The *in vitro* influence of norfloxacin nicotinate on the selected immune cell functions in rainbow trout (*Oncorhynchus mykiss*)

ANNA SIEROSŁAWSKA[1], CEZARY J. KOWALSKI [2], ANDRZEJ K. SIWICKI [3], ELŻBIETA TERECH-MAJEWSKA[4], ANNA RYMUSZKA[1]

[1]Department of Physiology and Ecotoxicology, The John Paul II Catholic University of Lublin, Poland; [2]Department of Pharmacology, Agriculture Academy in Lublin, Poland; [3]Department of Fish Immunology and Pathology, Inland Institute in Olsztyn, Poland; [4]Department of Epizootiology with Clinic of Infectious Diseases, University of Warmia and Mazury in Olsztyn, Poland

Abstract

The aim of the study was to assess the influence of norfloxacin nicotinate on the selected immune functions of the cells isolated from head kidney of rainbow trout (Oncorhynchus mykiss). To estimate the drug concentrations reached in the organ, fish were given norfloxacin nicotinate orally for five days or in a single intraperitoneal injection, and the drug residues in head kidneys were determined by HPLC. The highest concentration of norfloxacin, 25.60 μg/g, was found 24 h after the drug administration at 60 mg/kg in the single intraperitoneal injection. 7 days after application, the drug amounts in the tissue reached approximately 10% of the concentrations found 24 h after application, that is 1.41-2.38 μg/g. Then in the in vitro studies leucocytes isolated from fish head kidneys were tested on the lymphocyte proliferative response on mitogens and on respiratory burst activity of phagocytic cells after cell incubation with norfloxacin nicotinate at concentrations of 1, 5, 10, 20, 30, 40, 60 μg/ml. Suppressive effect of the drug was observed on B cell proliferation when the cells were exposed to the higher used concentrations, 30-60 μg/ml, T cells were less sensitive, the effect was seen only in the highest concentration. The drug caused stimulation of respiratory burst activity at 5 μg/ml. Other used concentrations had no effects on the studied parameter.

Key words: *norfloxacin nicotinate, fluoroquinolones, respiratory burst activity, lymphocyte proliferation, rainbow trout.*

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Introduction

Norfloxacin belongs to the group of fluoroquinolones, chemotherapeutics that are often used in farmed fish therapy because of their high potency against G(–) fish pathogenic bacteria, as *Escherichia coli*, *Salmonella sp*, *Aeromonas salmonicida*, *Edwardsiella tarda*, and against some G(+) bacteria. In the animal therapy two forms of the drug are used: norfloxacin ($C_{16}H_{18}FN_3O_3$) and norfloxacin nicotinate ($C_{16}H_{18}FN_3O_3 \bullet C_6H_5NO_2$).

Some antibiotics, except therapeutic effects, may also affect various immune mechanisms of the host organism. This negative influence can occur at different stages of the immune reaction. The effect mainly depends on the place of drug binding in the cell, drug concentrations reached in immunologically important organs or tissues, on maturity stage of the exposed immune cells and on the order and time between antigen and drug introduction. The influence of antimicrobial drugs on the immune cell functions can be considered in two basic aspects: modulating bacteria – immune cell interactions by changing bacterial surface antigens or direct affecting immune cell activity.

The aim of the study was to assess norfloxacin nicotinate (NFLXN) concentrations found in rainbow trout head kidney after the drug application and its *in vitro* effects on the basic immunological functions, as lymphocyte proliferative ability

Correspondence: Anna Sierosławska, Department of Physiology and Ecotoxicology, Faculty of Mathematics and Natural Sciences, The John Paul II Catholic University of Lublin, Norwida 4, 20-061 Lublin, Poland. Phone/fax number: +48 81 536 70 89, Email: ansie@kul.lublin.pl

and respiratory burst activity of phagocytic cells isolated from fish head kidney.

Materials and methods

Animals

The studies were conducted on 55 immunologically mature, weighting 200±50 g, healthy rainbow trouts (*Oncorhynchus mykiss*) of either sex. Fish were purchased from the Department of Salmonid Research Rutki, and maintained in 500 L tanks with aerated, flowing water at a temperature of 14°C and light-dark cycles at 8 h/16 h periods. Fish were fed with diet containing 45% of protein. In the studies the Principles of laboratory animal care and the national laws on the protection of animals were followed (Opinion and Approval of Local Committee of Ethics Nr. 490/2004).

HPLC determination of norfloxacin concentrations

45 rainbow trouts were divided into 3 groups (n=15). Fish in the 1st group were given pure NFLXN (99%, Polfa, Grodzisk) orally, via a syringe introduced into esophagus, at the dose of 40 mg/kg b.w. for 5 days. Fish in groups 2nd and 3rd were given NFLXN in Quinabic preparation (7%, Abic Ltd, Israel) once, intraperitoneally, at doses of 40 and 60 mg/kg b.w., respectively. Head kidneys from 5 fishes of each group were taken after 24, 72 h and 7 days after medication.

Each 0.2 g head kidney sample was added to 5 ml of extraction solution consisted of concentrated perchloric acid (HPLC grade, Veb. Laborchemie Apolda, Germany), concentrated phosphoric acid (HPLC grade, Merck, USA) diluted with water and methanol solution (1:1, v/v), homogenized and centrifuged for 15 min, 5500 x g. Supernatants were filtered on 0.45 µm diameter filters (Millex-HV_{13}, Millipore, Holland). The analysis were performed on the HPLC system consisted of STAR 9002 pump (Varian Analytical Instruments, USA), autosampler with a 20-µl injection loop (Varian Analytical Instruments, USA), the LiChroCARTR 250-4 Purospher STAR RP-18 column with RP-18 precolumn (Merck, Germany) and fluorescent detector (Varian Analytical Instruments, USA). Fluorescence at an excitation wavelength of 278 nm and an emission wavelength of 456 nm was used for detection. The mobile phase was water/methanol (4:1, v/v adjusted to pH 2.5 with H_3PO_4), which contained tetrabutylammonium hydrogen sulphate (Chemapol-Lachema, Czech Republic) as ion-pairing agent. The flow rate was 0.7 ml/min. The detection limit of norfloxacin in the tissues was 2.5 ng/ml of homogenate.

Head kidney cell isolation

Head kidneys were taken aseptically from 10 rainbow trouts euthanized with Propiscin (IRS, Żabieniec, Poland) at 2 ml/l of water and subsequently spine cord cutting. Isolation of leucocytes was performed according to Rowley [1]. Briefly, separating mixture was prepared with Gradisol L (1.077 g/ml, Aqua-Medica, Poland) for lymphocyte separation and Gradisol G (1.115 g/ml, Aqua-Medica, Poland) for phagocytic cells (neutrophils, monocytes/macrophages) isolation. Cell counts and viability testing by the dye exclusion method (0.1% trypan blue) were performed in a Bürker haemocytometer.

Lymphocyte mitogen-induced proliferation – MTT assay

The assay was performed following the method described by Mosmann [2]. Lymphocytes at a concentration of 3 x 10^6 cell/ml of RPMI 1640 without phenol red (BioMed, Poland) with 10% of inactivated FCS (foetal calf serum, Gibco, England) were incubated at 18°C with the NFLXN (99%, Polfa, Grodzisk) at concentrations of 1, 5, 10, 20, 30, 40, 60 µg/ml and with the mitogens: 50 µg/ml ConA (concanavalin A, Sigma) as a T cell stimulator, or 20 µg/ml LPS (lipopolysaccharide, Sigma) as a B cell stimulator. After 72 h MTT solution (methylthiazoltetrazolium, Sigma) at a concentration of 0.5 mg/ml was added and left for incubation for the next 4 h. Then the plates were centrifuged (1000 x g, 5 min) and cell-free supernatant was removed. DMSO (dimethyl sulfoxide, POCh, Poland) was added to the pellet and the plates were mixed. The optical density was measured at 630 nm on the microplate reader (BioRad, 550).

Phagocytic ability assay

Phagocytic ability was measured by the respiratory burst activity (RBA) with oxygen burst activator PMA (phorbol myristate acetate, Sigma) according to Chung and Secombes [3]. Briefly, pooled phagocytic cells, at a concentration of 5 x 10^6 cell/ml of RPMI 1640 (BioMed, Poland) with 0.1% of FCS were left for 2 h incubation with NFLXN (99%, Polfa, Grodzisk) at concentrations of 1, 5, 10, 20, 30, 40, 60 µg/ml at room temperature. Then the cell-free supernatant was removed and 0.1% NBT (nitrotetrazolium blue, Sigma) solution in RPMI 1640 without phenol red was added to the adherent cells with 1 µg/ml of PMA, as respiratory burst stimulator. After 30 min incubation at room temperature supernatant was removed and the adherent cells were fixed with ethanol. Plates were left to dry. Spectrophotometric measurement was performed on a microplate reader at 630 nm after 2M KOH and DMSO addition and mixing.

Statistical analysis of the results was performed by ANOVA. Differences on means were determined by Duncan test and considered statistically significant at $P<0.05$.

Results

The highest norfloxacin concentrations in fish head kidney were found 24 h after the drug application. After oral route of administration, corresponding with the route used while fish treating in practice, norfloxacin level was 19.95 µg/g tissue and was lower than after i.p. drug administration at a dose of 60 mg/kg (25.60 µg/g) and higher

Table 1. Norfloxacin residues in rainbow trout head kidney ($\bar{x}$±SD, n=5)

Time after application	Norfloxacin nicotinate dosage		
	1 x 60 mg/kg b.w. i.p.	1 x 40 mg/kg b.w. i.p.	5 x 40 mg/kg b.w. p.o.
	Norfloxacin concentrations (µg/g)		
24 h	25.60±5.01	16.93±5.24	19.95±3.74
72 h	5.86±3.86	6.59±2.56	12.44±5.65
7 days	1.41±0.61	2.04±0.22	2.38±2.18

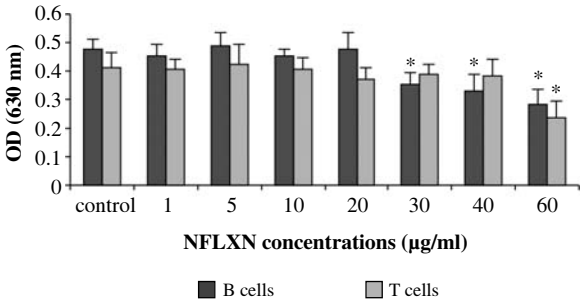

Fig. 1. The influence of NFLXN on the proliferative ability of T and B cells isolated from rainbow trout head kidney ($\bar{x}$±SD, n=15, *P<0.05)

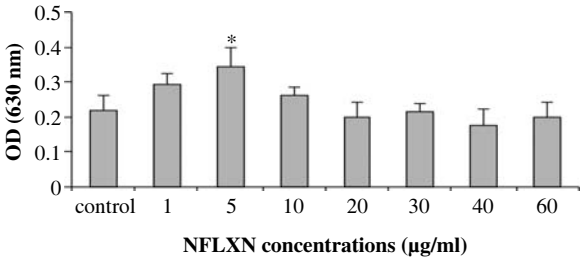

Fig. 2. The influence of NFLXN on respiratory burst activity of phagocytic cells isolated from rainbow trout head kidney ($\bar{x}$±SD, n=15, *P<0.05)

than after 40 mg/kg i.p. (16.93 µg/g). 72 h later the drug concentrations in head kidney decreased. However, it was found that the decrease of drug amount is the slowest in the group after oral administration. In that group norfloxacin concentration was 48% lower compared to the amount reached after 24 h, while in the groups in which the fish were injected with the drug, concentrations gained after 72 h were 3-4 folds lower that after 24 h.

After 7 days norfloxacin concentrations were reduced to approximately 10% of the amounts found after 24 h (table 1).

In our study the dose-dependent suppressive effects of norfloxacin nicotinate on B cell proliferative activity were seen at higher used drug concentrations. T cells were less sensitive to the toxic action of the drug and the statistically important decrease of proliferation was observed only at the highest NFLXN concentration, 60 µg/ml (figure 1).

Results of our study indicate that norfloxacin nicotinate may stimulate respiratory burst activity as seen at 5 µg/ml. Other used drug concentrations had no influence on the metabolic activity of phagocytes under described conditions (figure 2).

Discussion

Fish head kidney acts as the major haematopoietic and lymphoid organ. It has also analogical functions to mammalian lymph nodes [4]. Because of its significance, it is important to estimate the drug concentrations achieved in the organ and the drug influence on the immune cell activity during fish treatment.

In our study high concentrations of NFLXN were found in fish head kidneys after the two ways of application. All fluoroquinolones bind with blood proteins in low degree, have a large volume of distribution (3-4 l/kg for fluoroquinolones of 3rd generation) and high membrane permeability [5, 6], which enable them good tissue penetration and reaching high concentrations in organs. Fluoroquinolone concentrations found in fish head kidney during treatment are even higher than in liver [7]. Drug amounts found in the organs depend on environmental factors and can be up to five folds higher in fresh water fish than in marine ones [5, 7].

In vitro lymphocyte reactivity to mitogen stimulation gives information about their general status and ability to *in vivo* response to antigen. There are some reports in the literature, that 4-quinolones, which mechanism of action consists on inhibition of bacterial enzymes responsible for replication and transcription processes, may disturb DNA synthesis in fast dividing eucaryotic cells [8, 9]. There is no data referring to the fluoroquinolone influence on lymphocyte activity of poikilothermic animals.

In our study the dose-dependent suppressive effect of NFLXN on T and B cell proliferative activity was seen at higher used concentrations. It was also found that B cells were more sensitive to the drug than T cells. Toxic effects of higher concentrations of fluoroquinolones, including norfloxacin, on lymphocyte activity were also described in the studies on

mammalian cells. However the conducted studies generally concerned T cell reactivity, and the drug concentrations which reduced cell functions differ depending on the studied model, used mitogen or laboratory conditions [10].

Yu et al. [11] found that norfloxacin, ciprofloxacin and enoxacin at 40 μg/ml suppressed PHA induced human blood lymphocyte proliferation, with simultaneous increase of IL-2. Lower concentrations of fluoroquinolones (norfloxacin, ciprofloxacin, amifloxacin) were found not to change the cell reaction [12]. Impairment of lymphocyte proliferative ability by fluoroquinolones was also seen in the *in vivo* studies in mammals. Ciprofloxacin at doses of 5 and 10 mg/kg m.c. applied for 3 days to mice caused marked suppression of T and B cell proliferation and diminished delayed type hypersensitivity but only at the higher dose [13]. Moreover norfloxacin at a dose of 800 mg inhibited in humans 2-way mixed lymphocyte reaction [14] which suggests that the drug impairs lymphocyte proliferation ability induced by foreign MHC molecules.

Generally it is believed that high fluoroquinolone concentrations inhibit ^{3}H-thymidine uptake by lymphocytes [10, 15, 16] and at the concentrations ≥20 μg/ml may block human lymphocyte transformation from G_o/G_1 to S phase of cell cycle [17, 18]. It should be also considered that effects exerted by high concentrations of fluoroquinolones may be a consequence of interaction with eucaryotic topoisomerases I and II or DNA α-polymerase, although these enzymes possess different molecular conformation than bacterial ones. It is also suggested that mitochondrial but not nuclear DNA topoisomerases functioning is disturbed by these drugs [19] resulting in cell cycle inhibition at different stages, including energy consuming S and G_2/M phases [17].

Suppresion of proliferative activity of lymphocytes may be also caused by the chelating properties of quinolones. As while mitogen triggering calcium level inside the lymphocyte increases that allows the cell to proliferate, binding bivalent ions properties of fluoroquinolones [20] reduce calcium availability and thus may interfere with calcium-mediated processes. Another possible influence of ciprofloxacin on lymphocyte proliferation is modulation of cytokine production, especially IL-1 production in the cell culture [16].

Fluoroquinolone ability to accumulate in phagocytic cells allows to kill bacteria staying alive after being phagocyted [21]. Because of high concentrations reached by the drugs inside the cells (5-10 times higher than outside [22]) it is very important to determine their potential effects on phagocytic cell functions.

In our study NFLXN only at a concentration of 5 μg/ml changed respiratory burst activity of the head kidney phagocytes. Similar stimulatory effects were seen in the studies on human cells. Boogaerts et al. [23] described increase of phagocytic and killing ability of polymorphonuclear cells after incubation with norfloxacin at a concentration of 10 μg/ml, manifested as increase of phagocyted and intracellular killed bacteria *Staphylococcus aureus*. Also in the studies by Desnottes [24] stimulation of phagocytic activity, increase of chemiluminescence and killing activity of human neutrophils after *in vitro* exposure to therapeutically relevant concentrations of norfloxacin, pefloxacin and enoxacin were seen. The effects observed in our study are similar to Hoeben's et al. [25, 26] observations from the *in vitro* study on bovine polymorphonuclear cells, in which low concentrations of fluoroquinolones stimulated chemiluminescence of the cells after PMA stimulation, higher ones caused return of the parameter to the control level, while very high concentrations, 100-1000 μg/ml, had inhibitory effects.

In conclusion, during NFLXN fish treatment high concentrations of the drug are reached in head kidney. As B cells showed to be more sensitive to the drug, it should be taken under consideration, that some negative effects on B cell mediated immunity may occur. On the other hand NFLXN at lower concentrations may slightly stimulate respiratory burst activity of phagocytic cells.

References

1. Rowley AF: Collection, separation and identification of fish leucocytes. In: Techniques in Fish Immunology. Ed. JS Stolen, TC Fletcher, DP Anderson, BS Roberson, WB Van Muiswinkel. SOS Publications. USA. 1990, 113-136.
2. Mosmann T (1983): Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.
3. Chung S, Secombes CJ (1988): Analysis of events occurring within teleost macrophages during the respiratory burst. Comp Biochem Physiol 89b: 539-544.
4. Press CL, Evensen O (1999): The morphology of the immune system in teleost fishes. Fish Sellfish Immunol 9: 309-318.
5. Martinsen B, Horsberg TE, Burke M (1994): Multiple-dose pharmacokinetic and depletion studies of sarafloxacine in Atlantic salmon, Salmo salar L. J Fish Dis 17: 111-121.
6. Lewicki J (1994): Enrofloksacyna. Życie Wet 3: 81-84.
7. Posyniak A (2000): Farmakokinetyka leków przeciwbakteryjnych u ryb. Medycyna Wet 56: 296-299.
8. Hussy P, Maass G, Tummler B, et al. (1986): Effect of 4-quinolones and novobiocin on calf thymus DNA polymerase alpha primase complex, topoisomerases I and II, and growth of mammalian lymphoblasts. Antimicrob Agents Chemother 29: 1073-1078.
9. Miyazaki T, Nakauchi R, Kubota SS (1984): Toxicological examination of oxolinic acid and nalidixic acid in yellow tail. Bull Fac Fish 11: 15-26.
10. Shalit I (1991): Immunological aspects of new quinolones. Eur J Clin Microbiol Infect Dis 10: 262-266.
11. Yu CC, Kelly PA, Burckart GJ, et al. (2001): Sirolimus inhibition of lymphocyte proliferation is not antagonized by ciprofloxacin and other quinolone antibiotics. Transplant Proc 33: 2989-2991.
12. Manzella JP, Clark JK (1988): Effects of quinolones on mitogen-stimulated human mononuclear leukocytes. J Antimicrob Chemother 21: 183-186.
13. Jimenez-Valera M, Sampedro A, Moreno E, Ruiz-Bravo A (1995): Modification of immune response in mice by ciprofloxacin. Antimicrob Agents Chemother 39: 150-154.
14. De Simone C, Vullo V, Grassi M, et al. (1986): Assessment of immunomodulating activity of ofloxacin and three other quinolones in human plasma. Infection 14 (Suppl 4): S248-S249.

15. Riesbeck K, Forsgren A (1994): Limited effects of temafloxacin compared with ciprofloxacin on T-lymphocyte function. Antimicrob Agents Chemother 38: 879-882.
16. Roche Y, Fay M, Gougerot-Pocidalo MA (1987): Effects of quinolones on interleukin 1 production in vitro by human monocytes. Immunopharmacology 13: 99-109.
17. Forsgren A, Schlossman SF, Tedder TF (1987): 4-Quinolone drugs affect cell cycle progression and function of human lymphocytes in vitro. Antimicrob Agents Chemother 31: 768-773.
18. Riesbeck K, Forsgren A, Henriksson A, Bredberg A (1998): Ciprofloxacin induces an immunomodulatory stress response in human T lymphocytes. Antimicrob Agents Chemother 42: 1923-1930.
19. Castora FJ, Vissering FF, Simpson MV (1983): The effect of bacterial DNA gyrase inhibitors on DNA synthesis in mammalian mitochondria. Biochim Biophys Acta 740: 417-427.
20. Marshall AJ, Piddock LJ (1994): Interaction of divalent cations, quinolones and bacteria. J Antimicrob Chemother 34: 465-483.
21. Cacchillo DA, Walters JD (2002): Effect of ciprofloxacin on killing of Actinobacillus actinomycetemcomitans by polymorphonuclear leukocytes. Antimicrob Agents Chemother 46: 1980-1984.
22. Dębowy J (1997): Chinolony we współczesnej farmakoterapii weterynaryjnej. Medycyna Wet 53: 307-312.
23. Boogaerts MA, Malbrain S, Scheers W, Verwilghen RL (1986): Effects of quinolones on granulocyte function in vitro. Infection 14 (Suppl 4): S258-S262.
24. Desnottes JF (1987): Quinolones and phagocytosis. Pathol Biol (Paris) 35: 1426-30.
25. Hoeben D, Dosogne H, Heyneman R, Burvenich C (1997): Effect of antibiotics on the phagocytic and respiratory burst activity of bovine granulocytes. Europ J Pharmacol 332: 289-297.
26. Hoeben D, Burvenich C, Heyneman R (1997): Influence of antimicrobial agents on bactericidal activity of bovine milk polymorphonuclear leukocytes. Vet Immunol Immunopathol 56: 271-282.