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Two Short Peptides Including Segments of Subunit A of *Escherichia coli* DNA Gyrase as Potential Probes to Evaluate the Antibacterial Activity of Quinolones

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Abstract: Quinolones constitute a family of compounds with a potent antibiotic activity. The enzyme DNA gyrase, responsible for the replication and transcription processes in DNA of bacteria, is involved in the mechanism of action of these drugs. In this sense, it is believed that quinolones stabilize the so-called 'cleavable complex' formed by DNA and gyrase, but the whole process is still far from being understood at the molecular level. This information is crucial in order to design new biological active products. As an approach to the problem, we have designed and synthesized low molecular weight peptide mimics of DNA gyrase. These peptides correspond to sequences of the subunit A of the enzyme from *Escherichia coli*, that include the quinolone resistance-determining region (positions 75–92) and a segment containing the catalytic Tyr-122 (positions 116–130). The peptide mimic of the non-mutated enzyme binds to ciprofloxin (CFX) only when DNA and Mg^{2+} were present ($K_d = 1.6 \times 10^{-6}$ M), a result previously found with DNA gyrase. On the other hand, binding was reduced when mutations of Ser-83 to Leu-83 and Asp-87 to Asn-87 were introduced, a double change previously found in the subunit A of DNA gyrase from several CFX-resistant clinical isolates of *E. coli*. These results suggest that synthetic peptides designed in a similar way to that described here can be used as mimics of gyrases (topoisomerases) in order to study the binding of the quinolone to the enzyme–DNA complex as well as the mechanism of action of these antibiotics. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: affinity chromatography; DNA gyrase; *Escherichia coli*; peptide design; peptide synthesis; quinolones; solid phase; topoisomerases

INTRODUCTION

The three-dimensional structure of DNA plays a key role in biological processes such as replication, transcription and recombination. The enzymes responsible for maintaining the topological state of DNA are termed DNA topoisomerases. These proteins are essential to all cells and, as such, they are important targets for many clinically antibacterial drugs. DNA gyrase [1] is one of the most studied enzymes from bacteria and constitutes the intracellular target of quinolones, a group of drugs with a wide-spectrum of antibacterial activity.

Abbreviations: Ahx, ε-aminohexanoic acid; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; CFX, ciprofloxacin; DIEA, *N*,*N*′-diisopropylethylamine; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; *E. coli*, *Escherichia coli*; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; gyrA, subunit A of DNA gyrase; gyrB, subunit B of DNA gyrase; HOBt, 1-hydroxy-benzotriazole; *p*-MBHA, *p*-methylbenzhydrylamine; MCA, ε-maleimidocaproic acid; NMP, *N*-methyl-2-pyrrolidone; Pbs, pBlueScript; PyBOP, benzotriazol-1-yl-oxy-*tris*-pyrrolidino-phosphonium; rt, retention time; ssDNA, single-stranded DNA; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

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DNA gyrase is a type II topoisomerase consisting of two subunits, A (gyrA) and B (gyrB), which form the active complex A_2B_2 [2]. These subunits have a molecular weight of 97 kD (874 residues) and 90 kD (803 residues), respectively, in the case of *Escherichia coli*. This enzyme introduces negative supercoils into DNA in an adenosine triphosphate (ATP)-dependent manner, catalysing the cleavage of a double-stranded DNA (pBS, pBlueScript) segment and the formation of a transient DNA gate through which another segment is passed. It is known that quinolones inhibit the activity of DNA gyrase through the Mg^{2+}-mediated formation of a tertiary quinolone–enzyme–DNA complex as a key step of the process. However, the way in which the substrates interact [3–9], as well as the functions that the complex develops at the molecular level [10–16], are still matters of controversy.

Finding answers to these questions is crucial in order to design new antibiotic drugs which will prevent bacterial infection or avoid resistance problems. From the structural point of view, this study implies a tremendous difficulty because of the size of the complex. This observation prompted us to consider the possibility of using short peptides containing segments of gyrA involved in the recognition of DNA and quinolones as models to carry out binding studies. In this paper, we report on two 35-residue peptides formed by a *C*-terminal region including the catalytic Tyr residue at position 122 [17] and an *N*-terminal region containing residues 83 and 87, which are believed to be involved in the interaction with quinolones (Figure 1) [18]. Single-stranded DNA (ssDNA) and pBS were used in this work together with the antibiotic ciprofloxacin (CFX) as the quinolone model. Affinity chromatography [19] and membrane filtration techniques were exploited to perform the binding studies [20]. Evidence that these peptides might constitute suitable models to study gyrase–DNA–quinolone interactions as well as the mechanism of action of the drugs is presented.

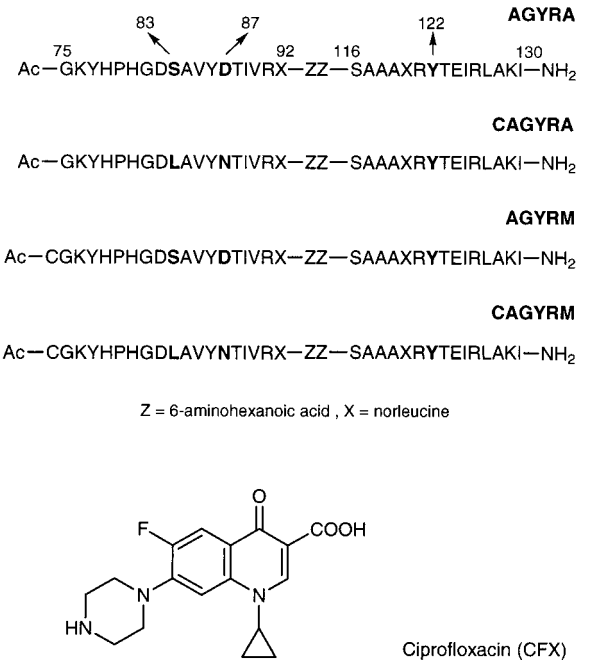

Figure 1

MATERIALS AND METHODS

Tert-butoxycarbonyl (Boc)-amino acids were supplied by Novabiochem AG (Läufelfingen, Switzerland), Bachem Feinchemikalien AG (Bubendorf, Switzerland), Adanced ChemTech (Maidenhead, UK), Propeptide (Vert-le-Petit, France) or Neosystem (Gennevilliers, France).

HF was from UCAR. *P*-Methylbenzhydrylamine (*p*-MBHA) resin (0.45 mmol g^{-1}) was purchased from Bachem, Sepharose 4B (7–12 μmol ml^{-1}) was supplied by Pharmacia Biotech (Sweden) and epoxy-activated Sepharose 6B (19–40 μmol ml^{-1}) was from Sigma–Aldrich Química (Madrid, Spain). Benzotriazol-1-yl-oxy-*tris*-pyrrolidinophosponium (PyBOP) was from Novabiochem. *N*,*N*′-diisopropylcarbodiimide (DIPCDI), 1-hydroxybenzotriazole (HOBt), Ac_2O and propionic acid were supplied by Fluka Chimie AG (Buchs, Switzerland). Dimethylformamide (DMF), supplied by Scharlau (Spain), was bubbled with nitrogen to remove volatile contaminants and kept stored over activated 4 Å molecular sieves. *N*-methyl-2-pyrrolidone (NMP) was from Applied Biosystems. HCl and Tris buffer were from Merck (Darmstadt, Germany). Thiophenol, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and ε-maleimidocaproic acid (MCA) were obtained from Sigma–Aldrich. *N*,*N*′-diisopropylethylamine (DIEA) was from Acros (Geel, Belgium). MeCN (Scharlau) was high performance liquid chromatography (HPLC) grade; dichloromethane (DCM) (Scharlau) and trifluoroacetic acid (TFA) (Solvay, Germany) were peptide synthesis grade and were used directly. $MgCl_2$-$6H_2O$, NaH_2PO_4 and Na_2HPO_4-$12H_2O$ were supplied by Fluka and NaCl was from J. Escuder (Spain). Bayer (Spain) supplied CFX as a kind gift.

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The supercoiled plasmid pBS was prepared in *E. coli* strain DH5α by conventional methods [21] and was purified by the optimized alkaline lysis method [22] followed by purification on a QIAGEN resin (purchased from Qiagen GmbH-Germany). Calf thymus ssDNA was purchased from Sigma–Aldrich. The pBS plasmid and ssDNA mother solutions were diluted with 5 mM Tris–HCl buffer (≈ 4 μg μl^{-1}, pH 7.2), frozen and stored.

Peptide-resins were hydrolysed using 12 M HCl/propionic acid (1:1) at 155°C for 2 h and peptides were hydrolysed in 6 M aqueous HCl solution at 155°C for 90 min. Amino acid analyses were performed on a Beckman System 6300 analyser.

Analytical HPLC was carried out on a Shimadzu apparatus comprising two solvent delivery pumps model LC-6A, automatic injector model SIL-6B69A, variable wavelength detector model SPD-6A, system controller model SCL-6B and plotter model C-R6A. Nucleosil C_{18} reverse-phase columns were used (25 × 0.5 cm, 5 μm). In general, peptides were eluted at a flow rate of 1 ml min^{-1} (A: water, 0.045% of TFA; B: MeCN, 0.036% of TFA) and detection was carried out at 220 nm.

Semi-preparative HPLC and medium performance liquid chromatography (MPLC) techniques were used for purification purposes. The former was performed in a Waters analyser consisting of a fluid unit model 600, automatic injector model 717, variable wavelength detector model 490E, system controller model 600E and a Shimadzu plotter model C-R5A. Nucleosil C_{18} reverse-phase columns were used (25 × 1 cm, 5 μm) at a flow rate of 3 ml min^{-1} and with the eluents mentioned above. Detection was carried out at 220 nm. Reverse-phase MPLC was carried out using a CFG-Prominent/Duramat pump, a 757 ABI variable wavelength detector, an automatic fraction collector model Gilson FC 205 and an Omniscribe rec 101 plotter (Pharmacia Biotech). A glass column (2.5 × 26 cm) packed with reverse-phase Vydac-C_{18} (15–20 μm) was used. A flow rate of 2 ml min^{-1} was utilized (A and B: 400 ml mixtures of water/MeCN with 0.05% of TFA) and the products were detected at 220 nm.

Binding data were determined by the membrane filtration method, using Microcon centrifugal filter units that utilize thin membranes ultracell-YM with a molecular weight cut-off of 50 kD. Centrifugations were carried out in a Beckman GS-15R centrifuge equipped with a fixed angle F2402H rotor. Fluorescence spectroscopy was performed using an Aminco–Bowman AB2 instrument. To carry out the experiments 1 cm-path length cells (1 ml) were used. Spectra were registered between 350 nm and 550 nm in the case of CFX (excitation, 330 nm; emission, 417 nm) and between 280 nm and 303 nm when AGYRA or AGYRM were used (excitation, 280 nm; emission, 303 nm). Ultraviolet spectroscopy was carried out in a Perkin–Elmer Lambda 5 apparatus, using a 1 cm-path length cell (2 ml). Spectra were registered between 230 nm and 280 nm, following the presence of DNA at 260 nm.

General Procedure for the Solid-phase Assembly of Peptides and their Acidolytic Cleavage from the Resin

Peptides were prepared by the solid-phase methodology and following the Boc/Benzyl strategy [23,24]. Protecting groups for amino acids were 2-Cl-Z (Lys), Tos (Arg), OcHx (Asp and Glu), Bzl (Ser and Thr), 2-Br-Z (Tyr), Dnp (His) and MeBzl (Cys). The syntheses were performed manually in polypropylene syringes fitted with a polyethylene disc. Prior to use, the polymeric support (*p*-MBHA resin, 1 g batches) was washed with (1) DCM, 1 × 5 min; (2) 40% TFA in DCM, 1 × 2 min + 1 × 20 min; (3) 5% DIEA in DCM, 3 × 2 min; (4) DCM, 3 × 1 min. Boc-amino acids were assembled using the following protocol: (1) DCM, 2 × 4 min; (2) 40% TFA in DCM, 1 × 2 min + 1 × 20 min; (3) DCM, 3 × 1 min; (4) DMF, 3 × 1 min or NMP, 1 min; (5) Boc-amino acid coupling and (6) DMF, 3 × 1 min; (7) DCM, 3 × 1 min. The fragment Nle-120/Ile-130 was assembled using DIPCDI/HOBt (Boc-AA-OH [5 eq], HOBt [5 eq], DIPCDI [5 eq]; 1 h) and the rest of the amino acids were coupled with PyBOP/DIEA (Boc-AA-OH [3 eq], PyBOP [3 eq], DIEA [6 eq]; 1 h). An additional treatment with 40% TFA in DCM (10 min) was needed in order to achieve α-amino deprotection from position 83. Acetylation was performed as follows: (1) Ac_2O (10 eq) and DIEA (10 eq), 30 min; (2) DMF, 3 × 1 min; (3) DCM, 3 × 1 min.

Deprotection of His residue was performed prior to the cleavage from the resin using the following protocol: (1) DCM, 3 × 1 min; (2) DMF, 3 × 1 min; (3) Thiophenol/DIEA/DMF (3:3:4), 1 × 30 min; (4) DMF, 3 × 1 min; (5) H_2O, 3 × 1 min; (6) MeOH, 3 × 1 min; (7) DCM, 3 × 1 min; (8) 40% TFA in DCM, 3 × 1 min; (9) neat TFA, 1 × 20 min; (10) DCM, 6 × 1 min.

Peptides were cleaved from the resins with HF on a Kel-F Toho–Kasei Ltd (Tokyo, Japan) apparatus. Peptidyl-resins (350–950 mg batches) were treated with 4.5 ml of HF and 500 μl of anisole during 1 h at 0°C. The resins were washed with 10 ml of AcOEt and the resulting suspension was washed with

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10 × 5 ml of 10% aqueous AcOH. The fractions were joined and lyophilized. Products were purified by MPLC and/or semipreparative HPLC, volatiles were removed under vacuum and the remaining solutions were lyophilized.

AGYRA

A total of 350 mg of peptidyl-resin were treated with thiophenol following the protocol described above to remove the Dnp protecting group. The acidolytic treatment of the resulting material afforded 118 mg (23 μmol) of the crude peptide (79%), which was eluted under MPLC conditions with a 20–48% convex gradient of organic component. The peptide was further eluted under semi-preparative HPLC conditions with a 25–45% linear gradient of organic component over 30 min to give 30.7 mg (5.1 μmol, 23% recovery; 13% overall yield). HPLC: rt, 20.3 min; 25–45% of B over 30 min. Matrix-assisted laser desorption ionization/time of flight-mass spectrometry (MALDI/TOF-MS): m/z 3952.5; $C_{180}H_{292}N_{51}O_{49}$ requires 3952.2.

AGYRM

A total of 838 mg of peptidyl-resin were treated with thiophenol following the protocol described above to remove the Dnp protecting group. The acidolytic treatment of the resulting material afforded 308 mg (56.9 μmol) of the crude peptide (85%), which was eluted under MPLC conditions with a 23–48% convex gradient of organic component. The peptide was further eluted under semi-preparative HPLC conditions with a 30–40% linear gradient of organic component over 30 min to give 46.5 mg (7.8 μmol, 21% recovery; 12% overall yield). HPLC: rt, 20.9 min; 25–45% of B over 30 min. MALDI/TOF-MS; m/z 3981.1; $C_{183}H_{299}N_{52}O_{47}$ requires 3977.3.

CAGYRA

A total of 957 mg of peptidyl-resin were treated with thiophenol following the protocol described above to remove the Dnp protecting group. The acidolytic treatment of the resulting material afforded 263 mg (56 μmol) of the crude peptide (80%), which was eluted under MPLC conditions with a 23–38% convex gradient of organic component. The peptide was further eluted under semi-preparative HPLC conditions with a 30–40% linear gradient of organic component over 30 min to give 8.3 mg (1.6 μmol, 15% recovery; 7% overall yield). HPLC: rt, 20.2 min; 25–45% of B over 30 min. MALDI/TOF-MS: m/z 4055.2; $C_{183}H_{297}N_{52}O_{50}S$ requires 4055.8.

CAGYRM

A total of 513 mg of peptidyl-resin were treated with thiophenol following the protocol described above to remove the Dnp protecting group. The acidolytic treatment of the resulting material afforded 146 mg (23.5 μmol) of the crude peptide (75%), which was eluted under MPLC conditions with a 18–40% convex gradient of organic component. The peptide was further eluted under semi-preparative HPLC conditions with a 30–40% linear gradient of organic component over 30 min to give 9.2 mg (1.4 μmol, 16% recovery; 6% overall yield). HPLC: rt, 21.5 min; 25–45% of B over 30 min. MALDI/TOF-MS: m/z 4080.5; $C_{186}H_{304}N_{53}O_{48}S$ requires 4080.3.

Affinity Chromatography

CFX Immobilized on Sepharose. CFX was immobilized on epoxy-activated Sepharose as previously reported [19]. To 2 g of the polymeric support were added 200 mg of CFX in 6 ml of 0.3 M carbonate buffer (pH 9.5). The mixture was left for 17 h at 37°C when 0.55 ml of ethanolamine were added and reacted for 4 h at 37°C. After washings with carbonate buffer (pH 9.5), water, acetate buffer (pH 4), water, 5 M urea and water, a 1-ml column was packed and equilibrated with standard buffer (5 mM Tris–HCl, pH 7.2/20 mM NaCl/5 mM $MgCl_2$).

To the column were added DNA (pBS or ssDNA; 20 μl), peptide (AGYRA or AGYRM; 200 μM standard buffer solution) or mixtures of both and the samples were left into the column for 1 h at room temperature under gentle rocking. Further washings with standard buffer were performed until no absorbance at 260 nm (DNA) or no fluorescence at 303 nm were detected. The column was washed with 4 M NaCl before equilibration.

Peptide Immobilized on Sepharose. Peptides CAGYRA and CAGYRM were anchored to a 2 ml batch of Sepharose resin previously functionalized with MCA, following the supplier's recommendations. Before coupling the linker, the polymeric support was put into a polypropylene syringe fitted with a polyethylene disc and washed as follows: (1) 0.5 M aqueous NaCl solution, 10 × 5 ml; (2) overnight equilibraiton with at 4°C with 0.5 M aqueous NaCl solution; (3) 0.5 M aqueos NaCl solution, 10 × 5 ml; (4) water, 10 × 10 ml. The resin was then transferred to a 50 ml round-bottom flask containing a solution of 63 mg (15 eq) of MCA in 4 ml of water/EtOH (2.5: 1.5) at pH 4.5. To this mixture were added, with smooth magnetic stirring, 58 mg (15 eq) of EDC. The pH was

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adjusted and kept between 4.5 and 6 during 1 h with diluted NaOH solution. After 14 h at room temperature, the slightly ninhydrin-positive functionalized polymeric support [25] was transferred to a syringe fitted with a polyethylene disc and washed as follows: (1) water/EtOH (2.5:1.5), 10 × 10 ml; (2) acetate buffer (0.5 M NaCl, pH 3.8) and Tris–HCl (0.5 M NaCl, pH 7.2), 10 × 10 ml alternatively; (3) water, 10 × 10 ml; (4) EtOH, 3 × 10 ml; (5) water, 10 × 10 ml. To the resulting resin were added 4 ml (2000 eq) of 1 M aqueous AcOH and 387 mg (100 eq) of EDC. A negative ninhydrin test was achieved after leaving the mixture for 5 h with smooth mechanical stirring at room temperature. Then, half of the resin was transferred to a 2 ml polypropylene syringe fitted with a polyethylene disc and 1.5 ml of a peptide (CAGYRA, 0.85 μmols; CAGYRM, 0.71 μmols) solution in phosphate buffer (pH 7.8) with a few drops of 20% aqueous AcOH were added. The mixture was left at room temperature for 16 h with smooth mechanical stirring, when the resin was filtered and washed with water and diluted aqueous AcOH. The resin was packed and equilibrated in a 1 ml column with standard buffer (5 mM Tris–HCl, pH 7.2/20 mM NaCl/5 mM $MgCl_2$). Peptides CAGYRA and CAGYRM were coupled with yields of 67% (0.6 μmols) and 71% (0.55 μmols), respectively, as determined by amino acid analysis of the filtrates. Both peptide-resins were stored in 2 ml of 5 mM Tris–HCl buffer (pH 7.2) at 4°C.

To the columns thus prepared were added the DNA samples (pBS or ssDNA; 20 μl) or the CFX samples (200 μl, 60 μM standard buffer solution), and they were left into the columns for 1 h at room temperature under gentle rocking. Further washings with standard buffer were performed until no absorbance at 260 nm (DNA) or no fluorescence at 417 nm were detected. CFX was added after 30 min of loading the column with DNA when mixtures of DNA and the quinolone were used to carry out the binding experiments. The column was washed with 4 M NaCl before equilibration.

Binding Experiments

The samples consisted of 500 μl reaction mixtures in standard buffer containing: (a) 5 pmol of DNA and different amounts of CFX (binding of CFX to DNA); (b) 5 pmol of DNA and different amounts of AGYRA or AGYRM (binding of the peptide to DNA); (c) 5 pmol of DNA, 1000 pmol of AGYRA or AGYRM and different amounts of CFX (binding of CFX to the DNA/peptide complex). Mixtures (a) and (b) were incubated at 32°C for 60 min and were transferred to the Microcon devices. In the case of mixture (c), DNA and the peptide were preincubated at 32°C for 30 min before adding CFX. A further incubation of 30 min at 32°C was performed and the mixture was transferred to the Microcon device. The membrane lots were tested prior to use following Shen and Pernet's recommendations [26]. Samples were centrifuged at 12.000 rpm (10483 × g) for 15 min and the filtrates were collected in the reservoir on the lower end of the device. The amount of ligand bound was calculated by subtracting the amount of free ligand in the reaction mixture from the initial amount of ligand (both of them determined by measuring the intrinsic fluorescence intensity of CFX and the peptide). All of the binding data were analysed using the Klotz plots [27] in which the apparent dissociation constant is the midpoint of the saturation curve.

RESULTS

Peptide Design

In order to design peptide mimics of DNA gyrase to be used in the study of quinolone/peptide–DNA complex interactions, we focussed our attention on gyrA, which is the subunit of the enzyme where quinolones display their inhibitory effect [2]. Reece and Maxwell [28] identified by protein engineering the fragment 7-523 as the smallest domain in *E. coli* gyrA with DNA-cleavage activity when complexed with gyrB. Recently, the crystal structure determination of this domain has revealed structural details about the sites that are believed to be involved in quinolone/DNA recognition and DNA cleavage [29]. Thus, a helix-turn-helix motif at the *N*-proximal head and a cluster of positively charged residues surrounding Tyr-122 have been proposed as the binding site and the active site of the breakage-reunion reaction, respectively, the two sites being close to each other. The helix-turn-helix motif is also considered the quinolone resistance-determining region since most mutations in resistant strains are located in its sequence (residues 66–92). In this connection, special attention has to be paid to the *C*-terminal helical fragment (residues 81–92) because of the high number of mutations that have been found in this segment so far [8,9,18,30–32].

According to these results, we thought that a good starting point for this study was to consider for peptide design the helix mentioned above and a

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short sequence of the native protein containing the active site Tyr-122. Therefore, two fragments of the protein sequence, one including the *C*-terminal helix of the helix-turn-helix motif and the other including Tyr-122, were chosen to be part of the primary structure of the model peptide. Their lengths were set according to the presence of basic residues in the corresponding sequences. In this sense, it has to be pointed out that there are several of such residues (Lys, His and Arg) in the protein at the *N*-proximal extreme of the helix and near Tyr-122 that could be involved in electrostatic interactions with the DNA phosphate groups. Finally, we decided to replace the 23 amino acid long native sequence that connects the two fragments by a flexible linker to simplify the synthetic problem. Taking into account all these considerations, the model peptide AGYRA was built using the natural fragments 75–92 and 116–130, and two residues of Ahx to connect them (Figure 1).

Quinolone resistance seems to be a consequence of the loss of binding of the drug to the gyrase/DNA complex induced by mutations in the enzyme sequence [8]. This observation prompted us to include in this work a comparative study between AGYRA and an analogue that mimic a mutated gyrA. According to what was described in the literature, mutations have been found at positions 83 and 87 in most cases [8,9,18,30,32]. In particular, mutations of Ser-83 to Leu and Asp-87 to Asn induce high levels of resistance to the quinolone CFX [18]. These results prompted us to consider for this study CFX and AGYRA together with its analogue AGYRM, the peptide that mimics the mutated gyrA (Figure 1). Both peptides were acetylated at the *N*-terminus and have a carboxamide group at the *C*-terminus in order to mimic the presence of amide bonds at these positions in the protein. Moreover, Met residues were substituted by Nle residues (Nle can be considered an isoster of Met) in order to prevent undesired oxidation and/or alkylation processes during the synthesis of the peptides.

Our goal was to determine whether CFX could stabilize a ternary complex with DNA and AGYRA, the gyrA model compound, and furthermore, whether such a complex could or could not form in the presence of AGYRM. To this purpose, we designed assays based on affinity chromatography. In doing that, we considered the possibility of having the peptide or the quinolone covalently anchored to the resin in order to know how the substrate bound to a polymeric support affects the experimental results. With the aim of immobilizing the peptides AGYRA and AGYRM on a polymeric support, their analogues with a Cys residue at the *N*-terminal position were also prepared (CAGYRA and CAGYRM, respectively). This residue would allow the binding of the peptide chain to the resin through the thiol function of the Cys side chain.

Peptide Synthesis

The peptides were synthesized by the solid-phase methodology using the Boc/Bzl strategy and *p*-MBHA as the polymeric support (Figure 2). Some difficulties were encountered during the assembling of the peptide chains under the standard conditions. Thus, the DIPCDI/HOBt coupling system proved to be efficient for residues 119–130, but PyBOP had to be used for residues 75 to 92, *ZZ*, and 116–118 in order to get acceptable coupling yields.

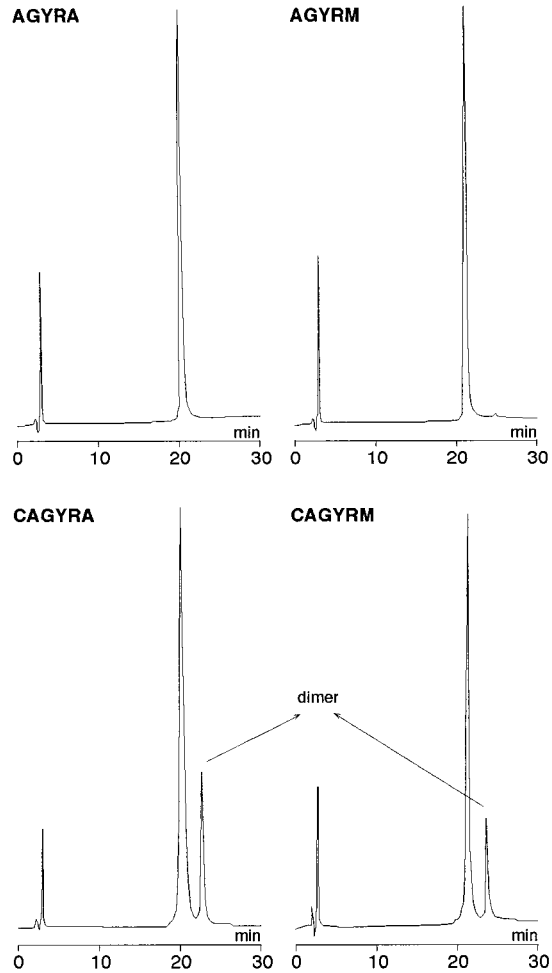

Figure 2 Chromatographic profiles of the peptides used in this study. See 'Materials and Methods' section for HPLC conditions.

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On the other hand, additional TFA treatments were necessary from residue 83 to achieve full N^{α}-amino deprotection.

As revealed by HPLC, in part the Cys containing peptides CAGYRA and CAGYRM underwent oxidation (dimerization) during the purification and the lyophilization steps (Figure 2). The dimers, $[CAGYRA]_2$ and $[CAGYRM]_2$, which were easily identified by MALDI/TOF-MS (8019 and 8162, respectively), had retention times higher than those found for the monomers (22.8 min and 24.0 min, compared to 20.2 min and 21.5 min, respectively). The fact that CAGYRA and CAGYRM have a tendency to aggregate, as revealed by circular dichroism (CD; results not shown), could explain the propensity of these peptides to dimerize.

CFX Affinity Chromatography

With the purpose of obtaining evidence on the formation of a ternary complex among CFX, DNA and the peptide model AGYRA, the first assays were performed using the quinolone bound to a polymeric support. To this aim an epoxy-activated Sepharose was utilized, to which the drug was anchored by alkylation of the secondary amino group under standard conditions (Figure 3(A)).

The peptides and DNA were detected following the fluorescence emission at 303 nm (excitation at 280 nm) and the UV adsorption at 260 nm, respectively. The chromatographic behaviours of peptide and DNA using the drug-immobilized column are shown in Figure 4 (A and B). Both DNAs, pBS and ssDNA (Figure 4(A), see inset for the latter), were retained in the column as demonstrated by their elution when the ionic strength was increased. On the contrary, AGYRA had no affinity for the CFX column in the absence of DNA (Figure 4(A)), but retention of both species was observed when a mixture of the peptide and DNA was utilized (Figure 4(B), see inset for ssDNA). The peptide mimic of the mutant gyrA AGYRM behaved as AGYRA in the absence of DNA (results not shown), but, unfortunately, the assay with a mixture of this peptide with DNA failed due to the insolubility of the components in the standard buffer. The assays with DNA were carried out in the presence of Mg^{2+} since it was reported that this cation stabilizes the quinolone–DNA complex through interactions with both species [4,6,7]. Experiments performed in our laboratory with the column mentioned above in the absence of Mg^{2+} did not show any retention of DNA (results not shown). The peptides and DNA were unable to bind

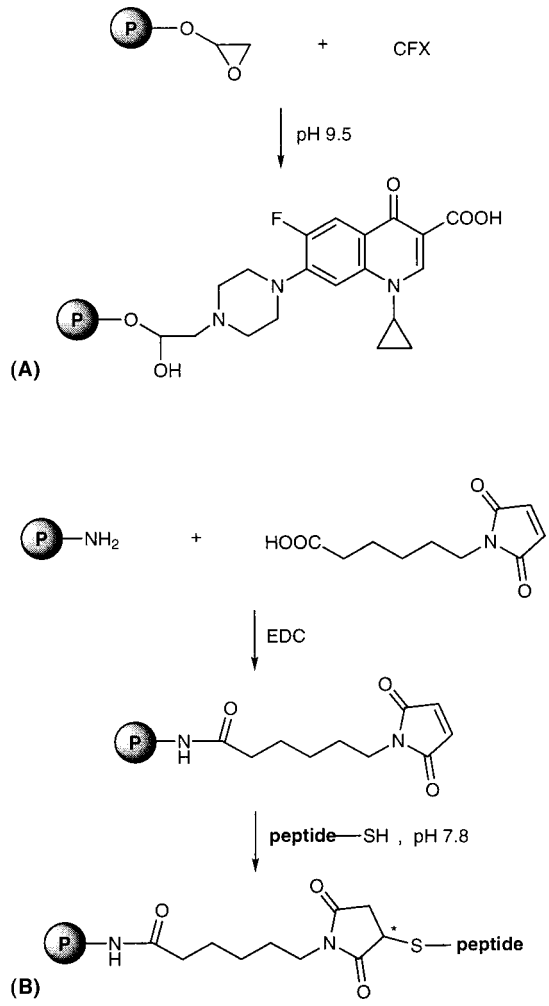

Figure 3 Anchorage of the quinolone CFX (A) and the peptides CAGYRA and CAGYRM (B) to Sepharose. * New chiral centre.

to the resin in a control experiment carried out using unmodified Sepharose.

It is interesting to point up the different chromatographic behaviour of pBS and ssDNA (see for example Figure 4(A)). Thus, DNA was retained in both cases but a higher ionic strength was needed for the total elution of ssDNA, which indicates a preferential binding of the quinolone to ssDNA rather than to pBS.

Peptide Affinity Chromatography

Peptide models were immobilized on a Sepharose resin that was previously functionalized by reaction with MCA (Figure 3(B)). Peptide chains were coupled to the polymeric support using CAGYRA or CAGYRM (Figure 1), by the nucleophilic addition of

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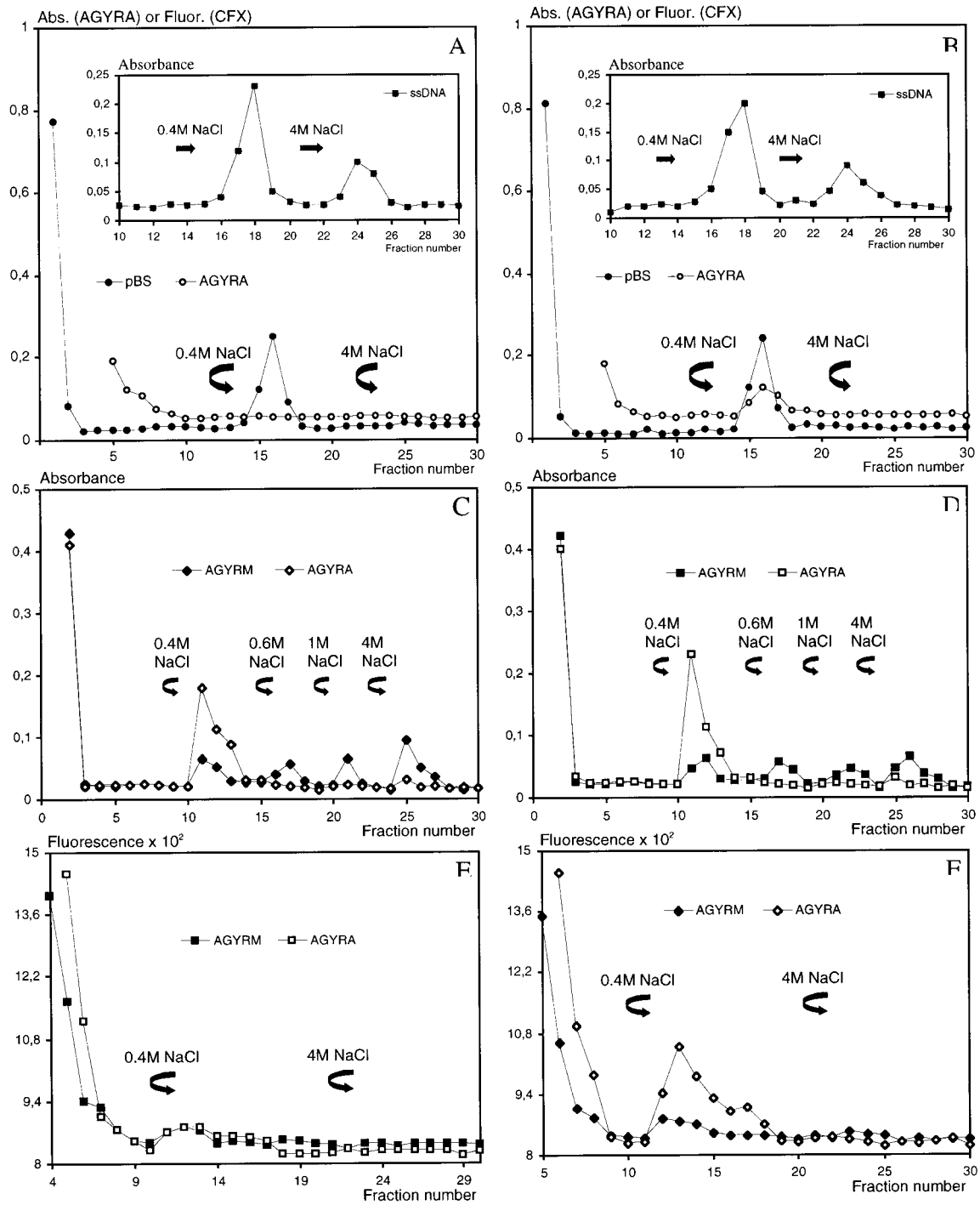

Figure 4 Affinity chromatography experiments with CFX (A and B) and AGYRA or AGYRM (C–F) bound to the polimeric support. (A) AGYRA or DNA alone. (B) A mixture of AGYRA and DNA. (C) pBS. (D) ssDNA. (E) CFX. (F) A mixture of CFX and pBS. The same scale applies for fluorescence and absorbance data in A and B (DNA, absorbance at 260 nm; peptide, fluorescence at 303 nm; CFX, fluorescence at 417 nm). Mobile phase: 5 mM Tris–HCl (pH 7.2), 20 mM NaCl/5 mM $MgCl_2$. Fractions 15–30 (A and B) and 11–30 (C–F) were eluted with the same buffer containing 0.4 or 4 M NaCl and 0.4, 0.6, 1 or 4 M NaCl, respectively, as indicated. Fractions having absorbance or flouresence values out of scale are not presented.

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the Cys side-chain thiol group to the double bond of the maleimido function. This process produces a new chiral centre in the pyrrolidinedione ring and, therefore, a mixture of two diastereomeric peptides.

Figure 4(C and D) shows the results concerning the affinity of DNA to the peptide-resins mentioned above. CFX and DNA were detected following the fluorescence emission at 400 nm (excitation at 330 nm) and as already described, respectively. Unlike the study carried out with the drug anchored to a resin, the fact of using the peptides bound to a polymeric support allowed us to determine from a qualitative point of view if they were able to interact with DNA. As indicated by the elution profiles, both DNAs, pBS and ssDNA, were retained in the columns in a similar way, a result that was independent of the presence of Mg^{2+}. On the other hand, DNA was eluted with 0.4 M NaCl when the AGYRA-resin was used, but higher salt concentrations had to be utilized to achieve the complete elution of DNA in the case of the AGYRM-resin (up to 4 M of NaCl). With regard to CFX, no appreciable affinity to the columns was observed even in the presence of Mg^{2+}, as it was found when the drug was bound to the resin (Figure 4(E)). However, CFX retention on the AGYRA-resin was detected when the drug was used together with DNA and in the presence of Mg^{2+}, as when CFX was anchored to the resin (Figure 4(F)). Interestingly, no retention of CFX on the AGYRM-immobilized column was observed under similar conditions (Figure 4(F), the corresponding assay with CFX-resin could not be done because of the insolubility of the DNA/AGYRM mixture). The chromatographic profiles presented in the Figure correspond to pBS, but identical results were obtained in the case of ssDNA. No retention of DNA was observed in a series of control assays that were performed using unmodified resin.

Binding Studies

The experiments carried out using affinity chromatography revealed interactions among DNA and the peptide or CFX. The most interesting result was that the three species studied were able to interact with each other in some way that could result in the formation of a ternary complex when the peptide mimic of gyrA (AGYRA) was utilized. However, this behaviour was not observed in the case of the peptide mimic of the mutant gyrA (AGYRM). This result prompted us to determine the binding parameters for the different systems mentioned above. The measurements were performed using the membrane filtration method [20,26]. The dissociation constants (K_d) were evaluated from the midpoint of the saturation curves resulting from Klotz plots of the amount of free ligand (peptide or CFX) against the molar binding ratio 'r' (bound peptide or CFX/total DNA) (Figure 5) [27].

As already found using affinity chromatography, CFX binds to both pBS and ssDNA in the presence of Mg^{2+} (Figure 5(A)). A preferential binding of the drug to ssDNA was also observed in this case (2–3-fold in relation to pBS, entry 'a' of the table). As shown in the table of Figure 5 (entries 'b' and 'c'), a similar result was obtained for the interaction of DNA with the model peptides. On the other hand, the peptide mimic of mutated gyrA, AGYRM, binds to DNA more efficiently than AGYRA (Figure 5(B and C); $K_d[\text{AGYRA} - \text{ssDNA}]/K_d[\text{AGYRAM} - \text{ssDNA}] = 7$, $K_d[\text{AGYRA} - \text{pBS}]/K_d[\text{AGYRAM} - \text{pBS}] = 4$), which is in agreement with our observations using affinity chromatography.

Regarding the interaction of CFX with DNA in the presence of peptide, the experiments carried out with mixtures of the three species yielded interesting results. Thus, the drug binds to both pBS and ssDNA when AGYRA is used, as it was expected from the results achieved with the peptide anchored to a polymeric support (Figure 5(D), entry 'd' of the table; the saturation curve that is shown corresponds to pBS). However, the K_d value for this process is similar to that found in the absence of peptide, suggesting that DNA might have different binding sites for CFX and the peptide mimic AGYRA (compare entries 'a' and 'd' of the table). Finally, no interaction of the drug with DNA was detected in the presence of the peptide mimic of the mutant gyrA (AGYRM) in agreement with the results obtained using affinity chromatography (Figure 5(D), entry 'e' of the table).

DISCUSSION

The binding assays carried out with CFX, DNA and the peptide models that were designed as mimics of gyrA (wild type and mutant) confirmed important features already known about the mechanism of action of quinolones. From the point of view of the experimental techniques used in this work, similar conclusions can be drawn from the results achieved with the affinity chromatography approach and the membrane filtration method.

Nowadays, it seems obvious that antibacterial quinolones inhibit DNA gyrase by trapping the

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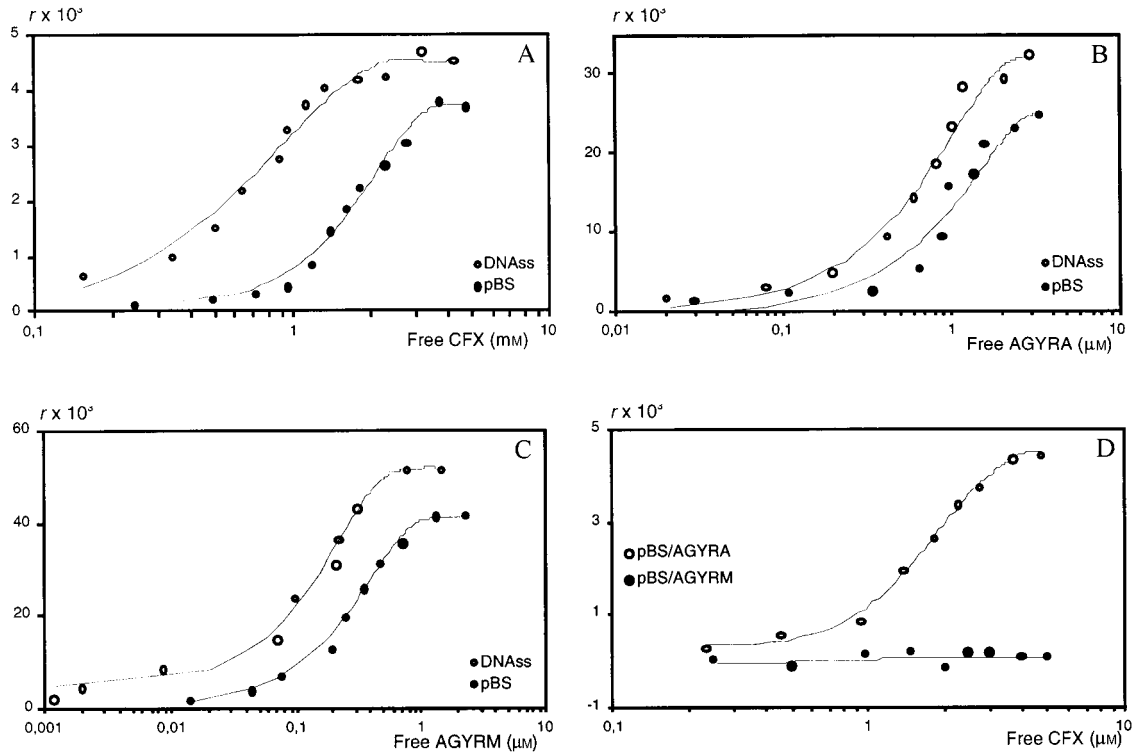

Entry	Ligand	K_d (x 10^{-7} M)	
		Supercoiled plasmid (pBS)	Single-stranded calf thymus DNA (ssDNA)
a	CFX	18.0	8.7
b	AGYRA	10.0	7.0
c	AGYRM	2.5	1.0
d	CFX/AGYRA[a]	16.0	6.9
e	CFX/AGYRM[a]	-[b]	-[b]

[a] CFX was added to a preincubated mixture containing DNA and a peptide mimic.
[b] No binding was observed.

Figure 5 Binding determinations using Klotz plots. (A) pBS (5 pmol) or ssDNA (5 pmol) and CFX (0.25–5 μM). (B) pBS (5 pmol) or ssDNA (5 pmol) and AGYRA (0.1–5 μM). (C) pBS (5 pmol) or ssDNA (5 pmol) and AGYRM. (D) pBS/AGYRA (5 pmol/1 nmol) and CFX (0.25–5 μM) or pBS/AGYRM (5 pmol/1 nmol) and CFX (0.25–5 μM). The midpoint of the saturation curve represents the apparent dissociation constant in all cases ('*r*' stands for molar binding ratio).

enzyme as a complex with DNA, the formation of which is the key event for the topoisomerase to develop its biological activity. The molecular details of the binding site in the ternary complex are unknown, but studies carried out in the absence of gyrase have revealed that the drug interacts with

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DNA [5,6,20,26,33]. On the other hand, it is known that the quinolone has low or no affinity for the DNA-free topoisomerase, [8,9,19,26,27] but how the drug binds to the enzyme in the ternary complex still remains unclear.

From a qualitative point of view, a parallel behaviour was found for AGYRA, the peptide mimic of gyrA. Thus, AGYRA is unable to bind CFX, no matter whether the quinolone (Figure 4(A)) or the peptide (Figure 4(E)) are attached to a polymeric support. However, both species bind DNA, as shown by the retention of pBS and ssDNA on the quinolone-immobilized (Figure 4(A)) and peptide-immobilized (Figure 4(C and D)) columns. The affinity chromatography also revealed the formation of a ternary complex when AGYRA (Figure 4(B)) and CFX (Figure 4(F)) were retained into the columns mentioned above, respectively, in the presence of DNA. As already reported in the literature, Mg^{2+} was required in order to promote the interaction of the drug with DNA [4,6,7,19,34,35]. These qualitative results were corroborated by the membrane filtration technique, which allowed K_d values to be determined for the binding of CLX and AGYRA to DNA and the binding of the quinolone to the peptide/DNA complex (table of Figure 5; entries 'b', 'a' and 'd', respectively).

The apparent dissociation constant for the binding of CFX to peptide-free DNA was of the same order of magnitude that the one obtained for the potent DNA gyrase inhibitor norfloxacin (an ethyl group at position N1, Figure 1) using a cooperative binding model (1×10^{-6} M) [26]. In this connection, it has to be pointed out that similar supercoiling inhibition constants have been described for both quinolones [3]. On the other hand, the affinity of AGYRA for DNA ($K_d \approx 10^{-6}$ M for pBS) proved to be much lower than the one reported for DNA gyrase ($K_d \approx 10^{-10}$ M) [36], which is not surprising considering the size of the natural protein.

CFX binds preferentially to ssDNA rather than to pBS, which is in agreement with the data described by other authors [20,26,36]. In this sense, it has been suggested that the drug binds to ssDNA in a non-intercalative way through hydrogen bonds that become available when the bases are unpaired by unwinding of the double strand upon binding of the DNA to gyrase [20,29,37]. The need of 4 M NaCl for full elution of ssDNA from the CFX-immobilized column also revealed a higher affinity of the quinolone for ssDNA since the same result was achieved with 0.4 M NaCl in the case of pBS (Figure 4(A)). However, both techniques afforded different results for the binding of AGYRA to DNA. Thus, the membrane filtration technique showed a preferential binding of the peptide to ssDNA, but similar chromatographic profiles were obtained for both DNAs with the peptide-immobilized column (full elution with 0.4 M NaCl in both cases, Figure 4(C and D)).

According to the K_d values for the AGYRA/DNA and CFX/DNA complexes, the peptide binds to ssDNA and pBS more efficiently than the quinolone, which could be explained in terms of the nature of the interactions that are responsible for binding. As in the case of the binding of basic proteins such as histones to DNA [38], the presence of seven basic residues in AGYRA is probably the key feature for the binding of the peptide to DNA through electrostatic interactions between these residues and the phosphate groups of the nucleic acid [29]. However, experimental data and molecular modelling studies [3,7,34] seem to indicate that the quinolone interacts with DNA through its carboxyl and carbonyl moieties (Figure 1) to form a complex in which Mg^{2+} acts as a bridge between a phosphate group of DNA and the drug.

The fact that AGYRA and CFX had affinity for the quinolone-immobilized and the peptide-immobilized columns, respectively, in the presence of DNA and Mg^{2+} (Figure 4(B and F)) revealed the formation of a ternary complex. This result is not surprising if the retention is a consequence of the simultaneous interaction of DNA with the peptide and the quinolone. It has to be pointed out that the specific interactions involved in the stabilization of the gyrase/DNA/quinolone complex at the quinolone-binding site are still a matter of controversy. In this connection, it has been speculated about the possibility that the binding of the drug to DNA gyrase through hydrogen bonding between the secondary amino group (Figure 1) and Asp-87 might play a crucial role [3,7]. However, AGYRA was retained on the column in spite of having CFX covalently bound to the polymeric support by the piperazine group at this position. Moreover, from a qualitative point of view, similar results were achieved with both columns under the conditions used to carry out the assays, that is, no matter the quinolone or the peptide was anchored to the polymeric support (full elution with 0.4 M NaCl in both cases).

Studies carried out with norfloxacin revealed the appearance of new binding sites for the quinolone in the enzyme–DNA complex as a consequence of the interaction between the two species [3,39]. Interestingly, the formation of a specific binding site by

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combination of a gyrase promoted unwound DNA region with number of residues on the protein has been recently suggested [37]. As shown in Figure 4(A and B), the affinity chromatography analysis did not bring any evidence about an increase of the binding of CFX to DNA in the presence of AGYRA (compare the chromatographic profiles for ssDNA and pBS). The results achieved with the membrane filtration technique were not conclusive either, in spite of the fact that the K_d values for the binding of the quinolone to the AGYRA/DNA complex were slightly lower than those found for the binding to the peptide-free DNA (Figure 5, compare entries 'a' and 'd' of the table for both DNAs).

The binding of the peptide mimic of mutated gyrA, AGYRM, to DNA proved to be more efficient than that of AGYRA (Figure 4(C and D) and Figure 5). The change of the effective charge of the peptide from $+2$ to $+3$ as a consequence of the mutation of Asp to Asn at position 87 could explain the higher affinity of AGYRM for DNA in terms of additional electrostatic interactions with the negatively charged phosphate groups. On the other hand, as it was already found for AGYRA, AGYRM had more affinity for ssDNA than for pBS according to the corresponding K_d values, although the chromatographic profiles obtained using the peptide anchored to the polymeric support were not conclusive on this point. However, the most intriguing results were obtained when CFX was used with the model peptide AGYRM. Unlike what was observed in the case of AGYRA, CFX did not bind to DNA in the presence of AGYRM, as revealed by the affinity chromatography and membrane filtration techniques (Figure 4(F) and Figure 5(D), entry 'e' of the table). This behaviour is in agreement with what was found by Willmott and Maxwell [8] for the quinolone norfloxacin and gyrase A with a mutation that confers quinolone resistance (Ser-83 to Trp). In that case, the enzyme showed greatly reduced drug binding. These results indicate that the model peptide AGYRA contains enough structural information to induce the formation of the ternary complex with CFX and DNA through mutual interactions among the three species. Mutations at the critical positions 83 and 87 produce structural changes that result in a destabilization of the complex, as it probably happens in the natural environment [40,41].

Many studies reported in the literature point to the fact that formation of the ternary complex is crucial for the drug to inhibit DNA gyrase activity. If so, according to the results obtained in this work, the use of short peptides such as AGYRA and AGYRM in DNA binding studies could be a suitable approach to a preliminary evaluation of quinolones as potential drugs.

In summary, the binding data reported in this work strongly suggest that the use of short peptides including sequences of gyrA provides an alternative way to study the interaction of quinolones with the DNA/gyrA complex, in order to get an insight into the mechanisms of antibiotic resistance. Moreover, we believe that AGYRA constitutes a starting point for the *de novo* design of peptides able to mimic the structural features of gyrA at the quinolone binding site and, therefore, it is potentially useful in the search for new drugs with antibiotic activity.

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