cancer cell lines to doxorubicin and docetaxel treatment on a schedule-dependent manner. *Cancer Chemother Pharmacol.* 2009;64(3):445-54.
19. Ebisuno S, Inagaki T, Kohjimoto Y, Ohkawa T. The cytotoxic effects of fleroxacin and ciprofloxacin on transitional cell carcinoma *in Vitro*. *Cancer.* 1997;80(12):2263-7.
20. Paul M, Gafter-Gvili A, Fraser A, Leibovici L. The anti-cancer effects of quinolone antibiotics. *Eur J Clin Microbiol Infect Dis.* 2007;26(11):825-31.
21. Kloskowski T, Olkowska J, Nazlica A, Drewa T. The influence of ciprofloxacin on hamster ovarian cancer cell line CHO AA8. *Acta Pol Pharm.* 2010;67(4):345-9.
22. Mondal ER, Das SK, Mukherjee P. Comparative evaluation of antiproliferative activity and induction of apoptosis by some fluoroquinolones with a human non-small cell lung cancer cell line in culture. *Asian Pac J Cancer Prev.* 2004;5(2):196-204.
23. Esmaeilzadeh A, Ebtekar M, Biglari A, Mohammad Hassan Z. Influence of ciprofloxacin on glioma cell line GL26: A new application for an old antibiotic. *Afric J Microb Res.* 2012;6(23):4891-6.
24. Dalhoff A, Shalit I. Immunomodulatory effects of quinolones. *Lancet Infect Dis*. 2003;3(6):359-71.
25. Dalhoff A. Immunomodulatory activities of fluoroquinolones. *Infection.* 2005;33 Suppl 2:55-70.
26. Riesbeck K, Forsgren A. Selective enhancement of synthesis of interleukin-2 in lymphocytes in the presence of ciprofloxacin. *Eur J Clin Microbiol Infect Dis.* 1990;9(6):409-13.
27. Norris MD, Madafiglio J, Gilbert J, Marshall GM,

Haber M. Reversal of multidrug resistance-associated protein-mediated drug resistance in cultured human neuroblastoma cells by the quinolone antibiotic difloxacin. *Med Pediatr Oncol.* 2001;36(1):177-80.
28. Herold C, Ganslmayer M, Ocker M, Blauberger S, Zopf S, Hahn EG, et al. Over additive anti-proliferative and pro-apoptotic effects of a combination therapy on colorectal carcinoma cells. *Int J Oncol.* 2003;23(3):751-6.
29. El-Rayes BF, Grignon R, Aslam N, Aranha O, Sarkar FH. Ciprofloxacin inhibits cell growth and synergises the effect of etoposide in hormone resistant prostate cancer cells. *Int J Oncol.* 2002;21(1):207-11.
30. FuY, Zhou S, Li D, Zhang Y, Li S, Li C. Ciprofloxacin inhibits proliferation and synergistic effect against hepatocellular carcinoma cancer lines with cisplatin. *Afric J Pharmacy and Pharmacol.* 2013;7(26):1793-801.