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^{1}H NMR studies of selective interactions of norfloxacin with double-stranded DNA

Karin Sandström,[a] Sebastian Wärmländer,[a] Mikael Leijon,[b] and Astrid Gräslund[a,*]

[a] *Department of Biochemistry and Biophysics, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden*
[b] *LightUp Technologies AB, Lunastigen 5, S-141 44 Huddinge, Sweden*

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Abstract

The interaction of the antibiotic drug norfloxacin with double-stranded DNA containing interior 5′-CpG-3′, 5′-GpC-3′, and 5′-GpG-3′ steps was studied by ^{1}H NMR. The drug is in fast exchange on the NMR timescale. A highly selective broadening of the imino proton resonances assigned to central CpG steps was observed after addition of drug, indicating an intercalation-like interaction. DNA sequences with central CpG steps also displayed broadening of non-hydrogen-bonded cytosine amino protons in the major groove upon addition of norfloxacin. Furthermore, a sequence-independent selective broadening of the adenine H2 resonance and an upfield shift of the guanine amino proton resonance, both protons located in the minor groove, was observed. Two-dimensional-NOESY spectra showed that no significant structural changes were induced in the DNA by the drug. The results suggest that the planar two-ring system of norfloxacin partially intercalates into CpG steps and that the drug also exhibits non-specific groove binding.

Quinolones are antibacterial agents that effectively inhibit DNA replication and are commonly used as treatment for many infections. The functional targets are the bacterial type II topoisomerases DNA gyrase and topoisomerase IV. DNA topoisomerases are essential enzymes that manipulate the DNA topology. DNA gyrase introduces negative supercoils into the DNA molecule that are important for the initiation of DNA replication and removes positive supercoils ahead of the replication fork. Topoisomerase IV separates interlinked daughter chromosomes in the terminal step of DNA replication. It has been proposed that quinolones inhibit the enzyme activity by stabilizing the DNA–enzyme complex so that resealing of the DNA strands never takes place [1]. The formation of the quinolone–DNA-topoisomerase ternary complex affects the enzyme conformation as well as the DNA structure in the complex [2].

One of the first clinically effective quinolones to be discovered was the molecule norfloxacin (Fig. 1). This three-ring zwitterion has been shown to interact with double-stranded DNA. However, the exact binding mode of the drug to DNA is not known. Nordén and coworkers used linear dichroism to detect a near perpendicular orientation of the plane of the drug and the DNA helix axis. They furthermore concluded that the drug does not bind via classical intercalation, nor by groove or surface binding modes [3]. Other studies by optical spectroscopy and molecular modelling indicate that the drug interacts with DNA via the minor groove and maybe also via the major groove [4–6]. It has also been shown that the drug prefers to interact with sequences with a high content of GC base pairs [4,7].

In the present work we have used ^{1}H NMR spectroscopy with the aim to characterize the specific interactions of norfloxacin with a series of different double-stranded DNA oligonucleotides of various sequences with GC-containing steps at different positions.

Materials and methods

Sample preparations and titrations. The self-complementary DNA oligonucleotides (DNA-1,2,3,4,5,6,7) in Fig. 2 were bought from Cybergene AB, Sweden, and dissolved in a 10-mM phosphate buffer

* Corresponding author. Fax: +46-8-155-597.
E-mail address: astrid@dbb.su.se (A. Gräslund).

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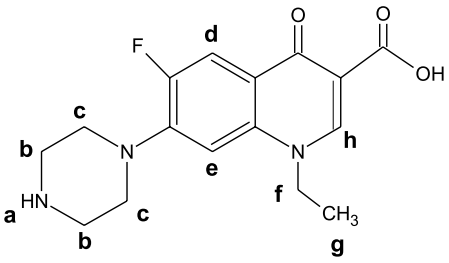

Fig. 1. Chemical structure of norfloxacin.

		1	2	3	4	5	5	4	3	2	1	
DNA-1	5′-	C	G	**C**	**G**	A	T	**C**	**G**	C	G	-3′
	3′-	G	C	**G**	**C**	T	A	**G**	**C**	G	C	-5′
DNA-2	5′-	C	G	C	A	**C**	**G**	T	G	C	G	-3′
	3′-	G	C	G	T	**G**	**C**	A	C	G	C	-5′
DNA-3	5′-	C	G	C	A	G	C	T	G	C	G	-3′
	3′-	G	C	G	T	C	G	A	C	G	C	-5′
DNA-4	5′-	G	C	G	C	A	T	G	C	G	C	-3′
	3′-	C	G	C	G	T	A	C	G	C	G	-5′
DNA-5	5′-	G	C	G	A	**C**	**G**	T	C	G	C	-3′
	3′-	C	G	C	T	**G**	**C**	A	G	C	G	-5′
DNA-6	5′-	G	C	G	A	G	C	T	C	G	C	-3′
	3′-	C	G	C	T	C	G	A	G	C	G	-5′
DNA-7	5′-	C	A	G	G	A	T	C	C	T	G	-3′
	3′-	G	T	C	C	T	A	G	G	A	C	-5′

Fig. 2. Investigated DNA sequences. Central 5′-CpG-3′ steps are indicated in bold (base pair 1 and 2 excluded).

containing 100 mM NaCl at pH 7.0 (90% H_2O and 10% D_2O). Norfloxacin was bought from Sigma and dissolved into a 60-mM stock solution. The norfloxacin concentration was determined with UV absorbance spectroscopy using a molar absorption coefficient of $\varepsilon = 37{,}500\,cm^{-1}\,M^{-1}$ at 273 nm. The duplex concentrations were in the range 0.5–1 mM, also determined by UV spectroscopy. At 30 °C, small aliquots of norfloxacin were added to the DNA sequences, yielding values of drug/DNA duplex (r) from 0 up to 3.

NMR spectroscopy. ^{1}H NMR experiments were carried out on a Varian Inova 600 MHz spectrometer. All spectra were obtained at 30 °C and a Jump-Return observe pulse was used to avoid excitation of the water resonance [8]. DNA resonances were assigned from 2D-NOESY ($\tau_{mix} = 250$ ms) experiments. The norfloxacin proton resonances were assigned from 2D-NOESY ($\tau_{mix} = 450$ ms) and TOCSY ($\tau_{mix} = 80$ ms) experiments. All 2D data processing was carried out with Felix97 (Molecular Simulations). The spectra are referenced to TSP.

Results and discussion

^{1}H NMR was used to probe the norfloxacin interaction with seven different self-complementary DNA dodecamers (Fig. 2). The sequences were chosen with a strategy to insert GC base pairs in different contexts; particularly positioning 5′-CpG-3′, 5′-GpC-3′, and 5′-GpG-3′ steps in the interior of the sequences. The aim was to probe in more detail the proposed preference of norfloxacin for GC base pairs [4,7].

The seven self-complementary DNA dodecamers (Fig. 2) were titrated with a norfloxacin stock solution. The DNA resonances of interest were assigned using standard methods including 2D-NOESY spectra. DNA-1 was also assigned in a previous study [9]. Fig. 3 shows the imino proton region (12.3–13.9 ppm) when norfloxacin was titrated to DNA-1 and DNA-4. Fig. 4 shows the aromatic proton region (7.2–8.7 ppm) and the amino proton region (6.3–7.2 ppm) when norfloxacin was titrated to DNA-1 and DNA-6. At every drug/DNA concentration ratio the spectra display single sets of well-resolved resonances. This shows that the drug is in

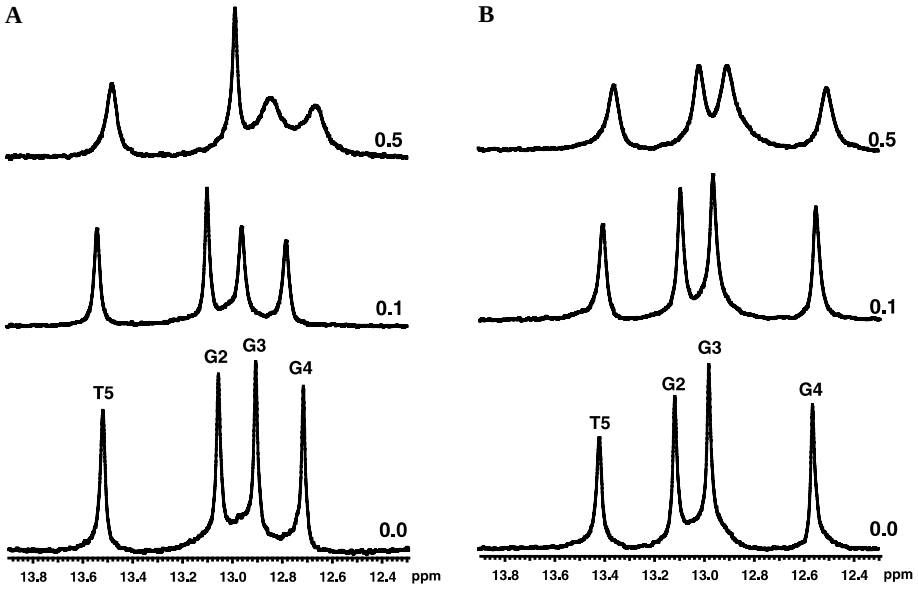

Fig. 3. 1D ^{1}H NMR spectra at 30 °C when norfloxacin was added to DNA-1 (A) and DNA-4 (B) showing the imino proton region (12.3–13.9 ppm). The imino proton resonances as well as the drug/duplex ratios (r) are indicated.

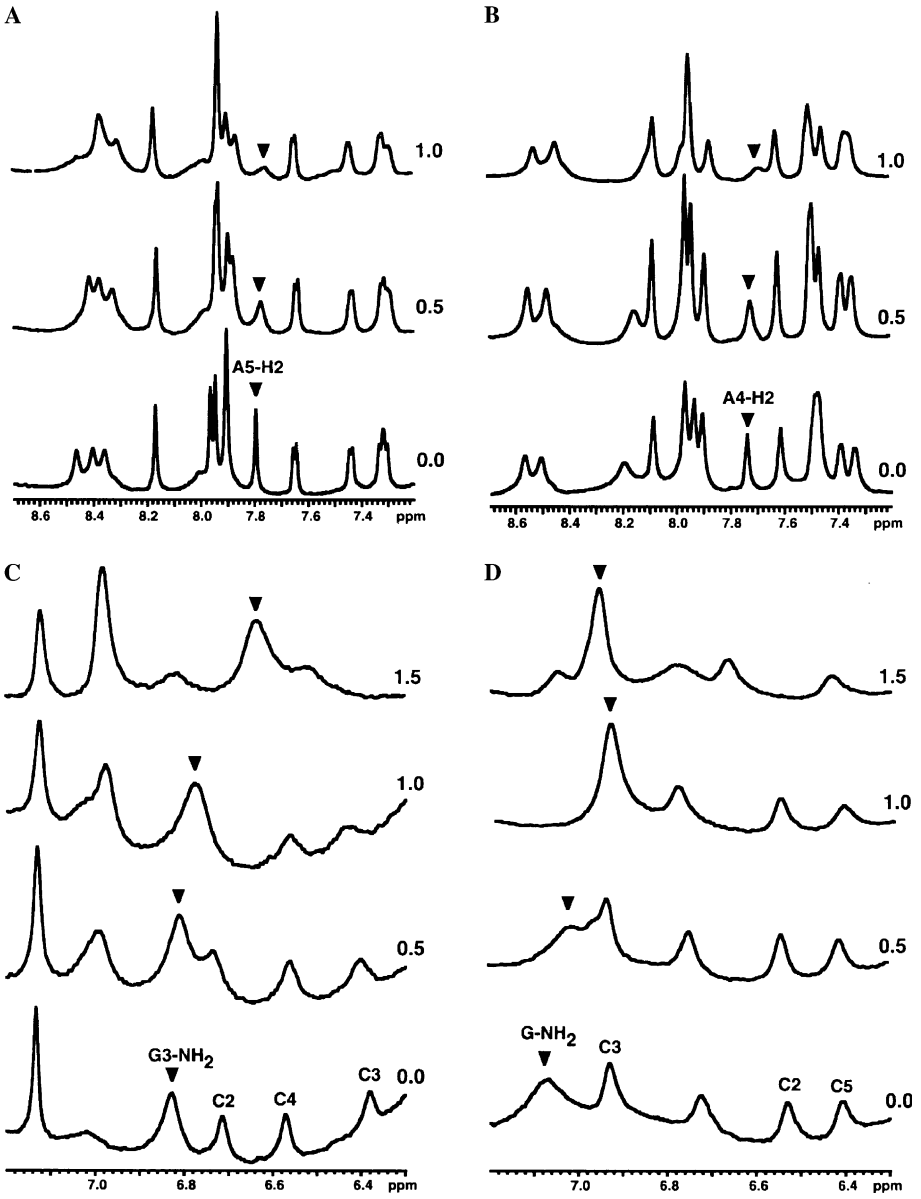

Fig. 4. The aromatic proton region (7.2–8.7 ppm) of the 1D ^{1}H NMR spectra at 30 °C when norfloxacin was added to DNA-1 (A) and DNA-6 (B), and the amino proton region (6.3–7.2 ppm) when norfloxacin was added to DNA-1 (C) and DNA-6 (D). Adenine H2 and guanine NH_2 resonances as well as drug/duplex ratios (r) are indicated. The guanine NH_2 resonance shown in (D) is not assigned to a particular guanine due to spectral overlap.

fast exchange on the NMR timescale under the experimental conditions ($k_{ex} \ll 1$ ms).

For the imino proton region of DNA-1 a highly selective broadening is observed for the imino protons of the interior CpG step, i.e., base pairs 3 and 4 (Fig. 3A). Also for the other sequences containing non-terminal CpG steps, i.e. DNA-2 and DNA-5, a highly selective broadening was found for the imino protons of these CpG steps (data not shown). The broadening is accompanied with an upfield shift at high drug concentrations. The imino protons of terminal CpG steps are difficult to monitor due to end-fraying effects, which generally cause broadening of the resonance lines. For sequences without non-terminal CpG steps, i.e. DNA-3, DNA-4, DNA-6, and DNA-7, no selective broadening of imino proton resonances was found (Fig. 3B). Instead, some nonselective broadening was displayed at high norfloxacin concentrations. These observations show that norfloxacin interacts preferentially with the imino protons of the non-terminal CpG steps. Since these imino protons are located at the center of the double helix an immediate interpretation is that the plane of the drug is inserted into the center of the helix, in an intercalation-like binding mode.

This interpretation is in agreement with the results of Nordén et al. [3] that the norfloxacin molecule is

oriented perpendicular to the DNA helix axis. The same authors also reported the binding constant of norfloxacin to DNA to be $2.8 \times 10^3\,M^{-1}$ at 25 °C, an order of magnitude different from that of a classical intercalator, e.g. $5 \times 10^4\,M^{-1}$ at 20 °C for ethidium [10]. The conclusion was that norfloxacin does not intercalate in a classical way. Our present observation of the drug being in fast exchange also agrees with a relatively low binding constant, quite different from what is expected for a typical intercalator, which usually leads to an intermediate or slow exchange situation with unresolved or double set of DNA resonances.

In Figs. 4A and B the aromatic proton region (7.2–8.7 ppm) of DNA-1 and DNA-6 is shown for different norfloxacin concentrations. It is seen that the resonance of the adenine H2 proton, situated in the minor groove, is strongly broadened upon addition of norfloxacin. This holds true for all investigated sequences.

The amino proton region (6.3–7.2 ppm) of DNA-1 and DNA-6 upon titration of norfloxacin is shown in Figs. 4C and D. The resonances of hydrogen-bonded guanine amino protons, located in the minor groove, are shifted upfield at high norfloxacin concentrations. This effect is also found in the other sequences. Furthermore, we observed that the resonances of the non-hydrogen-bonded cytosine amino protons in the major groove of the central CpG steps display a substantial broadening. This effect appears on the same base pairs whose imino proton resonances were preferentially affected by the added norfloxacin (see above). The effect is most prominent for the central cytosine in sequences DNA-2 and DNA-5 (data not shown) and less obvious for DNA-1 (Fig. 4C). For cytosines not involved in CpG-steps no large effect is seen in any sequence (Fig. 4D). As for the hydrogen-bonded amino protons of cytosine, no large effects were seen on any of the resonances, irrespective of sequence.

The observations in the 1D spectra titrations were further expanded in a series of 2D-NOESY experiments. Fig. 5 shows 2D-spectra resulting from NOESY experiments on sequences DNA-1 and DNA-5 in the presence and absence of norfloxacin. For all sequences the sequential pathway of connectivities between aromatic H6/H8 and ribose H1' protons is drawn in the spectrum and the assignment of the H6/H8 protons is indicated. Also the cytosine intranucleotide crosspeaks between, on the one hand, base-paired amino protons (8.3–8.5 ppm), and, on the other hand, H5 (5.2–6.0 ppm) as well as non-base-paired amino protons (6.3–7.0 ppm), are indicated. For all sequences, the intranucleotide crosspeaks of the outermost cytosine are lost due to end-fraying.

For DNA-1, an obvious effect of adding norfloxacin is the disappearance of the intranucleotide crosspeaks of the second outermost cytosine, C9 (Figs. 5A and B). Also the crosspeaks of the other two observable cytosines, i.e.

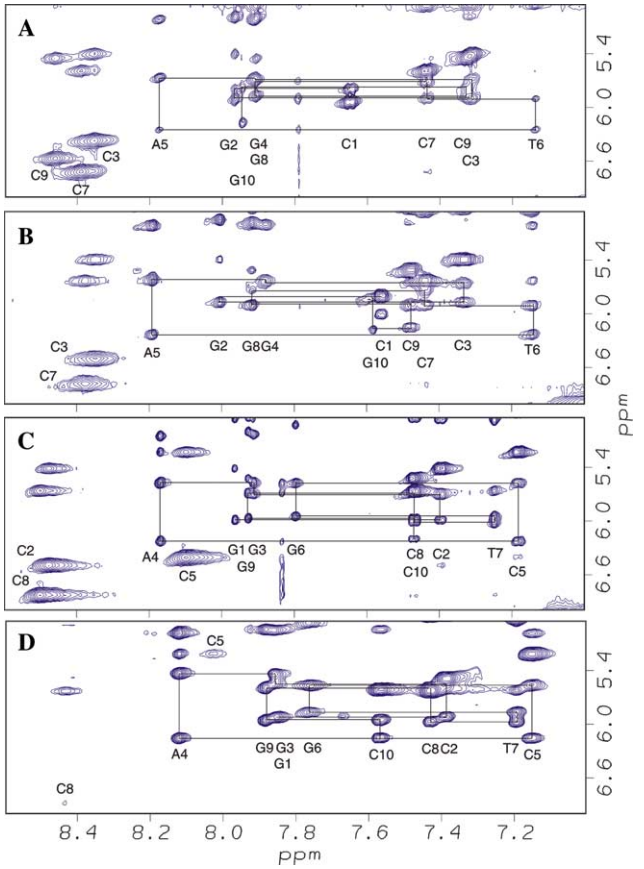

Fig. 5. 2D NOESY spectra of the sequence $C^1G^2C^3G^4A^5T^6C^7G^8C^9G^{10}$ without (A) and with (B) norfloxacin (drug/duplex ratio = 1.5), and $G^1C^2G^3A^4C^5G^6T^7C^8G^9C^{10}$ without (C) and with (D) norfloxacin (drug/duplex ratio = 1.5), obtained at 30 °C with a mixing time of 450 ms. In (D), the assignment of G^1 is ambiguous due to spectral overlap.

C3 and C7, have reduced intensities in the presence of the drug. Furthermore, the H6/H8 resonances of C1, C9, and G10 display large shifts after norfloxacin has been added.

For DNA-5, the crosspeaks of the three observable cytosines, i.e. C2, C5, and C8, all display a significant reduction in intensity after addition of norfloxacin (Figs. 5C and D). In fact, only weak crosspeaks between the resonances of the non-base-paired amino protons and the H5 protons of C5 and C8 remain visible. Again, the H6/H8 resonances of the terminal bases, i.e. G1 and C10, are shifted in the presence of the drug.

For both sequences, the crosspeaks corresponding to the central part of the DNA helix remain largely unaffected when norfloxacin is added. From this we conclude that the drug does not induce large structural changes upon the DNA helix. Therefore we rule out classical intercalation as a mode of interaction. Instead, these 2D data indicate an interaction between the drug and the major groove, as well as the ends, of the helix.

From our observations we conclude that there is an interaction between norfloxacin and the DNA taking

Table 1
Chemical shifts of norfloxacin protons at 30 °C

Proton resonance	Chemical shift (ppm)
g	1.52
b	3.52
c	3.60
f	4.48
e	7.10
d	7.63
h	8.70
a	8.75

place both in the major groove and the minor groove of the DNA helix. In addition, we have observed a selective intercalation-like interaction between norfloxacin and DNA CpG steps. Our results do not allow a clearcut distinction from which groove the intercalation-like binding originates, but the correlated broadening of CpG resonances from guanine imino protons and non-hydrogen-bonded cytosine amino protons suggest that the major groove is involved. Thus, several modes of interaction of norfloxacin with DNA occur simultaneously.

Alas, despite our best attempts, and despite the suitable binding constant of the drug, we were not able to observe any NOESY crosspeaks between drug and DNA resonances. Therefore our conclusions are based on observations of resonance broadening only.

The proton resonances of norfloxacin were assigned from 2D-NOESY and 2D-TOCSY experiments (Fig. 1, Table 1). We observed that particularly the resonance of proton d, but also the resonances of protons e and h (Fig. 1), are broadened when DNA-5 is titrated onto the drug (data not shown). This indicates that it is the planar two-ring system that interacts with the DNA helix, probably with the side of the ring-system not carrying the methyl group directed towards the DNA.

A general conclusion from this study is that the norfloxacin interaction with DNA does not lead to significant disturbance of the DNA structure, and that the binding kinetics is relatively fast on the NMR timescale ($k_{ex} \ll 1\,ms$). This conclusion is completely in agreement with previous proposals that the major drug activity in vivo is not directed towards naked DNA, but is most likely instead centered on the DNA topoisomerase complex [2,11,12].

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