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Full Length Research Paper

Influence of ciprofloxacin on glioma cell line GL26: A new application for an old antibiotic

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Ciprofloxacin HCL is a fluoroqinolone antibiotic, effective against a wide range of Gram positive and Gram negative bacteria, used mainly in the treatment of urinary tract infections, pulmonary tract, prostate gland, bone and bone marrow infections. Ciprofloxacin is also known for its *in vitro* anticancer properties. Several reports describe anti-proliferative and cytotoxic effects, apoptotic activity and tumour regression with various quinolones. In this study, murine glioma cell line GL26 has been used, for evaluation of cytotoxic properties of ciprofloxacin, against neoplastic cells. For this purpose, we used different concentrations of ciprofloxacin range from 10 to 600 µg/ml. Cell viability was counted using trypan blue assay. Ciprofloxacin induced morphological changes and decreased viability of GL26 cells in the concentration and time dependent manner. In low concentrations, a weak cytotoxic effect of ciprofloxacin was observed only after 24 h incubation. Observations indicated that in the highest concentration of ciprofloxacin, after 24, 48 and 72 h of incubation only a very small number of living cells (2%) remained. We can conclude that since ciprofloxacin displays anti-proliferative characteristics *in vitro*, this drug may operate as a novel treatment in glioma mouse model, and in the next step could lead to the design of a new clinical trials concerning this remedial strategy and its outcome in human glioma.

Key words: Ciprofloxacin, glioma, GL26 cell line, *in vitro*, cell proliferation.

INTRODUCTION

Ciprofloxacin is an antibiotic agent that belongs to Fluoroquinolones. This group of antibiotics has a broad spectrum of action against pathogens, especially Gram (-) aerobic bacilli, via inhibition of bacterial gyrase (Nelson et al., 2007; Kloskowski et al., 2010). Ciprofloxacin also inhibits topoisomerase II in eukaryotic, including mammalian cells (Kloskowski et al., 2010; Gootz et al., 1990). Ciprofloxacin is also known for its anticancer properties enabling cell cycle arrest and creating doublestrand breaks in nucleic acid, which triggers apoptosis of cancer cells *in vitro* (Kloskowski et al., 2010). Ciprofloxacin, inhibits activity of the topoisomerase II in high concentrations (Gurtowska et al., 2010), low concentrations do not always lead to the enzyme inhibition but cell proliferation is stopped. Therefore, there should be additional mechanisms responsible for ciprofloxacin cytotoxicity, which can be connected with its activity at the cell membrane and mitochondria levels (Holtom et al., 2000).

Ciprofloxacin has the ability to inhibit cell proliferation via mitochondrial DNA damage (Lawrence et al., 1993; Enzmann et al., 1999), and reacts with the mitochondrial topoisomerase II isoform. Due to this property, ciprofloxacin is able to inhibit mtDNA synthesis with mitochondrial damage, which leads to the reduction of

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the intracellular ATP (Kloskowski et al., 2010). Ciprofloxacin-induced cytotoxicity in cells may be a result of free radicals generation (Kloskowski et al., 2010).

According to *in vitro* and *in vivo* studies, ciprofloxacin phototoxicity during UVA irradiation was caused by arising reactive oxygen species such as hydrogen peroxide and hydroxylic radicals (Agrawal et al., 2007; Kloskowski et al., 2010). Ciprofloxacin induced oxidative stress in human fibroblasts and in cerebral and hepatic tissues of rats *in vivo* (Gurbay and Hincal, 2004; Kloskowski et al., 2010).

Glioblastoma Multiforme (GBM) is the most common primary and aggressive malignant brain tumor (Shabason et al., 2011). Conventional treatments have been largely ineffective and consist of surgical resections, followed by radiation and/or chemotherapy, radio surgery, corticosteroids, anti angiogenic therapies (Mamelak et al., 2007; Shelton et al., 2010). GBM has a poor outcome due to its invasive and aggressive nature (Shelton et al., 2010). Survival of patients affected by GBM has remained virtually unchanged during the last decades (that is, 6 to 12 months post-diagnosis) (Castro et al., 2003; Curtin et al., 2005).

The study of tumorigenesis and evaluation of new therapies for GBM requires accurate and reproducible brain tumor animal models, which ideally should recapitulate key features of the human disease, be reproducible and resemble progression kinetics and anti-tumor immune responses of spontaneous GBM (Maher et al., 2001; Kleihues et al., 1970).

Rodent glioma models, have been used in preclinical glioma research for over 30 years (Condolfi et al., 2007). *In vivo* tumor models for intracranial or subcutaneous implantation of glioma cell lines in rodents are widely used to test novel therapies that target different features of GBM, that is, angiogenesis, invasion, located within an immune privilege site (brain), secretion of immune-suppressive molecules, that is, TGF-*β*, amongst many others (Curtin et al., 2005; Castro et al., 2006; Fecci et al., 2003; Rainov and Ren, 2003). The advantages of these glioma models, are their highly efficient gliomagenesis, reproducible growth rates, and accurate knowledge of the location of the tumor (King et al., 2005). Human glioma xenografts implanted in immunocompromised mice are also extensively used. However, their xenogeneic nature impairs the study of immunemediated anti-tumor strategies. Syngeneic murine models, such as GL26 mouse glioma cells in C57BL6 mice are non-immunogenic. Thus, syngeneic glioma models are excellent for studying the response of brain tumors to immunotherapy (Condolfi et al., 2007; Kim et al., 2006). GL26 mouse glioma cell line is non-immunogenic, when injected in the brain syngeneic C57BL6 mice. This model is very useful in assessing immunotherapeutic approaches. For this reason, we have selected the Gl26 cell line for our study.

Since, the effect of ciprofloxacin on Gl26 glioma cell line remains unknown, we have undertaken this study to shed light on the matter.

MATERIALS AND METHODS

Cell line and cell culture

The GL26 murine glioma cell line was kindly provided by Dr. M Soleimani (Department of Stem Cells and Tissue Engineering, Stem Cell Technology Institute, Tehran, Iran.) The GL26 cells were cultured in Dulbecco modified Eagle medium (Gibco BRL Co., Grand Island, NY) supplemented with 10% heated-inactivated fetal bovine serum (FBS; Gibco), 2 mML-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in 5% CO_2 atmosphere and were used for assay, during the exponential phase of growth. GL26 cells were passaged routinely every 2 to 3 days.

Ciprofloxacin HCL

Stock solution of ciprofloxacin (Bayer Corporation) was 200 mg/ml. final concentrations of 10-600 µg/ml were produced by diluting in complete media prepared for GL26 cell lines.

Proliferation assay

The GL26 cells were seeded on 24-well plates, at density of 40000 cells on well. The plates were incubated overnight at 37°C. Cells were allowed to adhere for 24 h and were then exposed to ciprofloxacin. GL26 cells were exposed to various concentrations of ciprofloxacin (10, 60, 100, 200, 300, and 600 µg/ml). Cells were exposed to ciprofloxacin through 24, 48, 72, and 96 h. After each time of incubation, medium with antibiotic was removed from wells, each well was flushed with 0.5 ml sterile PBS and then cells were detached from wells using 0.5 ml 0.25% trypsin. After centrifugation at 1800 rpm for 5 min, cells were suspended in 1 ml of medium. Viability was based on the presence of living cells. After trypsinization and centrifugation of cells, 50 µL of the cells solution was taken and mixed with the same volume of trypan blue. Trypan blue dye is commonly used to estimate cell viability.

In assessing cell viability cell counting after 2 min incubation for fixation without any stain can be employed. Using trypan blue assay we were able to count all the cells from the sample, that is, cells growing in monolayer and detached cells as well. MTT assay -a method to examine cell proliferation within monolayers- does not apply when detached cells have to be taken into account (Kloskowski et al., 2010). Also, we used trypan blue assay, because we focused more on the examination of cell viability, than cell proliferation. Cells were counted in improved Neubauer chamber under the inverted microscope at 100x magnification. Application of the dye enabled us, to distinguish living cells from the dead cells. The number of living cells was calculated using the formula the average number of cells per square (total cells counted of squares used) multiplied by 10000 and the dilution factor to cells per ml.

Cytotoxicity assay

The cytotoxicity of ciprofloxacin against Glioma cell line was established on the basis of a comparison of viability of the cells in various concentrations of ciprofloxacin to lifetime of cells derived from control and calculated from the formula:

$$X = Lc/Lk \times 100\%$$

where X = cytotoxicity of ciprofloxacin, Lc = the average number of cells in the test sample, Lk = the average number of cells in control (Kloskowski et al., 2010). Each result was calculated from three independent measurements.

Analysis of data

The data are expressed as means ± standard deviations. The means were compared using *t*-Student test to detect statistically significant differences (P: 0.05) between treatment groups. All the figures shown in this article were obtained from at least three independent experiments. Values of $p < 0.05$ were considered significant.

Morphological study

GL26 cells were seeded (4×10^4 cells/well) in 24 well plates (Biofil) and after overnight culture, cells were exposed on various concentrations of ciprofloxacin (10, 60, 100, 200, 300 and 600 µg/ml) and visualized under inverted microscope (Nikon, Eclipse, Ti). Each experiment was conducted in triplicate and repeated three times.

RESULTS

Our results showed that ciprofloxacin HCL, induced morphological changes in GL26 cell lines (Figure 1). Ciprofloxacin is cytotoxic against GL26 cells and decreases their viability in vitro situations. Ciprofloxacin presented dose–depended and time–depended cytotoxicity (Figure 2). Gl26 cells without ciprofloxacin were used as the control. We showed that after incubation with ciprofloxacin, GL26 cells lost their regular shape, size and cell - cell contact. Many cells also lost their attachment to surface of plate, most cells were rounded in appearance and were aggregated.

In standard dose (10 µg/ml) and low concentration up to 60 µg/ml. Cytotoxic effect of ciprofloxacin is very weak after 24 h incubation. In the concentration of 100 µg/ml, viability of GL26 glioma cells decreased after 24 h of incubation time. In the highest concentration of ciprofloxacin, after 24 h incubation, only a very small number of living cells (2%) were observed.

DISCUSSION

This study proved that Ciprofloxacin HCL induces morphological alterations in GL26 cell lines. As we mentioned Ciprofloxacin is cytotoxic agent, against GL26 cells and reduced their viability in, *in vitro* systems. Ciprofloxacin demonsterates dose–dependent and time–dependent cytotoxicity. We are reporting for the first time on the influence of Ciprofloxacin HCL on viability and morphological changes of GL26 murine glioma cell lines.

This work presents the results of an experimental study of *in vitro* effects of ciprofloxacin on the GL26 glioma cancer cell line. The results of this study are similar to those, published by Kloskowski et al. (2010) in the hamster ovarian cancer cell line CHOAA8. In Kloskowski`s study they used different concentrations of ciprofloxacin in the range from 10 to 1000 µg/ml. cell viability (trypan blue) and morphological changes were evaluated. They showed that after incubation with ciprofloxacin CHO AA8 cells lost their regular shape, size and cell-cell contact. Many cells lost their attachment to surface of plate, a majority of cells were rounded in appearance, this subject suggested that the cell death process was going on. Similar results were reported by Mondal et al. (2004) on the NCI-H460 lung cancer cell line. In the study of Mondal et al. (2004) ciprofloxacin was used in concentration of 40 µg/ml through 24, 48, 72 and 96 h incubation times. They showed a decrease in viability correlating with dose of ciprofloxacin and incubation time.

According to Mondal et al. (2004) ciprofloxacin induced programmed cell death, inhibited proliferation of cells, increased population doubling time and reduced saturation density. In the present experiment, authors observed similar morphological changes, which occurred after ciprofloxacin exposure. Further studies are required to show whether similar mechanisms as mentioned in the Mondal work are involved as well.

In vitro studies shows cytotoxic properties on other cancer cell lines. Ciprofloxacin was tested for its effect on proliferation of a bladder carcinoma cell line, Jurkat Tcell leukaemia cell line and a normal human foreskin derived from a fibroblast cell line (Zehavi-Willner and Shalit, 1991). Hormone-refractory prostate cancer cell lines (HRPC) for for example PC-3 and LNCaP cell lines (Pinto et al., 2009), Two transitional cell carcinoma cell lines, MBT-2 and T24 (Ebisuno et al., 1997), PC3 cells and normal prostate epithelial cells (MLC8891) (Aranha et al., 2003) and transitional cell carcinoma, sarcoma, osteosarcoma, colorectal carcinoma (Paul et al., 2007), CHO AA8 ovarian cell lines (Kloskowski et al., 2010) and human lymphoidal cells (Jurkat cell line) (Koziel et al., 2010). In study of Koziel et al. (2010) they showed that, ciprofloxacin inhibits mitochondrial topoisomerase II and therefore affects cellular energy metabolism. They reported that concentrations exceeding 80 µg/ml ciprofloxacin induce apoptosis, while in 25 µg/ml, it inhibits proliferation of Jurkat cells without any symptoms of cell death. Exposition of Jurkat cells to ciprofloxacin inhibited cell proliferation, increased proportion of cells in the G2/M-phase of the cell cycle, compromised formation of the mitotic spindle and induced aneuploidy (Koziel et al 2010).

All studies showed that ciprofloxacin induced antiproliferative and apoptosis activity in tumor cell lines, antiproliferative effects of ciprofloxacin are confirmed by our study, which shows significant *in vitro* cell growth inhibition of GL26 cell lines.

Normal and non-tumorigenic cells are not targeted by

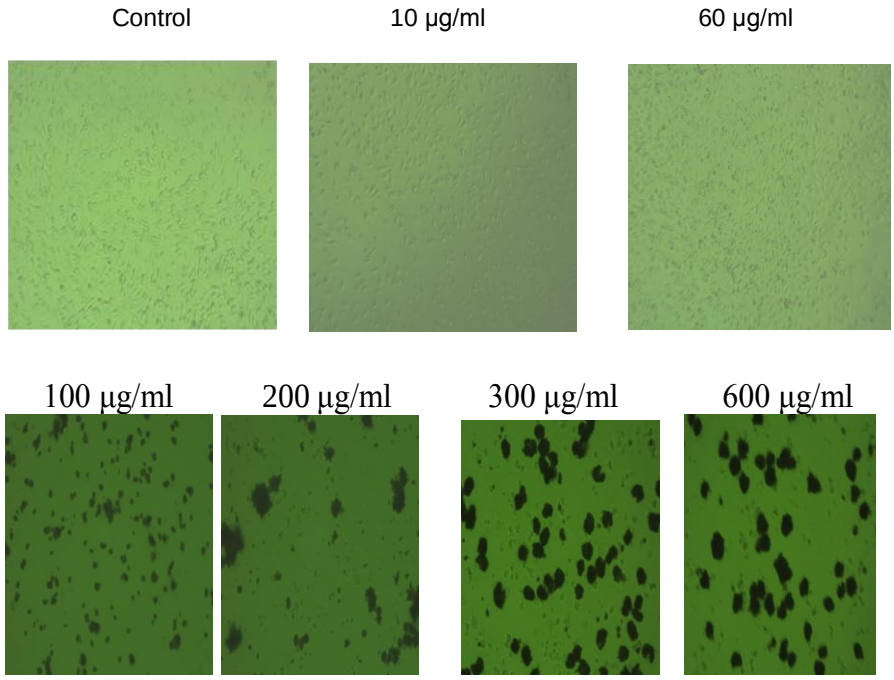

Figure 1. Morphological changes after ciprofloxacin incubation.

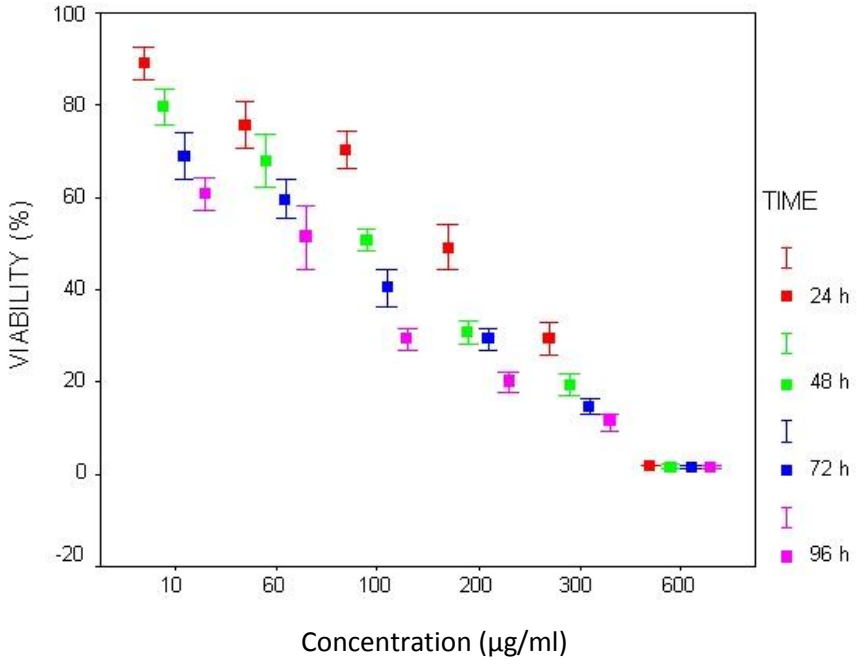

Figure 2. Viability of GL26 cell lines after treatment with ciprofloxacin.

ciprofloxacin. In the study of Aranha and et al. (2003) PC3 cells as well as normal prostate epithelial cells (MLC8891) were treated with 25 to 400 µg/ml ciprofloxacin, and cell counting was done during 3 days after treatment. This study showed that the fluroquinolone ciprofloxacin induced anti-proliferative and apoptosis activity on prostate cancer cells (PC-3) but not on non-tumorigenic prostate epithelial cells (MLC8891). These effects of ciprofloxacin were mediated by cell cycle arrest at S-G2/M phase of the cell cycle, Bax translocation to

mitochondrial membrane and by increasing the Bax/Bcl-2 ratio in PC3 prostate cancer cells (Aranha et al., 2003).

Results of the research of Tomasz (Kloskowski et al., 2010) and Aranha et al., (2003) also showed that ciprofloxacin displays anti-proliferative and apoptosis inducing activity only on malignant cells but not in normal cells. This scientific findings, support this point that, ciprofloxacin could be tested as a possible neoadjuvant for tumor therapy without any side effect on normal cells. Three broad mechanisms may underlie the benefits afforded by ciprofloxacin to cancer patients undergoing chemotherapy in addition to direct anti-bacterial activity (Paul et al., 2007). The first is immune modulation resulting in protection of the host against new infections, reduced cytokine production and improved immune response to infection (Dalhoff and Shalit, 2003; Dalhoff, 2005).

The second is an effect of quinolones on haematopoiesis. Quinolones by means of a cyclopropyl moiety at position N1 (for example ciprofloxacin) have been shown to increase production of colony-stimulating factors (Paul et al., 2007; Riesbeck et al., 1990), increased myeloid progenitors, white blood cell counts, haemoglobin and survival of sub-lethally irradiated mice and to shorten neutropaenia duration in cyclophosphamide-treated mice similar to that obtained with granulocyte colony-stimulating factor (Paul et al., 2007).

The third is a direct quinolone antitumor effect, mediated possibly by inhibition of mammalian topoisomerase I, topoisomerase II and DNA polymerase. Several reports described anti-proliferative and cytotoxic effects, apoptotic activity and tumour regression with various quinolones (Paul et al., 2007). Thus, *in vitro* and *in vivo* studies, corroborate possible immune-enhancing and anti-proliferative effects of quinolone antibiotics, supporting this fact, that quinolones reduce all-cause mortality among cancer patients. Further research on the glioma model can clarify the interaction of the drug with the blood brain barrier as well.

We can conclude that since ciprofloxacin displays anti-proliferative characteristics *in vitro*, this drug may operate as a novel treatment in glioma mouse model, and in the next step could lead to the design of a new clinical trials concerning this remedial strategy and its outcome in human glioma.

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