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Chemical reagents for immunodetection of fluoroquinolones with determinable specificity

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A new approach is proposed to obtain specific immunoreagents for detection of fluoroquinolones (FQs) that have a cyclopropyl radical in the 1st position of the quinolone core. The recommended immunogens should use coupling to a protein carrier via a carboxyl group in the 3rd position of the FQs, whereas solid-phase antigens for immunoassay should be obtained using the active groups of the radical in the 7th position. The given approach was applied to produce polyclonal antibodies against 3 FQs, danofloxacin (DAN), clinafloxacin (CLI), and moxifloxacin (MOX). The developed enzyme immunoassays based on their use are characterized by detection limits of 0.5 ng/mL for DAN, 1.0 ng/mL for CLI, and 0.5 ng/mL for MOX. Interactions of the antibodies with 25 representatives of FQs were studied, and the possibility of using the obtained reagents for immunodetection of FQs with broad cross-reactivity was shown.

Keywords*: antibodies, fluoroquinolones, 3D structures, enzyme immunoassay, cross-reactivity*

INTRODUCTION

Fluoroquinolones (FQs) are a class of antibiotics now widely used in veterinary medicine [1]. Consequently, the systematic intake of antibiotic-containing food may be accompanied by a number of undesirable effects such as disbacterioses, allergy, metabolic disorders, and selection of antibiotic-resistant strains [2]. Therefore, monitoring of FQs along food chains is an extremely important task. Immunoassays are among the leading approaches to antibiotics detection due to their simplicity, productivity, and relatively low cost [3, 4]. Antibodies are key reactants for immunoassays that determine sensitivity and specificity of analyte detection. Immunogens of different structures can be used to generate anti-FQ antibodies, and their structure may lead to narrow [5, 6] or broad [7-9] specificity of the obtained antibodies. Therefore, the choice of immunogens and protein-hapten conjugates for competitive assays needs detailed consideration to reach desirable immunoassay parameters.

The molecular structure of all FQs (Fig. 1) contains a carboxyl group, which is most often used for conjugation by carbodiimide activation or method of mixed anhydrides [10-14]. The carbodiimide syntheses may be realized in several ways depending on the presence or absence of the primary or secondary amino groups in the radical in the 7th position of the quinolone nucleus. The most common practices are FQ modification at the secondary nitrogen atom of the piperazine radical in the 7th position of the quinolone core [7-9], or obtaining recombinant antibodies [13, 15].

One of the promising approaches for studying the influence of the structure of the FQ derivatives on the specificity of antibodies is 3-dimensional (3D) modeling of chemical structures, and the FQ class is especially convenient for this in view of its vastness. Special software (HyperChem, Sybyl, etc.) allows not only building 3D models of compounds and optimizing them in space, but also identifying the structure-property correlations, and even simulating the structure of the immunogen to produce antibodies with the desired specificity [16]. In addition, the analysis of cross-reactions of antibodies to several structurally close compounds provides the possibility of determining the properties of the active center of antibodies to predict their specificity to other, not-experimentally-characterized compounds [17].

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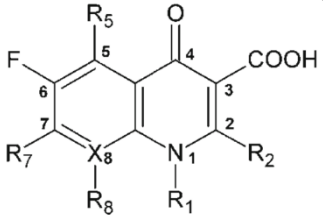

Fig. 1. General structure of fluoroquinolones.

The purpose of this work is to obtain reagents for immune detection of FQs with determinable specificity.

EXPERIMENTAL

Reactants

We used danofloxacin (DAN), clinafloxacin hydrochloride (CLI), moxifloxacin (MOX), ofloxacin (OFL), R-ofloxacin (R-OFL), garenoxacin (GAR), pefloxacin (PEF), gatifloxacin (GAT), sarafloxacin hydrochloride (SAR), lomefloxacin (LOM), sparfloxacin (SPA), difloxacin (DIF), pazufloxacin (PAZ), marbofloxacin (MAR), rufloxacin (RUF), norfloxacin (NOR), ciprofloxacin (CIP), enrofloxacin (ENR), pipemidic acid (PIP), nalidixic acid (NAL), oxinic acid (OXI), orbifloxacin (ORB), enoxacin (ENO), nadifloxacin (NAD), flumequin (FLU), bovine serum albumin (BSA), ovalbumin (OVA), casein, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (HSI), 3,3′,5,5′-tetramethyl benzydine (TMB), and N,N-dimethylformamide (DMF) from Sigma-Aldrich Chemical (St. Louis, MO, USA) plus horseradish peroxidase–labeled antibodies against rabbit IgG (H+L) from the N.F. Gamaleya Research Institute of Epidemiology and Microbiology (Moscow, Russia). All auxiliary reagents (salts, acids, alkalis, and organic solvents) were of analytical or chemical purity.

Synthesis of FQ conjugates by carbodiimide activation

FQ conjugates with carrier proteins were synthesized according to procedures [12, 18] with some modifications. 14.7 μmol of a FQ, 5.7 mg (30 μmol) of EDC, and 3.5 mg (30 μmol) of HSI were dissolved in 1.0 mL of DMF. The mixture was incubated for 2 hours with stirring. A solution of 10 mg of a protein was prepared in 8 mL of 100 mM sodium carbonate buffer, pH 9.5, with 50 μL of triethylamine, and the mixture was incubated for 1 h at +4°C. A solution of a FQ with an activated carboxyl group was added dropwise with constant slow stirring to the protein solution. Then the mixture was incubated with stirring for 5 h at room temperature in the dark. The synthesized conjugate was purified from low-molecular-weight substances against 0.01 M K-phosphate buffer, pH 7.4. Thus, the conjugates MOX-BSA, DAN-BSA, and CLI-BSA were obtained.

Synthesis of FQ conjugates using glutaraldehyde

The synthesis was carried out in accordance with previously published procedure [7] with some modifications. Five milligrams (0.11 μmol) of OVA and 2.2 mg (5.6 μmol) of CLI were dissolved in 8 mL of distilled water, and 20 μL of triethylamine was added. With vigorous stirring, 23 μL (5.6 μmol) of 2.5% glutaraldehyde was added. The solution was incubated for 1 h in the dark with constant vigorous stirring at room temperature. Five hundred microliters (30 μmol) of a 0.22% sodium borohydride solution in distilled water was added and the obtained mixture was incubated for 30 min. The synthesized conjugate was purified from low-molecular-weight substances by dialysis with an 0.01 M K-phosphate buffer, pH 7.4. Thus, the conjugate CLI-NH-C5-NH-OVA was synthesized.

Enzyme-linked immunosorbent assay (ELISA)

The conjugate KLI-NH-C5-NH-OVA (100 μL, 0.5 μg/mL) in 50 mM K-phosphate buffer, pH 7.4, containing 0.1 M NaCl (phosphate-buffered saline [PBS]), was immobilized in microplate wells overnight at 4°C. The wells were then washed 4 times with PBS containing 0.05% Triton X-100 (PBST). For analysis, 50 μL of a FQ solution in PBST (from 0.1 to 1000 ng/mL) and 50 μL of antibody solution (1:2000) were added to the wells. The mixture was incubated for 1 h at 37°C; then the wells were washed by PBST 4 times. One hundred microliters of the immunoperoxidase conjugate (1:6000 dilution in PBST) was added to the wells and incubated for 1 h at 37°C. After washing (3 times with PBST and once with distilled water), the activity of peroxidase bound to the carrier was determined. To do this, 100 μL of a 0.4 mM solution of TMB in 40 mM Na-citrate buffer, pH 4.0, containing 3 mM H_2O_2, were added to the wells and incubated for 15 min at room temperature. The reaction was stopped by the addition of 50 μL of 1 M H_2SO_4 per well. The optical density (D_{450}) of the reaction product was measured at 450 nm on a Zenyth 3100 microplate photometer.

Estimation of specificity

Cross-reactivity of the assay was evaluated as the percentage of cross-reaction with other compounds in comparison with the target analyte:

$$CR(X)\% = IC_{50}(\text{target analyte})/IC_{50}(X) * 100\%,$$

where IC_{50}(target analyte) is the concentration of the target substance at which the value of the analytical signal decreases by 50% from the

difference between the maximum and minimum signals, and $IC_{50}(X)$ is the concentration of the cross-reacting substance causing the same 50% decrease.

3D modeling

To calculate the conformation of FQ molecules in a vacuum, the Polak-Ribière algorithm was used. The chosen criterion for termination of the approximation cycles was a mean square gradient equal to 5 cal/(Å × mol). Then the conformations of the molecules in the solvated form were calculated. The obtained structures were compared using superposition between them.

RESULTS AND DISCUSSION

Structural analysis of FQs

Selectivity of immunoassays for FQs is connected to the following feature of the molecular structure of substances from this class (Fig. 1). For FQs, the carboxyl group in the 3rd position, the keto group in the 4th position, and the fluorine atom in the 6th position are constant structural compounds. Radicals in the 1st, 7th, and 8th positions form the variable region, being individual for different FQs. Therefore, for selective immunoassays of individual compounds, it is necessary to synthesize immunogens and protein conjugates using groups of the constant region (most often the carboxyl group in the 3rd position). To ensure group specificity of FQ immunoassays, it is necessary to use the conjugates synthesized via groups from the variable region. Most often [7-9], the radical with the primary or secondary amino group in the 7th position or the newly introduced carboxyl or amino groups are used for this purpose.

The combination of existing methods has allowed us to develop a new approach for immune detection of FQs with a certain radical in the 1st position. The approach consists of the use of immunogens synthesized via the carboxyl group in the 3rd position, and solid-phase antigens synthesized via active groups of the radical in the 7th position. The proposed approach was implemented to obtain antibodies and develop enzyme immunoassays of FQs with a cyclopropyl radical in the 1st position of the quinolone core (Fig. 2).

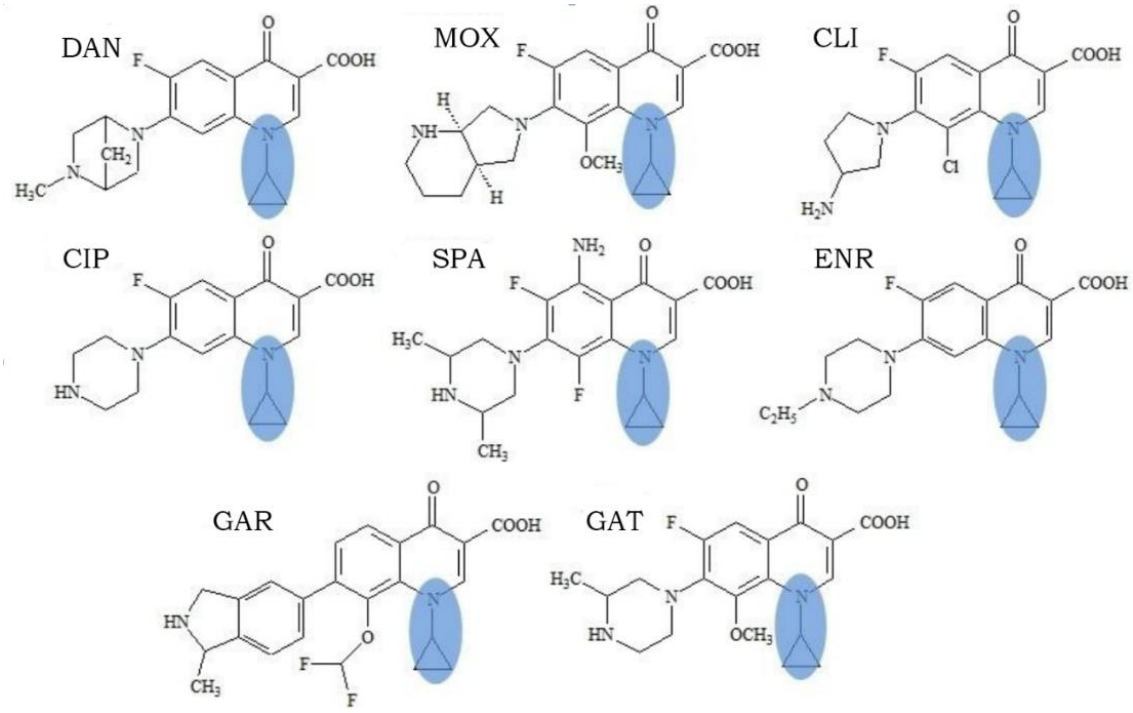

Fig. 2. Fluoroquinolones with a cyclopropyl radical in the 1st position of the quinolone core.

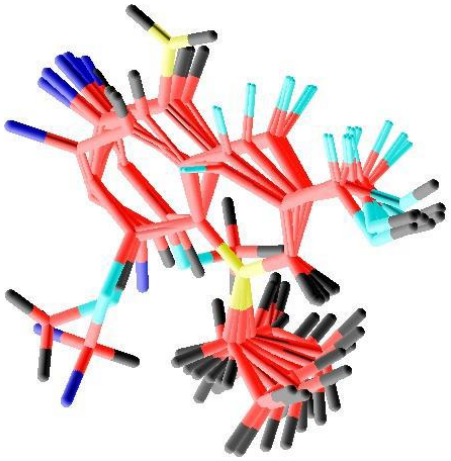

Fig. 3. Three-dimensional structures of 8 fluoroquinolones (danofloxacin, moxifloxacin, clinafloxacin, sparfloxacin, enrofloxacin, garenoxacin, and gatifloxacin) with a cyclopropyl radical in the 1st position of the quinolone core.

Using the HyperChem software and methods of molecular mechanics, the 3D structures of molecules in solvated form were optimized. After that, superposition on the nitrogen atom in the first position and 2 adjacent carbon atoms of the quinolone nucleus was applied, and the conformations of the FQs were combined, excluding the radicals in the 7th position (Fig. 3).

As you can see, the conformations of the FQ molecules with a cyclopropyl radical in the 1st position of the quinolone nucleus are variable, which can affect the specificity of their immunodetection. Note that even the constant region is characterized by a certain variation, for example, the spatial orientation of the keto group in the 4th position. The carboxyl group in the 3rd position and the fluorine atom in the 6th position have the most constant conformation, which suggests the absence of the influence of these radicals on specificity of immune recognition.

Production of antibodies to FQ

In the development of ELISA techniques, anti-DAN antibodies (obtained and characterized previously [19]), anti-MOX, and anti-CLI antibodies were used. CLI-NH-C5-NH-OVA was used as a solid-phase conjugate, in which the primary amino group of clinafloxacin at the 7th position of the quinolone core was conjugated with OVA via glutaraldehyde. Thus, the target fragments for antibodies are radicals in the 1st and 8th positions of the quinolone core.

Using these reagents, the conditions of the ELISAs were optimized to ensure maximum sensitivity of FQ detection. The calibration curves obtained under optimized conditions were characterized by the limits of detection of 0.5 ng/mL for DAN, 0.5 ng/mL for MOX, and 1 ng/mL for CLI (Fig. 4a-c).

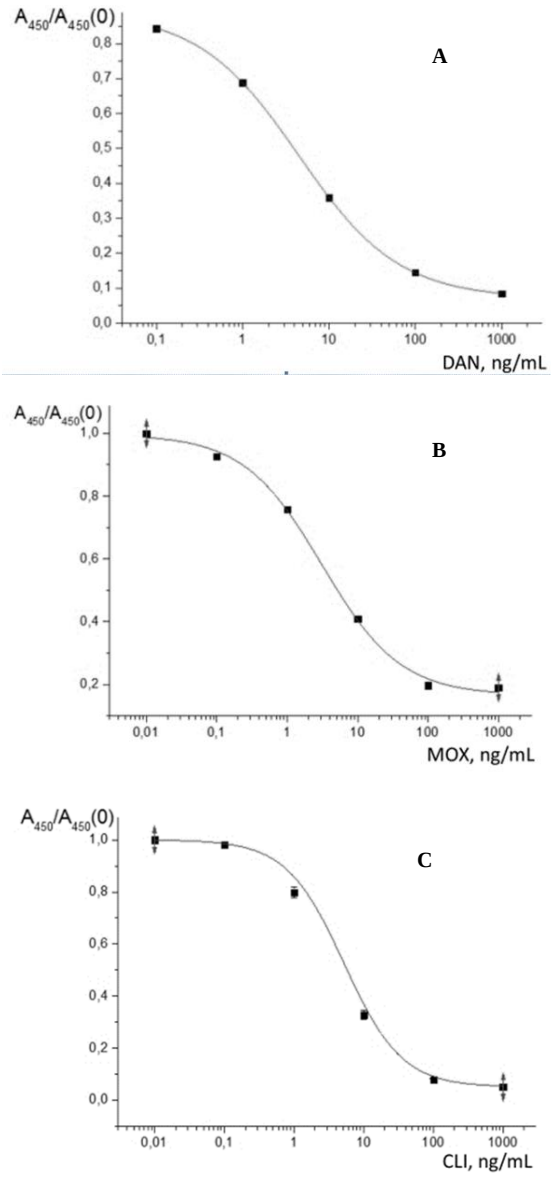

Fig. 4. Calibration curves for enzyme-linked immunosorbent assay (ELISA) of danofloxacin (a), moxifloxacin (b), and clinafloxacin (c).

Characterization of the specificity of antibodies to FQs

Evaluation of the specificity of the developed ELISA techniques was carried out using 25 compounds from the FQ class. Anti-DAN, anti-MOX, and anti-CLI antibodies are not cross-reactive with PEF, OFL, R-OFL, SAR, DIF, PAZ, MAR, RUF, NOR, PIP, NAL, OXI, and FLU. These 3 antibodies are all characterized by cross-reactivity with 12 other FQs (the cross-reactivity is more than 1%), 8 of which have a cyclopropyl radical in the structure and are characterized by maximum values of the cross-reactivity (Table 1).

Table 1. Data on the specificity of anti-DAN, anti-MOX, and anti-CLI antibodies in ELISA with the solid phase antigen CLI-NH-C5-NH-OVA.

Antigen	Antibodies		
	anti-DAN	anti-MOX	anti-CLI
	Cross-reactivity, %		
DAN	100	48	63
MOX	<1	100	10
CLI	15	120	100
CIP	38	20	73
SPA	5	5	10
ENR	25	10	33
GAR	5	100	92
GAT	5	27	20
NAD	130	67	90
ENO	5	40	5
LOM	5	80	<1
ORB	2	<1	10

CONCLUSIONS

The results obtained confirm the proposed approach for reactants obtained for immunodetection of FQs with a certain radical in the 1st position. The antibodies and conjugates obtained provide a wide selectivity and are the basis for the development of various formats of immunoassay systems for FQs.

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