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Influence of fluoroquinolones on viability of Balb/c 3T3 and HepG2 cells

Lidia Radko, Maria Minta, Sylwia Stypuła-Trębas

Department of Pharmacology and Toxicology,
National Veterinary Research Institute, 24-100 Pulawy, Poland
lidia.radko@piwet.pulawy.pl

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Abstract

The cytotoxic potential of fluoroquinolones (enrofloxacin, ciprofloxacin, difloxacin, sarafloxacin, danofloxacin, norfloxacin and marbofloxacin) was investigated using mouse fibroblasts Balb/c 3T3 and human hepatoma HepG2 cell lines. The cells were exposed for 24, 48, and 72 h to drugs at eight concentrations ranged from 0.78 to 100 µg/mL. Four independent cytotoxicity assays were applied, in which various endpoints were assessed: mitochondrial activity - MTT reduction, lysosomal activity - neutral red uptake, total protein content, and cellular membrane integrity - lactate dehydrogenase release. Mean effective cytotoxic concentrations (EC_{50}) calculated at different time points from concentration-response curves ranged from 10 to 100 µg/mL. The most affected endpoint in both cell lines was mitochondrial activity. The $EC_{50\text{-MTT-72 h}}$ <10 µg/mL was found for difloxacin, marbofloxacin (fibroblasts), sarafloxacin, and norfloxacin (HepG2). The data shows that cytotoxicity of the fluoroquinolones appears after longer exposure of both cell cultures to these compounds.

Key words: fluoroquinolones, cytotoxicity, Balb/c 3T3, HepG2, cell cultures.

Introduction

Fluoroquinolones (FQs) are common antibacterial agents used in human and veterinary medicine. FQs have satisfactory effect in the treatment of severe intestinal and respiratory infections in food–producing animals (20). They are excreted as parent compound conjugates, or as oxidation, hydroxylation, dealkylation, and decarboxylation products of the parent compound (26). Acute toxicity of FQs generally is low; however, their residues in food may pose a hazard to consumers through emergence of drug-resistant bacteria (5, 9, 21). The adverse effects associated with FQs include gastrointestinal effects (nausea, vomiting, diarrhoea), dermatologic effects (phototoxicity), tendinopathy, and CNS reactions (2). The hepatotoxicity of FQs has also been reported (18, 19). Although FQs-related hepatic injuries occur infrequently, their consequences can be severe and the mechanism of these effects has not been fully elucidated.

The aim of this study was to assess the potential cytotoxicity of FQs used in veterinary medicine: enrofloxacin, ciprofloxacin, difloxacin, sarafloxacin, danofloxacin, norfloxacin, and marbofloxacin. Two models, Balb/c 3T3 and HepG2 cell lines were applied. The mouse fibroblasts Balb/c 3T3 are mostly used for the assessment and comparison of general toxicity of xenobiotics. Human hepatoma HepG2 cells display many characteristics of normal liver cells and they are used as an alternative to animal models and isolated hepatocytes (25). Four established cytotoxicity assays used in *in vitro* toxicological studies were applied (7, 27). Additionally, the authors attempted to create the rank of FQs effects on each type of cells using a cell viability score (CVS) as a parameter expressing the cytotoxic potential of the drugs (1, 13).

Material and Methods

Reagents. Triton X-100, neutral red (NR), dimethyl sulfoxide (DMSO), foetal bovine serum (FBS), bovine calf serum (BCS), 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT), Coomassie Brilliant Blue R-250 dye (CBB), trypsin-EDTA, antibiotic solution (10.000 U/mL of penicillin, 10 mg/mL of streptomycin) were purchased from Sigma – Aldrich (USA). All other chemicals were

purchased from commercial suppliers and were of the highest available purity.

Drugs. Analytical standards of enrofloxacin (purity >99%, CAS 93106-60-6), difloxacin (purity >98%, CAS 91296-86-5), sarafloxacin (purity >97%, CAS 91296-87-6) were purchased from Fluka and ciprofloxacin (purity >98%, CAS 85721-33-1), danofloxacin (purity >99%, CAS 112398-80-0), norfloxacin (purity >99%, CAS 70458-96-7), marbofloxacin (purity >98%, CAS 115550-35-1) - from Riedel-de Haën.

Cell lines and cell culture conditions. Balb/c 3T3 clone A31 cell line was a gift from the Department of Swine Diseases of the National Veterinary Research Institute in Pulawy, Poland. The cells were cultured in Dulbecco`s Modified Eagle`s Medium (DMEM, GIBCO). HepG2 cell line was purchased from the American Type Culture Collection (ATCC HB-8065). These cells were cultured in Minimum Essential Medium Eagle (MEME) medium (ATCC). The media were supplemented with 10% of bovine calf serum (Balb/c 3T3 clone A31), 10% of foetal bovine serum (HepG2), 1% of L-glutamine, 1% of penicillin and streptomycin. The cells were maintained in 75 cm^2 cell culture flasks (NUNC) at 37°C in 5% CO_2 humidified atmosphere. The medium was refreshed every two or three days and the cells were trypsinised by 0.25% trypsin–0.02% EDTA after reaching 70%–80% confluence. Single cell suspensions were prepared and adjusted to a density of 2×10^5 cell/mL (HepG2) and 1×10^5 cell/mL for 24 h and 48 h exposition or 5×10^4 cell/mL for 72 h exposition (Balb/c 3T3). The cell suspension was transferred into 96-well plates (100 µL/well) and incubated for 24 h before the exposure to FQs.

Compound preparation. Stock solutions of the drugs were prepared in DMSO. The final concentration of DMSO was 0.1%. in the medium. The same final concentration of the solvent was used in the corresponding control. The medium used for test solutions and in control preparation did not contain serum and antibiotics (penicillin, streptomycin). All working solutions were freshly prepared and protected from light (covered with aluminium foil).

Exposure to drugs and cytotoxicity assessment. Each drug was tested in eight concentrations: 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 µg/mL ranging from 0.78 to 100 µg/mL. Each concentration was tested in six replicates. The cytotoxicity of FQs was assessed at three time points, after 24, 48, and 72 h of incubation, using four assays described below.

MTT reduction assay. The assay is based on the reduction of tetrazolium salt MTT in live cells to dark formazan product inside the cell *via* mitochondrial nicotinamide adenine dinucleotide phosphate (NADH) dependent dehydrogenases. The amount of generated formazan is assumed to be directly proportional to the cell density (24).

After the incubation, cells were incubated with 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT). MTT was dissolved in phosphate buffered saline (5 mg/mL), sterilised by filtration through a 0.22 µm Millipore® filter. A volume of 10 µL was added to each well of microplate and cultures were allowed to incubate for further 4 h at 37°C in 5% CO_2 humidified atmosphere. The medium was removed and the intracellular formazan crystals were dissolved with 100 µL DMSO. The plate was shaken for 15 min at room temperature and transferred to a microplate reader (Multiscan RC Labsystems) to measure the absorbance at 570 nm, using blank as a reference. The mean optical density (OD) was used to calculate the percentage of cell survival for each dilution of the test sample.

Neutral Red Uptake (NRU) assay. The assay is based on staining living cells by neutral red (NR, a weak cationic water-soluble dye), which readily diffuses through the plasma membrane and concentrates in lysosomes (3).

After the incubation, medium containing drug was removed and cells were washed with PBS-Ca^{2+}. Then 100 µL/well of NR solution (50 µg/mL in PBS) was added and incubated for 3 h. After this time, the cells were washed again with PBS-Ca^{2+}. The dye from viable cells was released by extraction with a mixture of acetic acid, ethanol, and water (1:50:49, v/v). After 10 min of shaking, the absorbance of the dissolved NR was measured at 540 nm using blank as a reference.

Total protein content (TPC) assay. The dye-binding (Coomassie Brilliant Blue (CBB), previously Kenacid blue) assay is based upon staining total cellular protein (proliferation) (4).

After the incubation, medium containing drug was removed and 100 µL of CBB R-250 was added to each well. The plate was shaken for 10 min. Then the stain was removed and the cells were rinsed twice with 100 µL of washing solution (glacial acetic acid/ ethanol/water – 5/10/85, v/v). Then the washing solution was replaced with 100 µL of the desorbing one (1 M potassium acetate), and plates were shaken again for 10 min. The absorbance was measured at 595 nm in microplate reader using blank as a reference.

Lactate dehydrogenase (LDH) leakage assay. LDH is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane (16).

The release of LDH from the cytoplasm into the surrounding culture medium was monitored using the commercially available Cytotoxicity Detection Kit (LDH) (Roche Diagnostics, Poland). Medium (100 µL/well) without cells and 100 µL freshly prepared reaction mixture were transferred into corresponding wells of an optically clear 96-well flat bottom microplate. Then the plates were incubated for 30 min at room temperature in dark. After that time, 1 M HCl (50 µL/well) was added to stop the reaction. The absorbance was measured at 492 nm in a microplate reader using blank as a reference. The wells

with cells and culture medium with 2% Trixon X-100 were the positive control.

Data analysis. Viability (percentage of control values) was expressed as mean ± SD of three independent experiments. Statistical evaluation was performed using one-way analysis of variance (ANOVA) followed by Dunnett`s *post-hoc* test. The mean effective cytotoxic concentrations (EC_{50}) at three time points (24, 48, and 72 h) were calculated according to the Hill's equation (sigmoidal model of concentration-response curve) and expressed as mean ± SEM of three independent experiments. The statistical evaluation of the effective cytotoxic concentrations (EC_{50}) between drugs in corresponding times was performed using Kruskal-Wallis and Mann-Whitney nonparametric tests for pairwise comparison of means. Differences were considered as statistically significant at $P \leq 0.05$.

Cell viability scores (CVSs). The $\%CVS_{50}$ was determined as the number of measurements showing viability ≥50%. The $\%CVS_{40/80}$ was calculated as the number of measurements showing viability >80% minus the number of measurements showing viability <40%. The values of $\%CVS_{50}$ and $\%CVS_{40/80}$ evaluated the cytotoxicity by change of cell viability (1, 13).

Results

Fig. 1 and Fig. 2 present the concentration and time-dependent response curves for seven FQs tested by four independent assays on two cell lines. The earliest effects of FQs on Balb/c 3T3 cells were observed after 24 h incubation, starting from the lower concentrations (3.13 and 6.25 µg/mL). The earliest effects of FQs on HepG2 cells were observed after 24 h exposure, starting from the higher concentrations (12.5, 25, 50 µg/mL).

The effective cytotoxic concentrations (EC_{50}) of the drugs on Balb/c 3T3 and HepG2 cells are presented in Tables 1 and 2. The EC_{50} values depended on the duration of exposure, the cell line, and the assay used. The EC_{50} values were calculated after 24 h exposure of Balb/c 3T3 cells to danofloxacin, norfloxacin, and marbofloxacin in MTT, NRU, and TPC assays. The EC_{50} for the other FQs were calculated only on the basis of TPC and NRU assays. The most dramatic decrease in cell viability was observed in Balb/c 3T3 line after 72 h exposure to difloxacin and marbofloxacin (Fig. 1), for which the lowest $EC_{50\text{-MTT-72 h}}$ values were established *i.e.* 4.2 µg/mL and 8.2 µg/mL, respectively (Table 1).

The EC_{50} values after 48 h exposure of HepG2 cells to enrofloxacin, ciprofloxacin, norfloxacin in NRU and LDH assay, to difloxacin in MTT and NRU assays, to sarafloxacin (MTT), danofloxacin (NRU), and marbofloxacin in LDH assay were calculated. However, the EC_{50} value for ciprofloxacin in LDH assay was calculated after 24 h. In HepG2, the lowest $EC_{50\text{-MTT-72 h}}$ were found for sarafloxacin (6.4 µg/mL) and norfloxacin (7.3µg/mL) (Table 2).

Table 3 summarizes the CVSs values of seven FQs tested on Balb/c 3T3 and HepG2 cells. The results were expressed as percentage of total number of measurements (288) for each drug (eight concentrations, three exposure times, four assays, three experiments). Based on the $\%CVS_{50}$, the order of the FQs from the highest to the lowest $\%CVS_{50}$ was as follows: sarafloxacin = difloxacin > danofloxacin > norfloxacin > marbofloxacin > ciprofloxacin > enrofloxacin. The fibroblasts and the human hepatoma cells viability ≤ 40% was for the sarafloxacin and difloxacin.

Discussion

In vitro test systems based on cellular models are considered as good alternatives for animal tests, and can contribute to the hazardous assessment of substances. The use of the battery of tests, which estimate different endpoints (27) *e.g.* metabolic and lysosomal activity (3, 24), membrane integrity (16), and proliferation (4), is the most practical approach for evaluating basal cytotoxicity.

In the study, the EC_{50} values of fluoroquinolones ranged from 10 to 100 µg/mL in both cell lines. However, values <10 µg/mL for difloxacin and marbofloxacin in Balb/c 3T3 cell cultures, and for sarafloxacin and norfloxacin in HepG2 cell cultures after 72 h incubation could be established.

The literature reports that inhibition concentrations of the FQs vary. It may result from the different experimental conditions. Among them, duration of exposure and cell line used are of a great importance. The inhibited proliferation of MC3T3-E1 osteoblast-like cells was observed at concentration of 40 µg/mL after 48 and 72 h exposure to ciprofloxacin (12). In the study performed using fibroblasts Balb/c 3T3, no decrease in cell viability after 24 h exposure to ciprofloxacin at concentrations 12.5, 25, 50 µM (4.14, 8.28, 16.57 µg/mL) was observed (23). Cytotoxic effects of ciprofloxacin against human fibroblasts were reported at concentration >50 µg/mL following longer (>48 h) exposure (6, 11).

In the study, fibroblasts were more sensitive than HepG2 cells. The differences in the sensitivity of cell lines were also reported in the literature. The mouse fibroblasts Balb/c 3T3 were more sensitive to levofloxacin than immortalized human endothelial cells (EA.hy926) (17). Herold *et al.* (10) found that colon cell carcinoma lines CC-531, SW-403 and HT-29 were sensitive to ciprofloxacin; whereas, the hepatoma cells remained unaffected. The newly differentiating cells are especially sensitive to that group of drugs (22). The FQs studied *in vitro* showed cytotoxicity on cancer cells, thus representing a potentially important source of new anticancer agents (14).

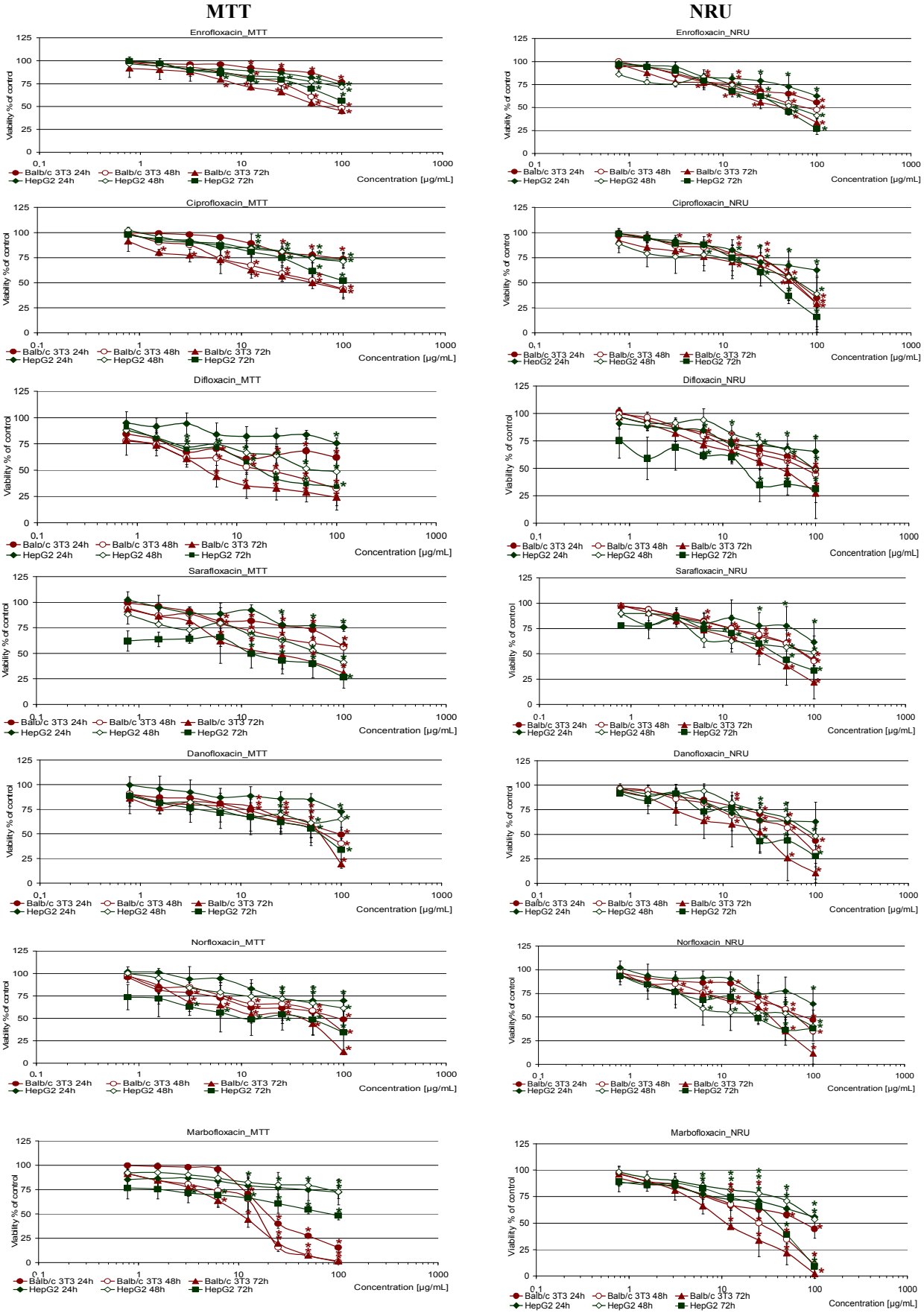


Fig. 1. Concentration and time–dependent response curves for FQs assessed by MTT and NRU assays on Balb/c 3T3 and HepG2 cells. The results are expressed as mean ± SD of three independent experiments. * $P \leq 0.05$ in comparison with control (Dunnett test)

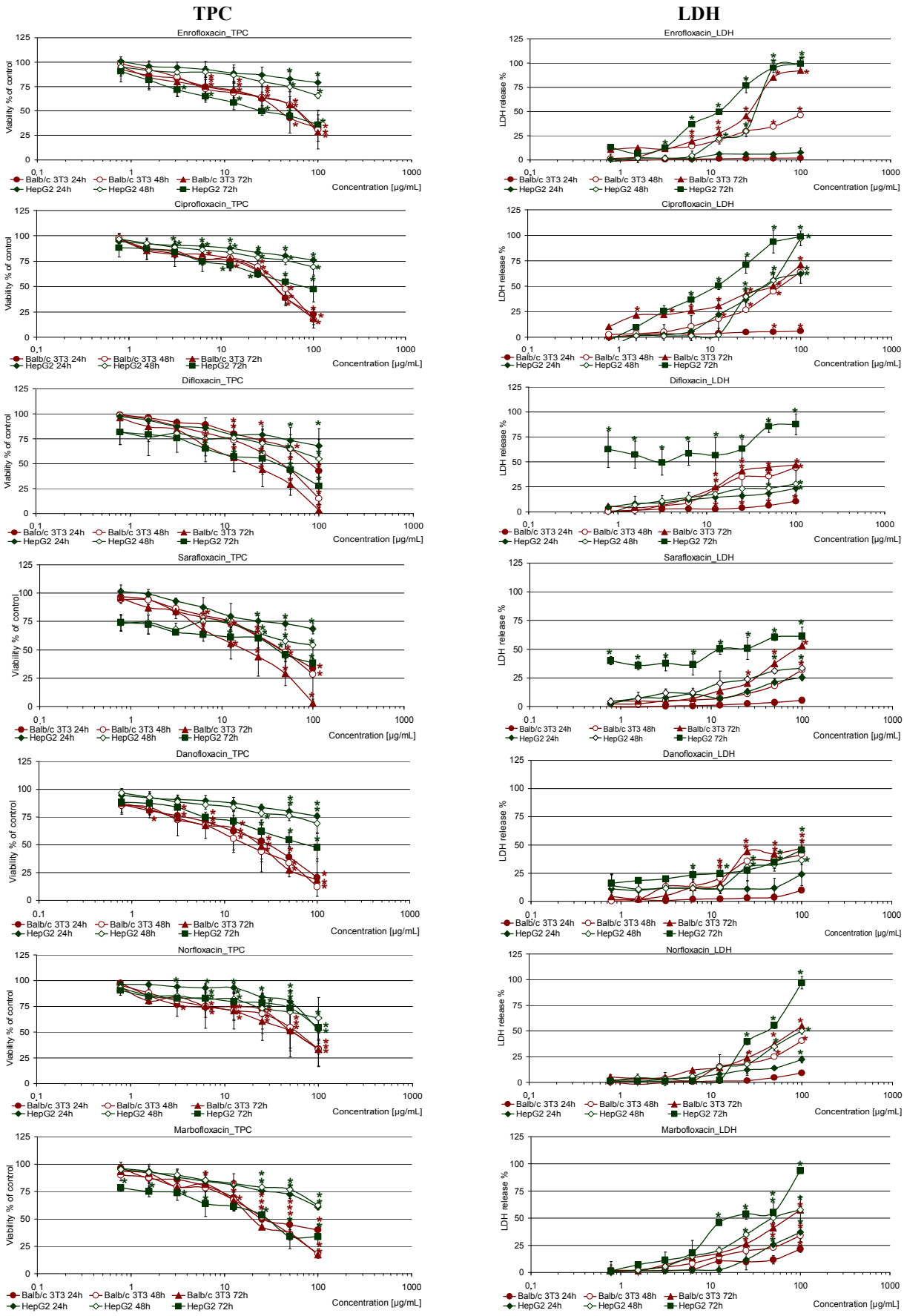


Fig.2. Concentration and time –dependent response curves for FQs assessed by TPC and LDH release assays on Balb/c 3T3 and HepG2 cells. The results are expressed as mean ± SD of three independent experiments. * $P \leq 0.05$ in comparison with control (Dunnett test)

Table 1. Effective concentrations (EC_{50}, µg/mL) determined in Balb/c 3T3 cells by MTT, NRU, TPC, and LDH assays after 24, 48, and 72 h exposure to enrofloxacin (ENRO-), ciprofloxacin (CIPRO-), difloxacin (DI-), sarafloxacin (SARA-), danofloxacin (DANO-), norfloxacin (NOR-), and marbofloxacin (MARBO-); data presented as mean (n = 3) ± SEM: standard error mean

Drug	MTT			NRU			TPC			LDH		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
ENRO	-	77.6 ± 2.8^{a}	57.1 ± 3.1^{a}	-	69.3 ± 4.2^{a}	31.6 ± 3.2^{a}	34.3 ± 7.1^{ad}	89.3 ± 5.5^{a}	87.3 ± 4.2^{a}	-	-	28.0 ± 2.1^{a}
CIPRO	-	86.5 ± 2.3^{b}	49.9 ± 0.8^{b}	95.1 ± 3.2^{a}	89.4 ± 3.0^{b}	39.8 ± 1.5^{a}	95.5 ± 1.5^{b}	96.8 ± 3.2^{a}	88.9 ± 1.7^{a}	-	-	64.2 ± 0.8^{b}
DI	-	17.1 ± 3.9^{c}	4.2 ± 1.3^{c}	94.0 ± 5.1^{a}	73.2 ± 11.2^{abc}	36.8 ± 6.9^{a}	66.5 ± 13.1^{c}	34.4 ± 8.3^{b}	18.9 ± 2.1^{b}	-	-	-
SARA	-	-	16.8 ± 7.4^{d}	68.9 ± 3.2^{b}	50.8 ± 4.1^{c}	45.9 ± 4.1^{b}	29.0 ± 1.8^{a}	27.6 ± 2.5^{b}	19.0 ± 1.2^{b}	-	-	90.0 ± 5.0^{c}
DANO	93.9 ± 2.1^{a}	81.1 ± 1.1^{b}	67.9 ± 3.2^{a}	87.9 ± 3.1^{ac}	57.3 ± 4.0^{c}	48.1 ± 2.1^{b}	44.1 ± 6.9^{d}	18.9 ± 2.1^{c}	13.4 ± 4.9^{b}	-	-	-
NOR	82.0 ± 10.2^{a}	57.1 ± 6.9^{d}	38.4 ± 5.2^{e}	82.1 ± 0.6^{c}	65.1 ± 9.0^{ac}	39.8 ± 1.5^{ab}	95.5 ± 1.5^{b}	96.8 ± 3.2^{a}	88.9 ± 1.7^{a}	-	-	64.2 ± 0.8^{b}
MARBO	19.6 ± 2.4^{b}	16.2 ± 7.4^{c}	8.2 ± 1.1^{d}	71.0 ± 10.8^{bc}	50.0 ± 11.4^{abc}	7.3 ± 1.5^{c}	22.4 ± 6.3^{a}	26.7 ± 5.5^{bc}	16.3 ± 3.2^{b}	-	-	76.7 ± 10.1^{b}

Different letters (a-e) within columns indicate significant differences ($P \leq 0.05$) between the drugs at the corresponding time (Mann-Whitney test). (-) indicates no EC_{50} value obtained for the corresponding incubation periods with the assay employed

Table 2. Effective concentrations (EC_{50}, µg/mL) determined in Hep G2 cells by MTT, NRU, TPC, and LDH assays after 24, 48, and 72 h exposure to enrofloxacin (ENRO-), ciprofloxacin (CIPRO-), difloxacin (DI-), sarafloxacin (SARA-), danofloxacin (DANO-), norfloxacin (NOR-), and marbofloxacin (MARBO-); data presented as mean (n = 3) ± SEM: standard error mean

Drug	MTT			NRU			TPC			LDH		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
ENRO	-	-	-	-	91.2 ± 11.0^{ab}	32.3 ± 6.0^{ab}	-	-	50.2 ± 7.7^{a}	-	32.8 ± 3.9^{a}	12.6 ± 3.2^{a}
CIPRO	-	-	-	-	82.9 ± 8.3^{a}	38.9 ± 4.3^{a}	-	-	68.1 ± 2.2^{b}	42.1 ± 9.9	41.2 ± 9.9^{ac}	12.1 ± 1.2^{a}
DI	-	74.4 ± 8.1^{a}	18.9 ± 5.8^{a}	-	95.3 ± 3.1^{b}	20.1 ± 7.9^{bc}	-	-	38.5 ± 7.8^{c}	-	-	-
SARA	-	89.2 ± 2.1^{b}	6.4 ± 3.1^{b}	-	-	34.5 ± 4.0^{a}	-	-	32.4 ± 1.9^{c}	-	-	12.4 ± 0.9^{a}
DANO	-	-	88.0 ± 6.9^{c}	-	55.3 ± 8.7^{c}	15.1 ± 6.1^{c}	-	-	68.1 ± 11.3^{ab}	-	-	-
NOR	-	-	7.3 ± 0.9^{b}	-	85.7 ± 8.6^{ab}	13.3 ± 3.2^{c}	-	-	-	-	99.4 ± 3.7^{b}	41.2 ± 9.0^{b}
MARBO	-	-	85.0 ± 8.2^{c}	-	-	35.2 ± 12.9^{ab}	-	-	45.5 ± 11.7^{ac}	-	49.5 ± 13.2^{c}	18.4 ± 1.1^{c}

Different letters (a-c) within columns indicate significant differences ($P \leq 0.05$) between the drugs at the corresponding time (Mann-Whitney test). (-) indicates no EC_{50} value obtained for the corresponding incubation periods with the assay employed

Table 3. $\%CVS_{50}$ and $\%CVS_{40/80}$ of FQs tested on Balb/c 3T3 and HepG2 cell lines

Drug	$\%CVS_{50}$		$\%CVS_{40/80}$	
	Balb/c 3T3	HepG2	Balb/c 3T3	HepG2
Sarafloxacin	81	82	26	34
Difloxacin	81	83	32	33
Danofloxacin	86	89	47	50
Norfloxacin	87	87	49	50
Marbofloxacin	90	90	34	43
Ciprofloxacin	91	91	52	52
Enrofloxacin	92	92	55	55

The results were expressed as a percentage of total number of measurements (288) for each drug (8 concentrations, 3 exposure times, 4 assays, 3 experiments)

The cytotoxic effects of fluoroquinolones on HepG2 cells were concentration- and time- dependent. In our study they were observed after longer exposure (>48 h). However, the EC_{50} after 24 h exposure of HepG2 cells to ciprofloxacin was calculated (42.1 ± 9.9 µg/mL). It is in agreement with Garcia-Kaufer *et al.* (8) study. They showed inhibition of HepG2 cells proliferation after treatment with ciprofloxacin for 24 h. Accordingly, in other studies on HepG2 cells, which are opposite to our results, the cytotoxicity of ciprofloxacin was independent of concentration (10–100 µg/mL) and exposure time (24, 48, and 72 h), or lack of effects on proliferation or apoptosis were shown (10, 15). However, it is necessary to remember that when hepatoma cell lines are used in the study, the ability of cells to metabolism drugs should be taken into consideration (19).

To conclude, the interpretation of the results and creation of the drug cytotoxicity rank were complicated in classical approach. Since series of assays under multiple conditions, including different drug concentrations, exposure times, and different cell lines give a useful but unequal volume of information, a summary of the data and the idea of cell viability score (CVS) was developed (1, 13). The values of $\%CVS_{50}$ and $\%CVS_{40/80}$ evaluated the cytotoxicity by change of cell viability, suggesting that the %CVS could be applied as an indicator of cytotoxicity of substances (1). The viability >80% was defined as "non toxic level" and <40% as a "significantly toxic level" (1, 13). The values of %CVS based on three times exposure (24, 48, and 72 h), eight concentrations, and four assays showed that sarafloxacin and difloxacin were cytotoxic for both cell lines. This rank can be comparable with the cytotoxic concentrations ($EC_{50_MTT\ 72\ h}$) for Difloxacin on Balb/c 3T3 cells and sarafloxacin on HepG2 cells.

The obtained results provide the evidence that cytotoxicity of fluoroquinolones is related to the duration of exposure. Taking into account all observations from *in vitro* and *in vivo* studies, fluoroquinolones should be used carefully during the long-term therapy.

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