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DRUG BIOCHEMISTRY

THE INFLUENCE OF CIPROFLOXACIN ON VIABILITY OF A549, HepG2, A375.S2, B16 AND C6 CELL LINES *IN VITRO*

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Abstract: Ciprofloxacin is a chemotherapeutic agent mainly used in the treatment of the pulmonary and urinary tract infections but is also known for its anticancer properties. The aim of these study was to check the anticancer effect of ciprofloxacin on selected five cell lines. Human non-small cell lung cancer line A549, human hepatocellular carcinoma line HepG2, human and mouse melanoma lines (A375.S2 and B16) and rat glioblastoma line C6 were used for evaluation of cytotoxic properties of ciprofloxacin (in concentration range: 10–1000 μg/mL). Viability was established using trypan blue assay and MTT.
Ciprofloxacin induced morphological changes and decreased viability of A549 cells in a concentration and time dependent manner. In case of A375.S2 and B16 cell lines, cytotoxicyty of ciprofloxacin was observed but we were not able to eradicate all cells from A375.S2 and B16 cultures. HepG2 line was sensitive to ciprofloxacin, but this effect was independent from concentration and incubation time. The C6 cells were insensitive to ciprofloxacin. Our results showed that ciprofloxacin can be potentially used for the experimental adjunctive therapy of lung cancer.

Keywords: ciprofloxacin, *in vitro* cytotoxiciy, lung cancer, hepatic cancer, melanoma, glioblastoma

Ciprofloxacin belongs to fluoroquinolones, the second group of synthetic quinolones. Ciprofloxacin has 6-fluoro-7-piperazinyl group, which is responsible for its antibacterial effectiveness (1). Ciprofloxacin is relatively non toxic, with broad spectrum especially to Gram (–) aerobic bacilli (2). Its bactericidal action depends on the inhibition of the bacterial DNA topoisomerase II. Ciprofloxacin is used for the treatment of wide variety of bacterial strains, like *Heamophilus influenzae*, *Salmonella sp.*, *Shigella sp.*, *Neisseria gonorrhoeae*, *Staphylococcus aureus* and *Escherichia coli* (3). The highest concentrations of ciprofloxacin can be obtained in the lung tissue, prostate gland and urine. These concentrations are several times higher than in serum (4, 5). Ciprofloxacin also inhibits topoisomerase II in eukaryotic, including mammalian cells (6, 7). Ciprofloxacin is also known for its anticancer properties enabling cell cycle arrest and creating double-strand breaks in nucleic acid, which triggers apoptosis of cancer cells (8, 9). There are many reasons indicating that ciprofloxacin can by used in adjuvant therapy of certain cancers (10, 11).

This paper is a continuation of a previous study on hamster ovarian cancer line CHO AA8 (12). In this study we examined influence of ciprofloxacin on viability of five cancer cell lines: human non-small cell lung cancer (A549), human hepatocellular carcinoma (HepG2), human and mouse melanoma (A375.S2 & B16), and rat glioblastoma (C6).

EXPERIMENTAL

Cell lines

The A549, HepG2, C6 and A375.S2 cell lines were purchased from ATCC. The B16 cell line was established from B16 tumors excised from C57BL/6J mouse. The A549, A375.S2, B16 and C6 lines were cultured in DMEM/HAM'S F-12 medium containing 10% of fetal bovine serum (FBS), supplemented with 5 μg/mL of amphotericin B, 100 μg/mL of streptomycin and 100 U/mL of penicillin. Medium for HepG2 was additionally supplemented with 1% of MEM (Minimal Essential Medium). All cell lines were grown in plastic tissue culture T-flasks 25 cm^2 (Nunc) at 36^oC and 5% CO_2.

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Drug

Stock solution of ciprofloxacin was 10 mg/mL (Polfa Warszawa, Poland). Final concentrations of 25–1000 μg/mL were produced by diluting initial concentration in complete growth medium.

Trypan blue assay

Viability was based on the presence of living cells. Cells were seeded on 24-well plates at a density of 5×10^4 cells on well and allowed to adhere for 24 h. Then, cells were exposed to ciprofloxacin. The A549, HepG2, A375.S2, B16 and C6 cells at various concentrations (10, 25, 50, 100, 200, 500, 800, and 1000 μg/mL) were exposed to ciprofloxacin for 24, 48, 72, and 96 h. After each time of incubation, medium with ciprofloxacin was removed from wells, each well was flushed with 0.5 mL PBS and then cells were detached from wells using 0.5 mL of 0.05% trypsin. After centrifugation at 300 × g for 5 min cells were suspended in 1 mL of medium. Then, 50 μL of the cell suspension was taken and mixed-up with the same volume of trypan blue. Cells were counted by two independent researchers in Neubauer chamber under the inverted microscope at 100× magnification. Application of the dye allowed to distinguish living from dead cells. The number of living cells was calculated using the formula:

$$\frac{A}{4}\times 2\times 10^4\times B$$

where A = number of cells counted in Neubauer chamber at 100× magnification, B = mL of volume of cell suspension.

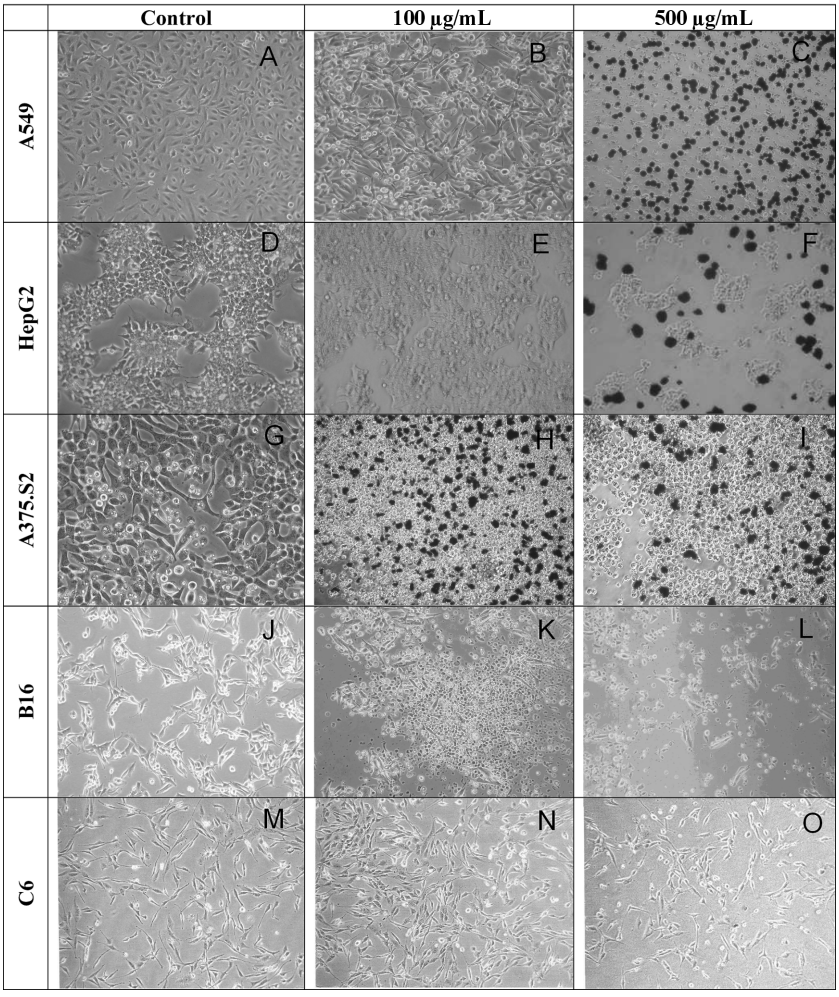

Figure 1. Morphology of cell lines: A549, HepG2, A375.S2, B16 andC6, light microscope at 100× magnification

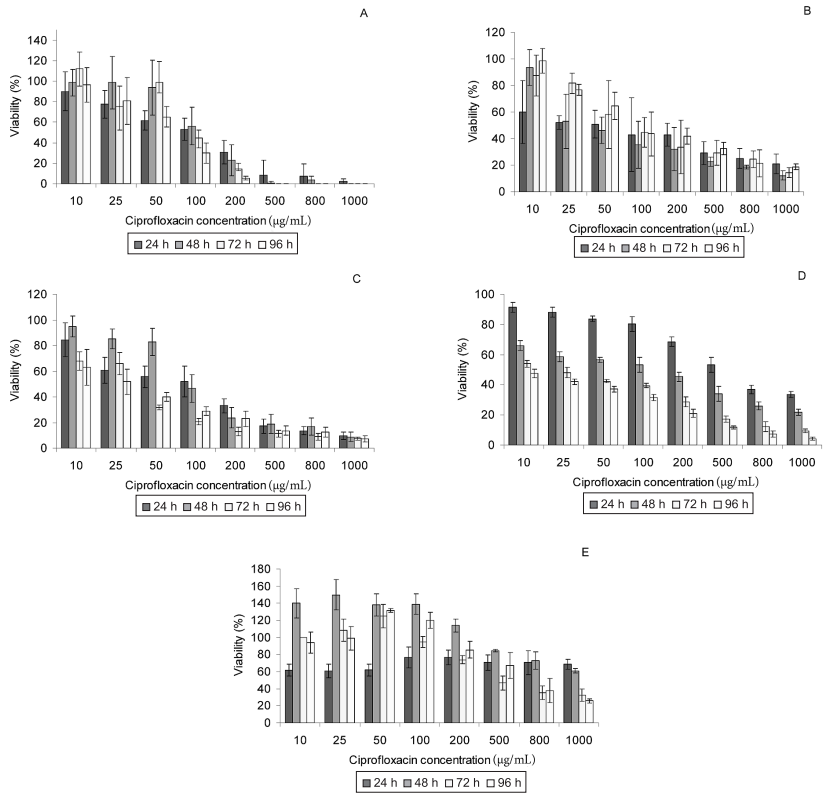

Figure 2. Viability of cells after treatment with ciprofloxacin, A – A549, B – HepG2, C – A375.S2, D – B16, E–C6

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

$$X = \frac{L_c}{L_k} \times 100\%$$

where X = cytotoxicity of ciprofloxacin, L_c = the average number of cells in the test sample, L_k = the average number of cells in control.

Each result was calculated from three independent measurements. Results were presented as the means ± standard deviations. The means were compared using Studnent *t*-test. Values of p lower than 0.05 were considered significant. The values of lethal concentration: LC_{10}, LC_{50}, LC_{90} were counted.

MTT assay

Viability was based on the presence of living cells. Cells were seeded on 24-well plates at a density of 5×10^4 cells per well. Cells were allowed to adhere for 24 h and were then exposed to ciprofloxacin in concentrations corresponding to LC values calculated earlier. After each incubation time, the medium was removed from wells and MTT solution was added. Cells were incubated with MTT for 3 h. The formazan crystals created after incubation were dissolved in DMSO (dimethyl sulfoxide). Absorbances of obtained colored solutions were measured under 570 nm wavelength (Cecil CE 2021, Cambridge, England). Each result was calculated from three independent measurements. The results of viability were presented in percentage compared to control (100%). The results were presented as the means ± standard deviations.

RESULTS

Morphological changes after incubation with ciprofloxacin

All cancer cell lines in the control had a regular shape and size (Fig. 1 A, D, G, J, M). The cells in A549, A375.S2, and B16 lines were elongated and spindle-shaped. Cells tightly covered wells area with numerous cytoplasmic lamellipodia (Fig. 1 A, G, J). HepG2 cells have grown in clusters and had a

regular hexagonal shape (Fig. 1 D). The C6 cells have the characteristic shape resembling neuronal-like phenotype (Fig. 1 M). After incubation with ciprofloxacin, the A549, HepG2, A375.S2 and B16 cells lost their regular shape and size, they lost their cell-cell contact (Fig. 1 C, F, I, L). Many cells lost their attachment to surface of culture wells, a majority of cells were rounded in appearance (Fig. 1 C, H, I, K, L). The C6 cells do not show any changes after incubation with ciprofloxacin (Fig. 1 N, O).

Cytotoxic influence of ciprofloxacin on viability of cancer cell lines

Trypan blue assay

A549 cell line viability decreased with increasing concentration of this chemotherapeutic agent. At low concentrations (10–50 μg/mL) cytotoxic effect of ciprofloxacin was weak; cells may even surpass the number of cells in control. At concentration 100 μg/mL, the number of cells decreased by about half compared to the control (24, 48, 72 h incubation time), after 96 h of incubation cell viability was 30%. In the highest concentration of ciprofloxacin, after 24 h incubation, only a very small number of living cells (2.2%) was observed. No living cells were observed after 48 and 72 h of incubation times and ciprofloxacin concentrations of 1000 and 500 μg/mL, respectively (Fig. 1, Fig.2 A, Tab. 1).

In the case of hepatocellular carcinoma line HepG2, cytotoxic influence of ciprofloxacin was independent of incubation time and concentration of antibiotic. In all cases a decrease in viability was observed, but full cytotoxic effect was never reached (Fig. 1, Fig. 2 B, Tab. 1).

The B16 cell line viability decreased with increasing concentration of antibiotic, but in concentration of 50 μg/mL cell viability was 56% and 40% after 48 and 96 h of incubation, respectively. The highest concentrations of ciprofloxacin (500 and 1000 μg/mL) acted less effectively; even after 72 and 96 h of incubation viability of cells was about 20% (Fig. 1, Fig. 2 D, Tab. 1).

The A375.S2 cell line viability decreased with increasing concentration of antibiotic, but similar to mouse melanoma cell line viability was still about

Table 1. Values of lethal concentrations for A549, A375.S2, B16, HepG2, and C6 cell lines (n.a. – not assessed).

Cell line	Time of incubation with ciprofloxacin (h)	LC values (μg/mL)		
		LC_{10}	LC_{50}	LC_{90}
A549	24	27.7	133.3	593.2
	48	18.2	102.1	389.5
	72	11.7	84.5	243.8
	96	7.63	54.8	164.9
HepG2	24	12.5	60.5	1279.2
	48	10.9	59.8	959.4
	72	12.5	105.0	1096.5
	96	15.4	155.8	1211.8
A375.S2	24	40.5	266.6	885.6
	48	40.5	266.6	885.6
	72	32.9	216.6	719.6
	96	35.1	231.1	767.5
B16	24	23.8	409.5	n.a.
	48	12.0	81.0	n.a.
	72	9.0	27.3	n.a.
	96	7.2	24.0	471.4
C6	24	n.a.	5915.0	n.a.
	48	572.0	1092.0	n.a.
	72	174.0	652.0	n.a.
	96	235.0	699.0	n.a.

Table 2. MTT values for cell lines A549, A375.S2, B16, HepG2 and C6 (n.a. = not assessed).

Cell line	Time of incubation with ciprofloxacin (h)	Viability (%)		
		LC_{10}	LC_{50}	LC_{90}
A549	24	115 ± 22.7	62 ± 3.1	52 ± 3.1
	48	85 ± 10.9	45 ± 0.8	0
	72	102 ± 8.5	62 ± 6.1	3 ± 0.5
	96	79 ± 5.5	51 ± 3.0	1 ± 0.5
HepG2	24	76 ± 6.2	64 ± 12.5	14 ± 0.8
	48	76 ± 5.0	47 ± 7.8	4 ± 0.9
	72	71 ± 8.3	9 ± 2.9	3 ± 0.5
	96	43 ± 3.6	9 ± 0.7	3 ± 0.6
A375.S2	24	86 ± 17.9	19 ± 5.1	0.5 ± 0.7
	48	72 ± 19.6	2 ± 0.8	0
	72	58 ± 14.6	0.6 ± 0.2	0.4 ± 0.2
	96	39 ± 1.6	0.9 ± 0.2	4 ± 1.6
B16	24	158 ± 6.7	84 ± 19.7	n.a.
	48	58 ± 3.5	32 ± 5.7	n.a.
	72	39 ± 11.1	43 ± 16.3	n.a.
	96	41 ± 18.8	44 ± 17.3	4 ± 0.9
C6	24	n.a.	60 ± 17.9	n.a.
	48	12 ± 8.1	7 ± 0.7	n.a.
	72	38 ± 0.8	9 ± 0.8	n.a.
	96	13 ± 1.2	8 ± 5.3	n.a.

20% in concentrations of 500 and 800 µg/mL and 10% in 1000 µg/mL (Fig. 1, Fig. 2 C, Tab. 1).

The C6 cells were insensitive to the influence of ciprofloxacin. Cell viability was higher than in control after 48, 72 and 96 h, even in concentration of 200 µg/mL (Fig. 1, Fig. 2 E, Tab.1).

MTT assay

MTT assay was performed to confirm results obtained after trypan blue assay. MTT determined metabolical activity of cells, which is related with mitochondrial action. The faster cells proliferate, the more formazan crystals are created. The obtained results are not exactly similar to those obtained in trypan blue assay. The greatest similarity was observed in case of A549 and B16 cell lines. In case of other tested lines differences were observed, mainly in LC_{50} and LC_{90} concentrations (Tab. 2). These differences can be caused by slower cell growth and smaller number of created formazan crystals, because each cell line proliferates with characteristic rate.

DISCUSSION

Our results showed that ciprofloxacin was cytotoxic to A549, it induced morphological changes in those cancer cells and decreased their viability *in vitro*. Glioma C6 line was resistant to the action of ciprofloxacin. In case of HepG2, A375.S2 and B16 cell lines cytotoxicyty of ciprofloxacin was observed but we were not able to eradicate all cells from the culture.

The results of MTT assay may differ from trypan blue assay. MTT method is not appropriate, when detached cells have to be taken into account. The medium with detached cells over monolayer is routinely removed before MTT will be added. The percentage of alive cells within the population of detached cells is presumed to be as high as 30% (13–15). There is a probability that cancer stem cells can be found within the population of detached cells (16). The trypan blue assay was more appropriate in our case because we focused more on the examination of cell viability than on the cell proliferation.

An increase in cell viability compared to control, even in concentration 200 μg/mL after 48 h incubation in glioma cell line was probably caused by the predominance of activation of prosurvival pathways over the apoptotic pathways at this concentration of chemotherapeutic agent (17). This phenomenon is described as hormesis, which is characterized by the fact that low dose of test compound stimulates cell growth, and high dose causes a reduction in cell viability (18). This effect was shown in the study of Gürbary et al. on rat astrocytes. They showed that ciprofloxacin in low doses (< 50 μg/mL after 24 h, < 100 μg/mL after 48 h and < 125 μg/mL after 72 and 96 h) increases the survival of cells up to 150% compared to control (19). In higher concentration of ciprofloxacin and after longer incubation times we observed a decrease in viability, but the lowest viability was about 25%. This negative effect can be potentiated by low levels of ciprofloxacin that can be reached in cerebrospinal fluid (0.15–0.56 μg/mL, depending on the mode of dosage) (20). Ciprofloxacin also accumulates in the brain and reaches a concentration at least as high as the levels in serum and up to 19.4% times higher than in the cerebrospinal fluid (21).

Ciprofloxacin also showed cytotoxic properties in relation to other cancer cells. Herold et al. studied the influence of ciprofloxacin on three colorectal cancer cell lines (CC-531, SW-403, HT-29). Ciprofloxacin caused in all these cancer cell lines morphological changes, inhibition of DNA synthesis, cell cycle arrest and cell death, mitochondrial membrane damage and induction of apoptosis (22). Ciprofloxacin after oral administration can achieve higher concentrations in urine than in serum, that is why most research is conducted into the use of potential anticancer activities of ciprofloxacin against bladder cancer cells (11). Say et al. were examined influence of ciprofloxacin on three different human bladder cancer cell lines (TCCSUP, T24, J82). Mortality of all cell lines were high, under 96% (23). Studies on the influence of ciprofloxacin on bladder cancer cell lines were carried out by Aranha et al. (HTB9) and Kamat and Lamm (TccSup, HTB9 and T24) (24, 25). The results from bladder and colorectal cancer lines are similar with our results, we observed similar morphological changes and cellular growth inhibition in a concentration and time-dependent manner.

The most effectively ciprofloxacin acted on human non-small cell lung cancer (NSCLC) line A549. Similar results were obtained in a study conducted on a different NSCLC line NCI-H460 (26). Mondal and coworkers established LC_{50} value after 48 h incubation in *in vitro* conditions which was 19.5 μg/mL, in our work this value was 102.1 μg/mL. This indicates that differences can be observed between NSCLC strains. The anticancer effect can be enhanced due to the ability of ciprofloxacin to accumulate in higher concentrations in lung tissue than in serum after intravenous administration. Haraguchi et al., in their *in vivo* examination showed that after administration of ciprofloxacin at a dose of 300 mg 2 times a day, the concentration of this antibiotic in the lung parenchyma reached the value higher than 2 μg/g (5). Similar results were obtained by examining different fluoroquinolone, pulrifloxacin (27).

Multhaupt et al. examined the influence of ciprofloxacin on normal cell line (human chondrocytes) and its malignant counterpart (human chondrosarcoma cell line). Ciprofloxacin was toxic in both, but in case of normal chondrocytes there was only a decrease in proliferation but no induction of apoptosis. In case of chondrosarcoma, cell growth was strongly inhibited and apoptosis was triggered (28).

Our results support the supposition that ciprofloxacin could be tested as possible neoadjuvant or adjuvant experimental therapy for lung cancer. Three properties of ciprofloxacin give confirmation of this thesis. Ciprofloxacin accumulates in lung tissue after intravenous administration. It is toxic against lung cancer cell lines in a concentration and time dependent manner. Finally, ciprofloxacin inhibits topoisomerase II, which leads to cell death by apoptosis in malignant but not in normal cells. Future experiments are needed to investigate ciprofloxacin activity against lung cancer.

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