
Paper 3

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" Antibacterial Fluoroquinolone Agents - Interaction with DNA"

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Antibacterial Fluoroquinolone Agents - Interaction with DNA.

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Abstract

The interaction of the antibiotic drug levofloxacin (levo) with single-stranded and double-stranded DNA and ATP in the presence and absence of Mg(II) was studied by ^{1}H and ^{31}P NMR and restrained molecular dynamics (MD). At the present conditions, levo does not interact with ss-DNA in the presence or absence of Mg(II), while levo does interact with ds-DNA. Two self-complementary ds-DNA sequences were investigated, 5′-d(TATGGTACCATA)-3′ (**1**) and 5′-d(GATCGGCCGATC)-3′ (**2**). For both duplexes interaction was, as expected, enhanced by the addition of Mg(II) ions. Levo binding to both the major (for **1**) and the minor groove (for **1** and **2**) was observed, with a preference for the 5′-TAT or 5′-GAT terminal section of the minor groove. Molecular modelling shows an optimal van der Waal's complementarity of levo in the minor groove. At higher levo:DNA ratio levo was shown to intercalate between a central GpG base step for **2**. The results suggest that at lower drug:DNA ratios, non-specific minor groove binding is preferred, while intercalation occurs at higher ratios. The binding modes seem to be non-cooperative.

Introduction

Quinolones are an important group of antibiotics and several quinolones are in common clinical use. Ciprofloxacin (cipro), Levofloxacin (levo) and Ofloxacin (oflo) are the top-selling antibiotics worldwide. Resistance against antibiotics is on the rise and multi-drug resistance will probably become a major problem in the 21st century. It is therefore vital to gather knowledge about the molecular mechanisms of action for antibiotics. The quinolones act by inhibiting the function of the enzyme DNA gyrase, a type II topoisomerase found only in prokaryotic cells. DNA gyrase introduces negative supercoils in DNA[1] by wrapping the DNA around the enzyme. The enzyme then catalyses the breakage of a segment of the wrapped DNA, the passage of a segment of the same DNA through the break and finally the religation of the break.[2] In this way, DNA 'knots' are resolved and the DNA is exposed for replication processes. The enzyme is essential for all bacteria and is therefore an excellent target for antibiotics. Quinolones are assumed to act by blocking the strand ligation and thereby preventing proper replication of DNA, eventually leading to cell death.

Several structural models have been suggested to elucidate the mechanism of action of fluroquinolones. All these models imply direct interaction between the drug and either single- or double-stranded DNA.[3-6] Some also suggest that Mg(II) ions play an important role for the drug binding.[4, 7-10] Palú and co-workers reported a reasonably strong interaction between quinolones and plasmid or single-stranded DNA that occurs only in the presence of physiological concentration of Mg(II) (1-2 mM).[4] A good relationship was found for the binding constants of the ternary DNA-drug-Mg(II) complex and gyrase poisoning activity. The authors did not suggest a structural model for the ternary complex, except reporting that the data did not support quinolone intercalation into DNA.[11] This conclusion is in agreement with results based on CD and LD measurements of a calf thymus DNA - norfloxacin system where the drug chromophore is significantly tilted with respect to the DNA helical axis.[12]

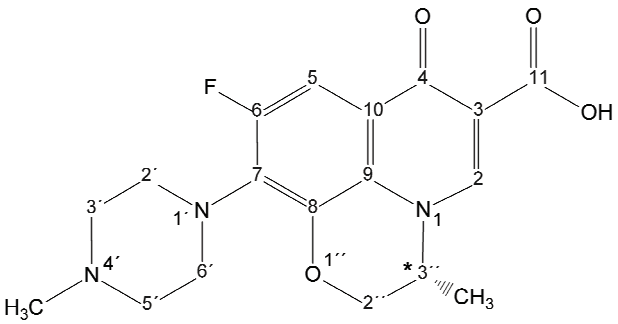
On the contrary, Son et al.[13] found by CD/LD experiments a near perpendicular orientation of the norfloxacin chromophore plane relative to the DNA helical axis which excludes classical groove or surface binding. However, fluorescence spectroscopy showed low unwinding of the DNA and consequently the possibility of classical intercalation was ruled out. The equilibrium constant of the norfloxacin - DNA complex formation was estimated to $2.8 \cdot 10^3$ M^{-1}. Previously, we have investigated the interaction of ciprofloxacin with ds-DNA by ^{1}H NMR and molecular docking calculations.[10] Ciprofloxacin was found to interact both in the minor and major groove as evident from NOESY cross-peaks and chemical shifts. The results suggested that in the presence of Mg(II), minor groove binding was favoured. Later, Sandström et al.[14] investigated norfloxacin binding to 7 different oligonucleotides by ^{1}H NMR. They did not observe any cross-peaks between norfloxacin

and the oligomers, but selective line broadening and chemical shift differences suggested significant interaction. As in the study of ciprofloxacin with DNA, titration of norfloxacin induced upfield shifts for guanine amino protons and selective line broadening of adenine H2 indicating interaction in the minor groove. Additionally, selective broadening of non-bound cytosine amino signals suggested that also major groove interaction took place. Furthermore, selective broadening of imino signals belonging to central CpG steps suggested intercalation. The latter was not observed for the ciprofloxacin – DNA system.

To our knowledge, only two molecular modelling studies have been performed on fluoroquinolone binding to DNA. In one study, the interaction of ciprofloxacin with ds-DNA in the presence and absence of Mg(II) was investigated by high-field NMR and modelled by molecular docking.[10] Minor groove binding was favoured over major groove with no apparent sequence specificity. In another study, norfloxacin binding to ds-DNA was modelled by non-restrained energy minimisation and short MD simulations (100 ps).[15] Here both intercalation and minor groove binding were modelled. The results were compared to previously reported CD/LD and fluorescence spectroscopy experiments [16] by the following criteria: i) the angle between the molecular plane of norfloxacin and the DNA helix should be in the range 65°-80°, ii) negligible amount of unwinding of the DNA helix, iii) interaction between norfloxacin and the amine group of the guanine base. The intercalation structures were excluded because of too high unwinding of the DNA helix and lower bending angles than for the minor groove structures. The minor groove models formed hydrogen bonds between the carboxylic and carbonyl groups and the amino group of guanine, which the authors related to an observed preference for poly[d$(GC)_2$] over poly[d$(IC)_2$] and poly[d$(AT)_2$].[16] They concluded that norfloxacin binds to the minor groove in a non-classical manner.

Various techniques have been used to investigate the sequence selectivity of fluoroquinolones for DNA. However, the published results do not represent a consistent bonding picture. The group of S.K. Kim have performed a series of CD, UV and fluorescence studies on the sequence selectivity of norfloxacin, ofloxacin and levofloxacin binding to polynucleotides.[6, 13, 16-18] Results for norfloxacin and levofloxacin are very similar and show that the fluoroquinolones adapt a different binding mode towards poly[d(A-T)·d(A-T)] as compared to poly[d(G-C)·d(G-C)]. The same observations were found by Ulrih and co-workers[19] for ciprofloxacin, but at higher drug:polynucleotide ratios. From molecular modelling studies it was suggested that norfloxacin bound to poly[d(G-C)·d(G-C)] in the minor groove via a hydrogen bond between the guanine amine group and norfloxacin oxygen atoms with the possibility of partial intercalation, while binding to poly[d(A-T)·d(A-T)] or poly[d(I-C)·d(I-C)] was "near the minor groove" and stabilized by electrostatic interaction with the phosphate group.

In summary, the large amount of work published does not represent a consistent picture of the mode of quinolone-DNA interactions. In the present work, we have studied the interaction between levofloxacin and DNA mono- and oligonucleotides using NMR spectroscopy and molecular modelling. We have focused on the following aspects: (i) the preference for single- or double-stranded DNA, (ii) the role of Mg^{2+} ions, (iii) groove binding versus intercalation.



Scheme 1. Schematic drawing of the levofloxacin molecule.

Experimental

Sample preparation: ATP was purchased from Sigma-Aldrich and used without further purification. From a 12.6 mM stock solution, 4 mM NMR solutions in 99.96 % D_2O were prepared with 40 mM phosphate buffer, 50 mM NaCl at pH 6.95 (pD 7.35). Additions of levofloxacin (Fluka) were made from a stock solution of 30.5 mM, additions of $MgCl_2$ (Merck) were made form a 50 mM stock solution. All stock solutions were dissolved in 99.96 % D_2O. Spectra were recorded at 305 K. Assignments for levo[10, 20, 21] was made in accord with previous publications.

The octamer 5′-d($C_1C_2T_3A_4A_5T_6C_7C_8$)-3′ (ssTAAT) was purchased from DNA Technologies and purified by HPLC (Waters 626 LC instrument using Millenium 32 software) using an XTerra MS C_{18} 2.5 µm column (Waters). Eluents for HPLC were A: 5 % acetonitrile in 0.1 M TEAA (Triethylammonium Acetate) and B: 30 % acetonitrile in 0.1 M TEAA. A linear gradient from 8 % B to 36 % B in 20 mins at 2.0 ml/min flow, room temperature was applied. TEAA and acetonitrile were removed by lyophilizing the sample five times; first without changing the pH, then at pH 3.3, 12.2, 3.3 and finally at 12.7. Due to the accumulated salt from the pH adjustment (by HCl and NaOH), the sample was desalted on a Sephadex G-25 column (NAP-10, Pharmacia Biotech) using phosphate buffer as eluent (10 mM, pH 7.02). Final concentration was 0.9 mM ssTAAT in 480 µl 99.96% D_2O (Aldrich) with 70 mM phosphate buffer, 45 mM NaCl at pH 7.24 (pD 7.64). To increase the stability of the single-strand, 14 mM $MgCl_2$ was added. TSP was used as chemical reference. The single-strand DNA was titrated with a stock solution of 23.5 mM 1:1 levo:Mg(II) dissolved in 99.96 % D_2O (Aldrich) at pH 7.25 (pD 7.65). The titration was monitored by ^1H and ^{31}P 1D and 2D NMR.

Self-complementary 5′-d(TATGGTACCATA)-3′ (**1**) was purchased from DNA Technologies and purified by dialysis against 0.2 mM NaCl bulk over 27 hours at 4 °C with three changes of bulk solution. A 1000 MWCO "Float-A-Lyzer" dialysis membrane (Spectrum Labs) was used. The sample was then lyophilized to dryness and dissolved in NMR buffer with levofloxacin added. Final concentration was 4 mM duplex and 4.4 mM levofloxacin in 0.5 ml 99.96 % D_2O with 20 mM NaCl, 10 mM Tris at pH 8.8 (pD 9.2). Spectra recorded at 301 K or 285 K.

Self-complementary 5′-d(GATCGGCCGATC)-3′ (**2**) was purchased from DNA Technologies. The sample was desalted on a Sephadex G-25 column using 10 mM phosphate buffer as eluent. The collected fractions were lyophilized, dissolved in 1 ml doubly distilled deionised water (Millipore) and dialysed against 0.2 mM NaCl bulk solution over 22 hours at 4 °C with three changes of bulk solution. A 1000 MWCO "Float-A-Lyzer" dialysis membrane (Spectrum Labs) was used. The sample was then lyophilized to dryness and dissolved in NMR buffer. Final concentration as measured by UV was 0.45 mM duplex in 450 µl 99.96 % D_2O with 24 mM NaCl,

0.01 mM EDTA, 30 mM cacodylic buffer at pH 7.03 (pD 7.43). Chemical shift references TSP 0.75 mM and TMP ca. 25 mM were added (the TMP concentration was too high by mistake, but the TMP chemical shift at 3.74 ppm did not interfere with key resonances in the 2D NMR spectra) Spectra were recorded at 305 K or 281 K. At higher concentrations of levo, some precipitate was observed (see text).

NMR measurements: The 1H NMR spectra were recorded on Bruker DRX 400, 500 or 600 spectrometers operating at ^{1}H proton frequencies 400, 500 and 600 MHz, respectively. The chemical shifts were referred to TMS or the water resonance at 4.760 ppm, 298 K. Two-dimensional NOESY spectra were recorded with mixing times between 200 and 350 ms, TOCSY with 70 ms mixing time. Typically, a total of 512 t_1 increments, each with 2048 t_2 complex points, were collected with each FID as the average of 32 - 80 transients. In the t_1 dimension, linear prediction and zero filling was applied to reach a size of 2048 data points, equal to the t_2 dimension. For resolution enhancement, a 90°-shifted squared sine bell apodization function with line broadening of 0.3 – 3.0 Hz, depending on the signal-to-noise ratio, was used in both dimensions. For sequences **1** and **2**, the assignments were made from a combined use of NOESY and TOCSY spectra. Distances were derived from 2D NOESY spectra using the isolated spin-pair approximation (ISPA) method. The cytosine H5-H6 distance (2.45 Å) was used as reference. The NOESY mixing time for **2** was 300 ms. NMR processing was performed using XWIN-NMR (Bruker Biospin), Topspin (Bruker Biospin), SPARKY[22] and MESTRE-C 2.3a[23] software.

Energy minimisation and molecular dynamics simulations: Energy minimisation (EM) and molecular dynamics (MD) simulation studies were performed using AMBER 6.0[24] with the parm94 force field. The Mg-levofloxacin complex was modelled using the gaff force field[25] which is fully compatible with parm94. Both **2** and the Mg-levo complex were built with xleap of the AMBER suite of programs. The MD simulations were performed in vacuo using the generalized Born solvation model with no periodicity and a dielectric constant of 1.0. The time step was 1 fs for the initial simulations and 2 fs for the longer simulations of the final structures. The electrostatic energy was calculated using the particle-mesh Ewald method. During whole MD simulations, Lennard-Jones interactions were computed with an infinite cut-off distance, the non-bonded interactions list was updated every 10 steps, and the SHAKE algorithm was applied to all X-H bonds (tolerance criterion of 0.0005). Two initial structures were created with the levo-Mg(II) complex positioned in the major and the minor groove, respectively. The initial structures were heated over 100 ps to 300 K by the Berendsen algorithm and extended for a 20 ps equilibration period. The structures were subsequently energy minimised (500 cycles in vacuo with the first 200

steps using the Steepest Descent algorithm) before submitted for MD simulation. The MD was consisted of four steps: i) 100 ps applying harmonic restraints of 50 kcal/mol for one key levofloxacin – DNA distance and 20 kcal/mol for the terminal WC hydrogen bonds to avoid fraying, ii) 100 ps production period with no restraints, iii) 300 ps applying all NOE derived restraints for major and minor groove, respectively and iv) 250 ps production period with no restraints. The average structure for each of the production periods (steps ii and iv) was energy minimised (500 steps of in vacuo geometry optimisation for hydrogen atoms only) and then submitted to unrestrained MD of 800 or 900 ps. The complete trajectories for the final MD simulations were used for comparison with the NMR data.

The analysis of trajectories used the programs CURVES[26] for the calculation of helical parameters and the backbone torsion angles, and the CARNAL module of AMBER6.0 to determine average. PTRAJ was used for distance calculations. The averaged structures were submitted to energy minimization before structural analysis (500 steps of in vacuo geometry optimisation for hydrogen atoms only). VMD[27] was used for structural visualization and generation of figures. The Grace[28] program was used for graph plotting.

Results and discussion

Levofloxacin interaction with ATP : ATP is required for DNA Gyrase function and provides energy for the structural rearrangement necessary for the binding of DNA around the GyrA unit and subsequent cut and strand passage.[5, 29] It was therefore interesting to investigate whether ATP could be a target for fluoroquinolones. ATP was titrated with levofloxacin in the presence and absence of Mg(II) and followed by ^{1}H and ^{31}P NMR (Table S-1, Supplementary Information). Levo interacted with ATP in the absence of Mg(II) as shown by ^{1}H chemical shift differences for the aromatic signals of both levo and ATP. The addition of Mg(II) induced selective line broadening for levo aromatic signals showing that Mg(II) was bound to the keto-carboxyl group as expected.[21] Line broadening of the α and γ phosphates in the ^{31}P NMR spectrum showed Mg(II) binding to ATP. Increased line broadening of α, β and γ phosphate signals for ATP in the presence of both Mg(II) and levo at a 1:1:1 ratio (Figure S-1, Supplementary Information), selective broadening of ATP-H2 (+3.4 Hz) and slightly larger shift differences for ATP and levo suggested that a ternary ATP-Mg(II)-levo complex is formed. However, the lack of significantly downfield shifted aromatic signals ruled out the possibility of a stacking arrangement for the ternary complex.

Levofloxacin interaction with single-stranded DNA: Several groups have investigated the possible binding preference of fluoroquinolones for single- or double-stranded DNA.[6, 11, 12, 17, 19, 30] All studies have employed fluorescence and/or CD/LD spectroscopic techniques. To our knowledge, no high resolution NMR study has so far been reported and to gain further insight into the mechanisms of binding to either ss- or ds-DNA, the interaction of levofloxacin with a short ss-DNA was investigated. The sequence 5′-d($C_1C_2T_3A_4A_5T_6C_7C_8$) (**3**) was chosen as model since binding preference for ciprofloxacin binding to ss-DNA containing solely TA-base pairs in the central region has been reported.[19]

The levo:Mg(II) complex was added until a final ratio of 3:1:19 (1D ^{1}H spectra in Figure S-2, Supplementary Information). Both 2D ^{1}H-^{1}H and 1D ^{31}P NMR showed no significant interaction. This was surprising taking into account the reported preference for ss-DNA[30] and especially for sequences containing central TA-base pairs.[19] Mg(II) is bound to the keto-carboxylic group as seen by the broadness of the aromatic levo signals H2 and H5.[21]

The groups of Kim[6] and Ulrih[19] have reported no preference for either ss- or ds-DNA at intermediate to high salt concentration (> ~5 mM), while at low concentration (1.8 mM) or in the absence of NaCl, a ~4-5 fold preference for ss-DNA was observed. Possibly, the high salt concentration could be one reason for the discrepancy between the NMR data and the fluorescence, CD and LD data.

Magnesium(II) titration of levofloxacin/d($T_1A_2T_3G_4G_5T_6A_7C_8C_9A_{10}T_{11}A_{12}$)$_2$ (1)

It is known that a majority of small molecules that bind in the minor groove of duplex DNA, e.g. distamycin and Hoechst 33258, have a marked preference for AT-rich sequences.[31-33] Quinolones are found to interfere with DNA topoisomerases and transcription factor binding.[34-37] AT-tracts in B-DNA result in a narrowing of the minor groove and it has been suggested that sequence specificity may be dictated by groove width, allowing optimum van der Waal's complementarity and hydrophobic surface burial, rather than specific hydrogen bonding interactions with the groove floor.[38] This was observed for ciprofloxacin interaction with a DNA oligomer[10] which showed that the van der Waal's complementarity in the minor groove gave a significant contribution to the stabilization energy.

A solution of the self-complementary oligomer 5′-d($T_1A_2T_3G_4G_5T_6A_7C_8C_9A_{10}T_{11}A_{12}$) (**1**) and levofloxacin at a 1:1 ratio was titrated with magnesium(II). The combination of 2D NOESY and TOCSY spectra allowed the assignment of all resonances, except H4′, H5′ and H5′′. The spectra showed that little or no interaction took place between levo and **1** in the absence of magnesium as evident from the lack of chemical shift changes and the absence of NOE cross-peaks.

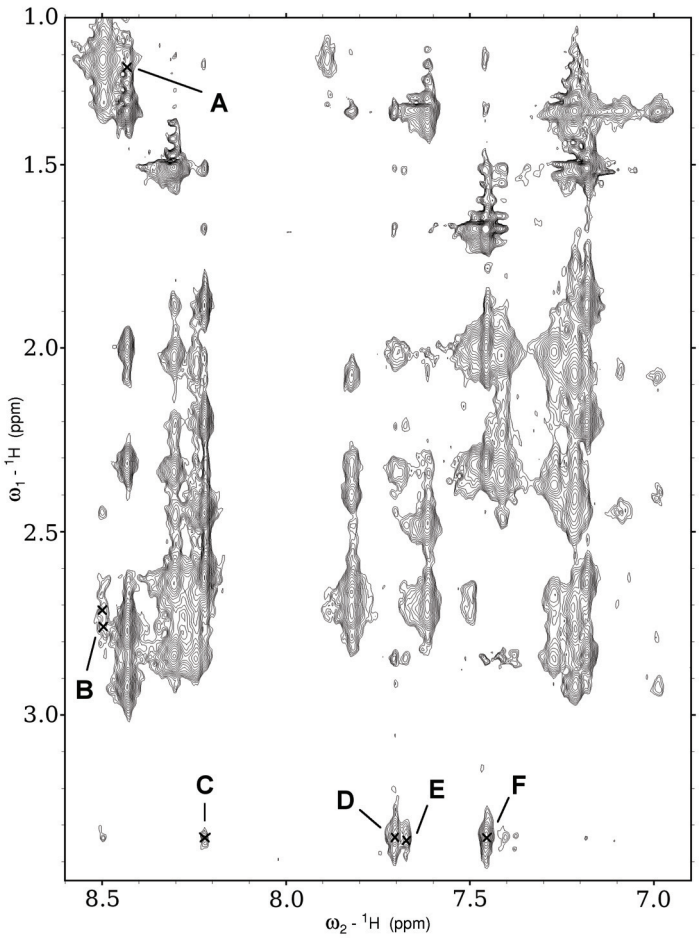


Figure 1. The aromat-H2′/2′′region of a 2D NOESY spectrum showing intermolecular cross-peaks between levofloxacin and **1** at a ratio 1:1:4 (levo:DNA:Mg(II)). NOESY mixing time 250 ms, 20 mM NaCl, 10 mM Tris at pH 8.8 (pD 9.2), 301 K. Marked cross-peaks: (A) Lev-Me3′′ - A2-H8, (B) A2-H2′ - Lev-H2, (C) Lev-H2′/6′ - A12-H8, (D) Lev-H2′/6′ - A2-H2, (E) Lev-H2′/6′ - A10-H2 and (F) Lev-H2′/6′ - T1-H6.

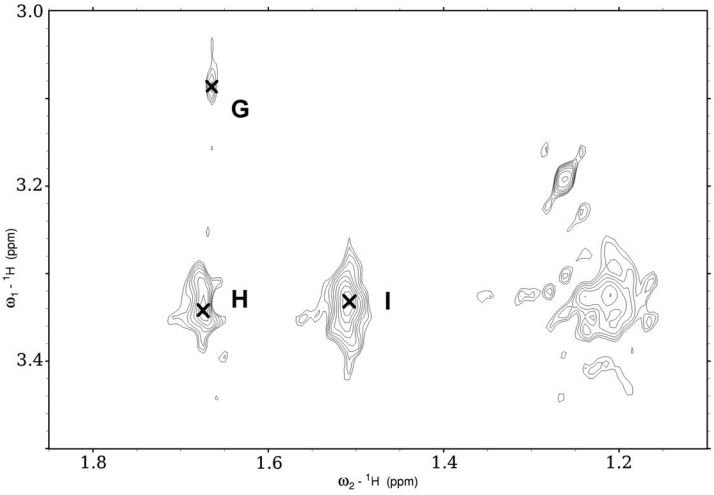


Figure 2. Region of a 2D NOESY spectrum showing the cross-peaks between the levo piperazine moiety and the T1 and T11 methyl groups at ratio 1:1:4 (levo:DNA:Mg(II)). Same conditions as Figure 1. Marked cross-peaks: (G) Lev-H3′/5′ - T1-Me, (H) Lev-H2′/6′ - T1-Me and (I) Lev-H2′/6′ - T11-Me.

The addition of Mg(II) did not significantly alter the B-DNA conformation for **1**, as seen from the small chemical shift differences and the presence of only one complete sequential walk in the aromatic-anomeric region of the NOESY spectrum (Figure S-3, Supplementary Information). Some minor peaks could not be assigned. A complete sequential walk could be performed also in the aromatic-aromatic and H2′/H2′′-aromatic regions. However, the sequential walk was broken in the H3′-H6/H8 region for G5 as the G4-H3′ - G5-H8 and G5-H3′ - H8 cross-peaks were missing. No other spectral observations suggested an interaction at this site. The major interaction site seemed to be the terminal TAT region as demonstrated by the observation of several medium and weak intermolecular NOE cross-peaks between levo and **1**, found mainly within this context (Figures 1 and 2, Table 1). Additionally, major chemical shift changes were observed for T1 (H2′, H2′′, H6, CH_3) and for A2 (H1′) and multiple sets of signals were observed for T1, T3, C9 and A12. One may notice that not all the observed NOE contacts between levo and DNA (Table 1) are compatible with a one-site binding model. At least 4 sets of mutually consistent NOE distances may be distinguished representing both minor and major groove interaction. From the 1D and 2D NMR data, no clear preference for either major or minor groove could be found. Restrained molecular modelling was carried out based on distances derived from the NOE cross-peaks (*vide infra*).

Table 1. Intermolecular cross-peaks between levo and **1** from 2D NOESY spectra at ratio 1:1:4 (levo:DNA:Mg(II)).

Major groove restraints [Å]				**Minor groove restraints [Å]**			
Levo	**Base**	**Atom**	**NOE[a]**	**Levo**	**Base**	**Atom**	**NOE[a]**
H2	A12	H2′	w	H5	G4	H1′	vw
H2	A12	H8	w	H2	G4	H1′	w
H2	A2	H2′	vw	H3′′	T1	H1′	m
H2	T1	H6	vw	H2′/6′	A10	H2	w
H2′′[b]	T3	Me	m	H2′/6′	A12	H1′	w
H3′′	T1	H6	w	H2′/6′	A2	H2	m
H3′′	T3	Me	m	H2′/6′	C9	H1′	w
H2′/6′	A12	H8	m	H2′/6′	T11	H1′	m
H2′/6′	T1	H6	m	H2′/6′	T3	H1′	w
H2′/6′	T1	Me	w	Me3′′	A2	H1′	vw
H2′/6′	T11	Me	m	Me3′′	G4	H1′	m
Me3′′	A2	H8	vw	Me3′′	G5	H1′	vw
Me3′′	T1	H6	w	Me4′	T1	H1′	w

[a] The NOE derived distances are divided into three groups: s = < 2.5 Å, m = 2.5-4.0 Å, w = 4.0-5.5 Å (referenced to $d_{\{C8\ H5\text{-}H6\}}$=2.45 Å), [b] Ambiguous assignment for H2′′a and H2′′b.

The terminal T1 and A12 residues were seen to adopt *syn* conformations, as indicated by strong internal H1′-H8 cross-peaks and H3′-H8 cross-peaks.[39] It is not unusual that terminal residues adopt *syn* conformations due to base pair opening, so-called fraying. However, several cross-peaks between the piperazine ring and the T1-A12 nucleotides were consistent with a separate class of cross-peaks, indicating that fraying could be important for one mode of levo - ds-DNA interaction. A second class of cross-peaks was consistent with minor groove interaction centred at T3. In this case, molecular modelling (*vide infra*) showed that the binding of levo demanded no increase in the minor groove width, indicating a different mode of interaction than for former class. This suggests the presence of three different interaction modes between levo and **1**: major groove, minor groove and interaction with the terminal residues.

Titration of magnesium(II) and levofloxacin to d($G_1A_2T_3C_4G_5G_6C_7C_8G_9A_{10}T_{11}C_{12}$)$_2$: The self-complementary dodecamer 5′-d($G_1A_2T_3C_4G_5G_6C_7C_8G_9A_{10}T_{11}C_{12}$), (**2**) was used as a model to investigate possible sequence specificity for groove binding and intercalation. Initially, levo was added to **2** until a 0.8:1 (levo:DNA) ratio. A general weak line broadening was observed for all signals, except for the G6-H8 signal which experienced a larger broadening and for the levo signals which were very broad (Table 2). The addition of Mg(II) to a ratio of 0.8:1:20 (levo:DNA:Mg(II)) induced insignificant changes in the ^{1}H spectra. Increasing the levo concentration to r = 1.6:1:20 resulted in the observation of weak cross-peaks in the 2D NOESY spectrum between levo and the minor groove of **2**: Lev-H2′/6′ - A2-H2, Lev-H2′/6′ - (A10/C12)-H1′ and Lev-Me3′′ - A2-H4′. At r = 3.3:1:20 an additional cross-peak, Lev-H2′/6′ - A2-H2′ appears. Surprisingly, no significant shift differences were observed and the NOESY sequential walk was complete, indicating that no large structural perturbation of the B-DNA conformation take place . A general line broadening was observed for all signals, except A2-H2 and C12-H5 which were sharper and G6-H8 and C7-H5 which were broader. Further addition of levo to r = 5:1:20 resulted in one additional minor groove cross-peak, Lev-H2′/6′ - A10-H2. The A2-H8 and A10-H2 signals were selectively broadened and A2-H2 was shifted upfield. The Lev-H2 signal shifted downfield suggesting a change in the equilibrium of free *vs.* bound drug.

Table 2. ^{1}H chemical shift values of **2** and shift differences for levo:DNA:Mg(II) at the ratio 7:1:20 (given in *italic*). Temperature was 305 K, pD 7.5. More details are given in Experimental.

	H6/H8	H2/H5/Me	H1′	H2′	H2′′	H3′	H4′
G1	7.90		5.68	2.56	2.74	4.86	4.20
	-0.05		*-0.08*	*-0.04*	*-0.04*	*0.00*	*n.a.*
A2	8.33	8.00	6.35	2.76	2.99	5.05	4.49
	-0.03	*-0.10*	*-0.06*	*-0.04*	*-0.05*	*-0.01*	*0.00*
T3	7.20	1.38	5.96	2.08	2.49	4.87	4.23
	0.00	*-0.01*	*-0.02*	*-0.04*	*-0.04*	*-0.01*	*n.a.*
C4	7.40	5.56	5.69	1.94	2.37	4.85	4.12
	0.00	*0.01*	*-0.02*	*0.02*	*-0.04*	*n.a.*	*n.a.*
G5	7.84		5.63	2.66	2.74	4.99	4.34
	0.04		*n.a.*	*0.03*	*0.02*	*n.a.*	*-0.01*
G6	7.72		5.87	2.54	2.69	4.96	4.39
	0.02		*-0.04*	*0.00*	*-0.03*	*n.a.*	*n.a.*
C7	7.30	5.24	5.91	2.02	2.42	4.80	4.17
	0.04	*0.00*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*
C8	7.36	5.51	5.50	1.96	2.33	4.82	4.05
	0.02	*0.02*	*0.01*	*0.03*	*-0.01*	*n.a.*	*n.a.*
G9	7.88		5.66	2.72	2.80	5.01	4.35
	-0.01		*-0.04*	*0.01*	*-0.03*	*n.a.*	*-0.01*
A10	8.16	7.86	6.26	2.62	2.90	4.99	4.44
	0.01	*-0.06*	*-0.02*	*0.02*	*-0.01*	*0.00*	*-0.01*
T11	7.19	1.43	6.02	2.05	2.46	4.85	4.16
	0.01	*-0.04*	*-0.04*	*-0.03*	*-0.03*	*n.a.*	*n.a.*
C12	7.63	5.77	6.28	2.27	2.27	4.56	4.04
	0.01	*0.00*	*-0.05*	*0.00*	*0.00*	*0.01*	*-0.02*

With further increase of the levo concentration to r = 7:1:20 a second interaction site become apparent. The NOESY spectrum showed that the sequential walk was broken for the context - $C_4G_5G_6C_7$ -, suggesting intercalation of levo at this site (Figure 3). The G5pG6 step seems to be the predominating intercalation site as both the C4pG5 and G6pC7 steps exhibit weak inter-nucleotide cross-peaks in the H2′/2′′-H6/8 region of the NOESY spectra. Intercalation at the G5pG6 step could lead to disruption of the W-C bonds to the complementary C7pC8 step. This could be one reason why the aromatic signals of the C7 and C8 bases are strongly and selectively broadened (e.g. the C7-H5 – H6 cross-peak in figure 3, top, right). No inter-molecular cross-peaks between levo aromatic signals and those of the intercalated bases were observed. However, it is unlikely that such cross-peaks could be observed due to the broadness of the signals. All the intra-molecular cross-peaks were observed for the - $C_4G_5G_6C_7$ - context. It could be argued that the break was a consequence of partial denaturing of the duplex, but the lack of major chemical shifts as well as the non-perturbed sequential walk for the terminal ends strongly suggests that the duplex conformation was intact. Interestingly, minor groove binding is also present as evident from several

cross-peaks between the levo piperazine ring and sugar protons of **2** (see Table 3). The 1D ^{1}H spectrum showed selective line broadening for G6-H8, C7-H5, C8-H1′, C8-H5, A10-H2 and A10-H8. Upfield shifts (0.05-0.10 ppm) were observed for A2-H2 and G5-H8. Interestingly, the bases T3, G9, T11 and C12 did not seem to be affected by shift or line broadening.

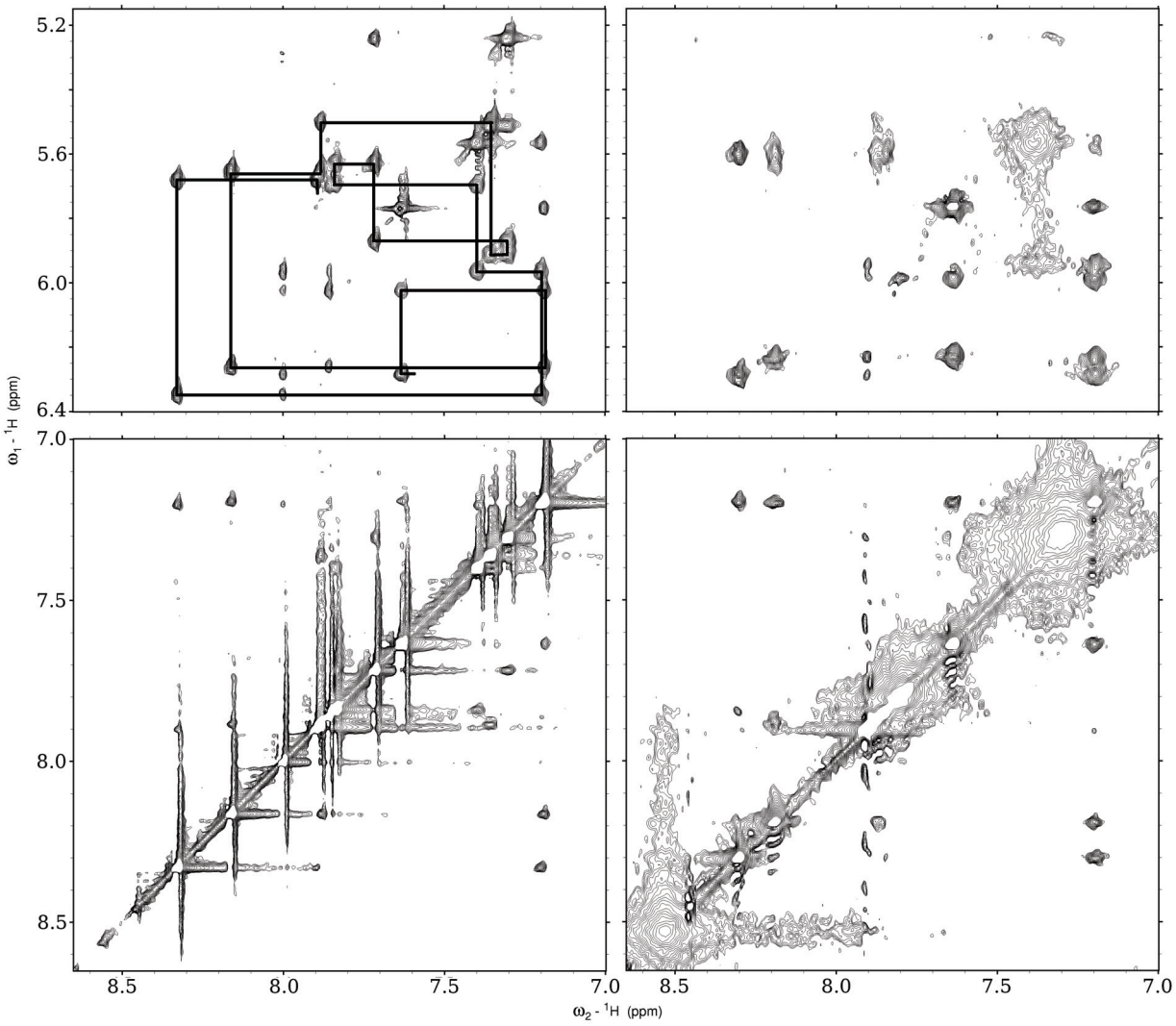


Figure 3. Selected regions of 2D NOESY spectra at r = 0:1:0 (left) and r = 7:1:20 (right) (r = levo:DNA:Mg(II)). The sequential walk in the anomeric-aromat region is indicated for r = 0:1:0 (top left). Spectra were recorded at 305 K in 99.96 % D_2O with 24 mM NaCl, 0.01 mM EDTA, 30 mM cacodylic buffer at pH 7.03 (pD 7.43).

The NMR data are consistent with minor groove binding at the terminal end as evident from the NOE cross-peaks and the selective broadening of A10-H2 and upfield shift of A2-H2. In addition, a break in the sequential walk and selective line broadening of G6-H8 may be due to intercalation at the G5pG6 step. CD spectra of levo interaction with poly[d(G-C)·d(G-C)] showed

that a transition from predominantly one-site binding to multiple-site binding started at a ratio of one levo molecule per five DNA bases.[17] For our system significant intercalation takes place at levo:DNA ratios higher than ~5:1 with the most pronounced interaction at 7:1, corresponding to levo:base-pair ratios of 1:5 and 1:3, respectively, in good agreement with the CD observations. This further strengthens the hypothesis that groove binding is the predominant interaction mode at low levo:DNA ratios, while intercalation occurs only at higher ratios.

Table 3. Observed intermolecular cross-peaks between levo and **2** from 2D NOESY spectra at ratio 7:1:20 (levo:DNA:Mg(II)).

Levo	**2**		NOE[a]
H2′/6′	A2	H2	m
H2′/6′	T3	H1′	w
H2′/6′	C7	H1′	w
H2′/6′	A10	H2	m
H2′/6′	A10/C12	H1′	m
H2′/6′	T11	H1′	w
H2′/6′	C12	H2′/2′′	w
Me4′	G1/G6	H4′	m
Me4′	G1/G9	H1′	w
Me4′	T3	H1′	w
Me4′	T3/T11	H4′	m
Me4′	C4/C8	H4′	m
Me4′	G5/G9	H4′	w
Me4′	T11	H1′	w

[a]The NOE derived distances are divided into three groups: s = < 2.5 Å, m = 2.5-4.0 Å, w =4.0-5.5 Å

Molecular modelling of Mg(II)-levofloxacin interaction with d($T_1A_2T_3G_4G_5T_6A_7C_8C_9A_{10}T_{11}A_{12}$)$_2$: Energy minimisation (EM) and molecular dynamics (MD) simulation studies were performed for levo adduct of the self-complementary oligomer 5′-d($T_1A_2T_3G_4G_5T_6A_7C_8C_9A_{10}T_{11}A_{12}$) using Amber 6.0[24] with force fields parm94 and General Amber Force Field (gaff). Two initial starting structures were built with the levofloxacin-Mg(II) complex placed in the major and the minor grooves, respectively. Distance restraints were derived from 2D NOESY spectra at ratio 1:1:4 (levo:DNA:Mg(II)) by the isolated spin-pair approximation (ISPA) method[40] and characterized as major and minor groove restraints, respectively. Energy minimization was performed to initialise the structures and a three-step MD simulation was applied as follows: i) 100 ps with one key levo-**1** NOE restraint; ii) 100 ps with no NOE restraints; iii) the averaged structure from step (ii) was subjected to a 800 or 900 ps non-restrained MD (details in Experimental).

For the major groove MD, initally the T3-Me - Lev-H3′′ distance was restrained to 3.2±1.0

Å. The levo-Mg(II) complex was orientated with the Mg(II) atom facing the phosphate backbone in the major groove for electrostatic interaction (Structure I, Figure 4). In step (ii), the piperazine ring buried deeper into the groove, allowing the aromatic moiety of levo to form stacking interaction with the base of residue G4. In step (iii), the conformation was stabilised by Mg(II) interaction with the phosphate group of T6. The W-C base pairing for the three first bases was broken, but stacking was retained for A10 and T11. The helix showed an overall 35±15º bending towards the major groove (Structure II, Figure 4). Comparison with the NOE distances show that structures I and II satisfy the same restraints, while a second class of restraints indicate binding to the terminal base pairs.

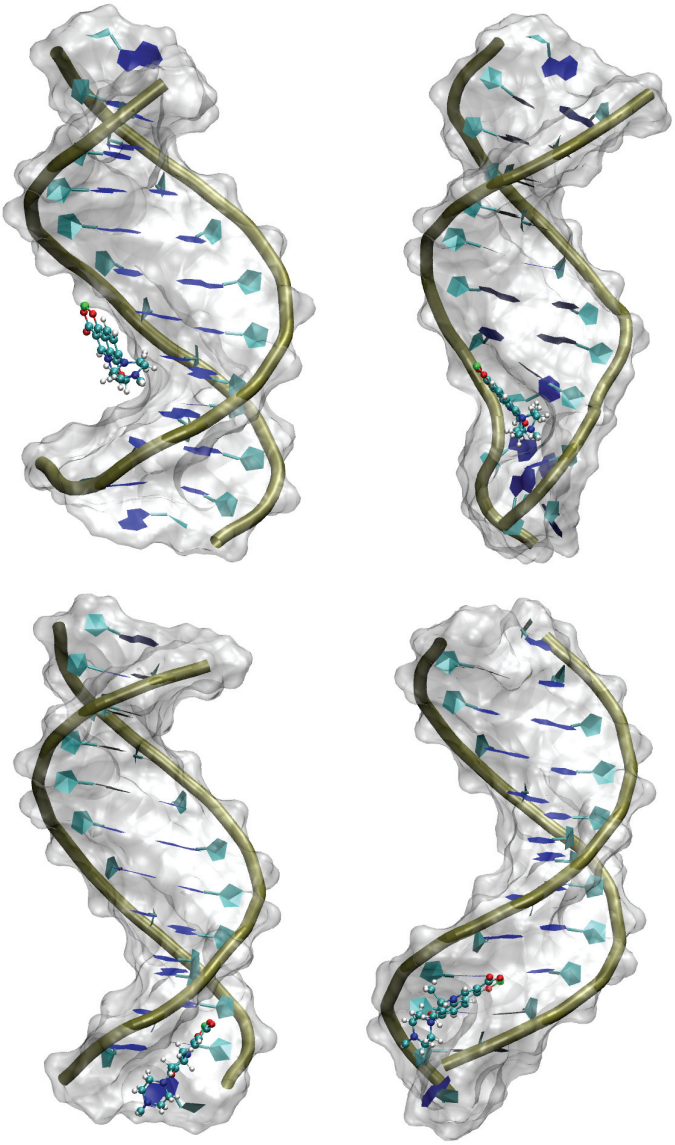


Figure 4. Averaged and energy minimized structures from the MD trajectories of the binding of the levofloxacin-Mg(II) complex in the major and minor groove of **1**. Details for structures I (left) and II (right), III (bottom left) and IV (bottom right) are given in the text. Levofloxacin is presented in a

CPK-model, the phosphate backbone in golden tube and the DNA surface in transparent grey.

For the minor groove MD, the distance A2-H2 – Lev-H2′/6′ was restrained to 3.5±1.0 Å in the first step (Structure III, Figure 4). This pulled the levo-Mg(II) complex into the minor groove oriented with the piperazine ring towards the terminal end. In step (ii), this restraint was removed, allowing levo to move along the minor groove towards the central region of the duplex. The conformation in step (ii) was averaged and subjected to a non-restrained 900 ps MD. Levo remained stable in the minor groove with only small fluctuations throughout the simulation (Structure IV, Figure 4). This structure is in agreement with most of the piperazine and H5 cross-peaks observed, except cross-peaks to the terminal base pair. This suggests, as for sequence **1**, that multiple binding sites are present. Structure IV had the lowest energy of all the conformations investigated (see Supplementary Information). The B-DNA structure is mostly unperturbed due to the near-perfect fit of levo in the groove (Figure 5) and the conformation is very similar to the model for ciprofloxacin binding to DNA derived from docking calculations[10] and to the model of norfloxacin binding to DNA derived from MD simulations and CD and LD spectroscopy.[15] No widening of the minor groove width beyond the normal range was observed. This suggests that the optimal van der Waal's complementarity of fluoroquinolones in the minor groove is important for drug affinity and is a common feature for all quinolones. Figure 5 shows that while the aromatic rings of levo are positioned in the minor groove "sandwiched" between the phosphate backbone of each strand, the piperazine ring is positioned at the end of this tract where the backbone of one strand ends. The piperazine ring stays at this position throughout the 900 ps simulation without entering the narrower part of the groove. This conformation could be relevant for the structure of the quaternary DNA Gyrase-levo-Mg(II)-DNA complex. It is known that DNA Gyrase cuts the phosphodiester bond of each DNA strand 4 base pairs apart and fluoroquinolones are suggested to act by blocking the consecutive ligation process.[41] This could imply that fluoroquinolones interact with the dangling 4-base end of the cut DNA in a fashion similar to the binding mode seen in Figure 5. The optimal van der Waals complementarity of the levo-Mg(II) complex in the minor groove as well as the presence of distinct groups of NOE cross-peaks, suggests that levo-Mg(II) complex can easily translate along the minor groove tract.

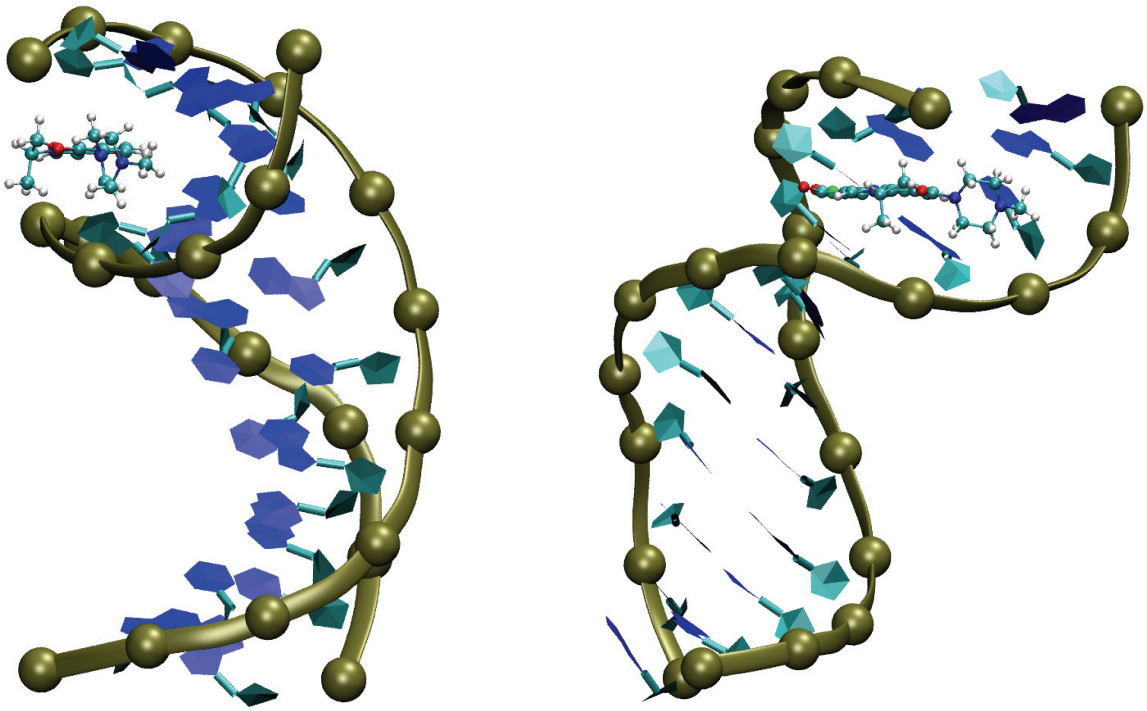


Figure 5. Levofloxacin-Mg(II) complex bound in the minor groove of **1**. The structure is a snapshot from a 900 ps non-restrained MD simulation based on a 100 ps restrained MD where the distance A2-H2 – Lev-H2′/6′ was restrained to 3.5±1.0 Å (Structure IV). The view is along the minor groove (left) and rotated anti-clockwise 90º (right). DNA bases are blue, sugars cyan, the phosphate backbone is represented by ribbons and the phosphate atoms are exaggerated as gold coloured balls for clarity.

The NMR and MD results show that the ss-DNA-like environment is not a necessity for the ability of levofloxacin to bind to ds-DNA. Furthermore, it is likely that several binding modes are present simultaneously. For sequence **1**, at levo:DNA ratios up to 1:1, no intercalation was observed for the GG site, while significant groove binding was observed for the terminal 5′-TAT end. Similarly, for sequence **2** binding in the minor groove was observed for the terminal 5′-GAT region at levo:DNA ratios 3.3:1 and higher. Contrary to the interaction of levo with **1**, no binding to the terminal base pair G1•C12 was observed. This could be explained by the fact that 5′-GAT is less prone to fraying than 5′-TAT and might also be the reason why no interaction is observed for **2** at the 1:1 ratio. At a levo:DNA ratio of 7:1 intercalation at the G5-G6 step was observed. In summary, this shows that levo can interact both in the major and minor groove, with the open terminal end and by intercalation between DNA bases. The chemical and structural environment of the trapped DNA-enzyme complex is unknown and thus it is difficult to speculate which binding mode is the most relevant at physiological conditions.

Conclusions

At the present conditions, levo does not interact with ss-DNA with or without Mg(II). This result is in disagreement with the dominating view that fluoroquinolones bind preferentially to ss-DNA.[3, 6] We have found, on the other hand, that ds-DNA form adducts with levo as demonstrated by the presence of significant cross-peaks in the 2D NOESY maps. The interaction is as expected enhanced in the presence of Mg(II) ions and the NOESY data show that the interaction site is localized at the terminal part of the duplexes; 5′-TAT (**1**) and 5′-GAT (**2**). Molecular modelling shows optimal van der Waal's complementarity of levo in the minor groove. The minor groove conformation is very similar to the model for ciprofloxacin binding to DNA derived from docking calculations[10] and to the model of norfloxacin binding to DNA derived from MD.[15] Thus, the conformational fit in the minor groove seems to be a common feature for all fluoroquinolones. There seem to be no clear sequence-dependent difference in binding affinity between duplex **1** and **2**. However, the first direct observation of fluoroquinolone intercalation was observed for **2**. A break in the sequential walk in the NOESY map shows that levo intercalates between GG steps in the central GGCC region at higher drug:DNA ratios.

Acknowledgements

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Supplementary information

Antibacterial Fluoroquinolone Agents - Interaction with DNA.

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Table S-1. ^{1}H chemical shifts and line widths of ATP and levofloxacin at pH 7.4, 305K in 40 mM phosphate buffer. ATP, levo and Mg(II) concentrations either 0 or 4 mM. [i]Ambiguous assignment.

			ATP+Mg(II)		Levo+ATP		Levo+ATP +Mg(II)	
ATP	ppm	(Hz)	ppm	(Hz)	ppm	(Hz)	ppm	(Hz)
H8	8.54	(2.0)	-0.01	(2.6)	-0.02	(1.9)	-0.05	(2.5)
H2	8.28	(1.8)	-0.01	(2.3)	-0.05	(1.9)	-0.09	(5.7)
H1′	6.15	(2.1)	0.00	(2.7)	-0.04	(2.2)	-0.05	(2.7)
H2′	4.81		0.00		-0.03		0.01	
H3′	4.62		-0.03		-0.02		-0.01	
H4′	4.41		0.00		-0.01		0.00	
H5′	4.30		-0.01		0.00		0.00	
H5′′	4.22		0.02		0.01		0.03	
Levo								
H2	8.35	(3.6)	-		0.11	(2.4)	0.20	(17.5)
H5	7.46	(4.0)	-		0.08	(2.2)	0.02	(18.8)
H3′′	4.70		-		-0.03		-0.02	
H2′′a/b[i]	4.47		-		0.08		0.06	
H2′′a/b[i]	4.38		-		0.03		0.03	
H2′6′	3.52	(11.9)	-		0.07	(10.3)	0.04	(18.1)
H3′5′	3.35	(28.1)	-		0.10		0.04	
Me4′	2.89	(4.0)	-		0.08	(2.1)	0.08	(2.3)
Me3′′	1.46	(5.4)	-		0.07	(2.7)	0.05	(4.3)

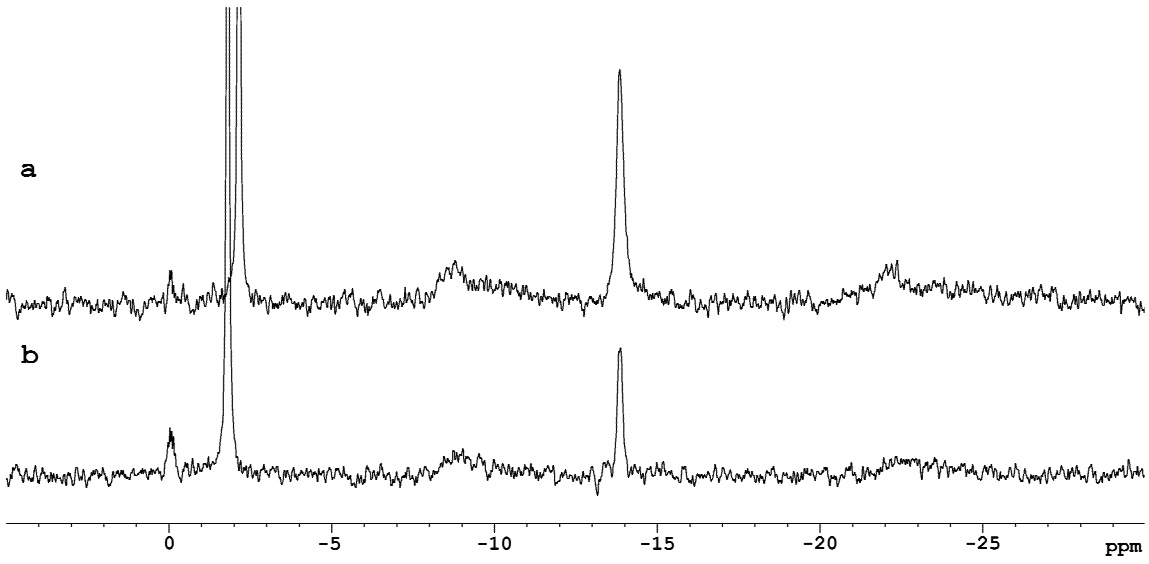

Figure S-1. 1D ^{31}P NMR spectra of 1:1 ATP:Mg(II) (a) and 1:1:1 levo:ATP:Mg(II) (b). Assignments: -1.9 (phosphate buffer), -8.8 (α) , -13.2 (β) and –22.2 ppm (γ). Conditions were 4 mM ATP, 4 mM $MgCl_2$, 4 mM levo, 40 mM phosphate buffer, pD 7.1, 305 K.

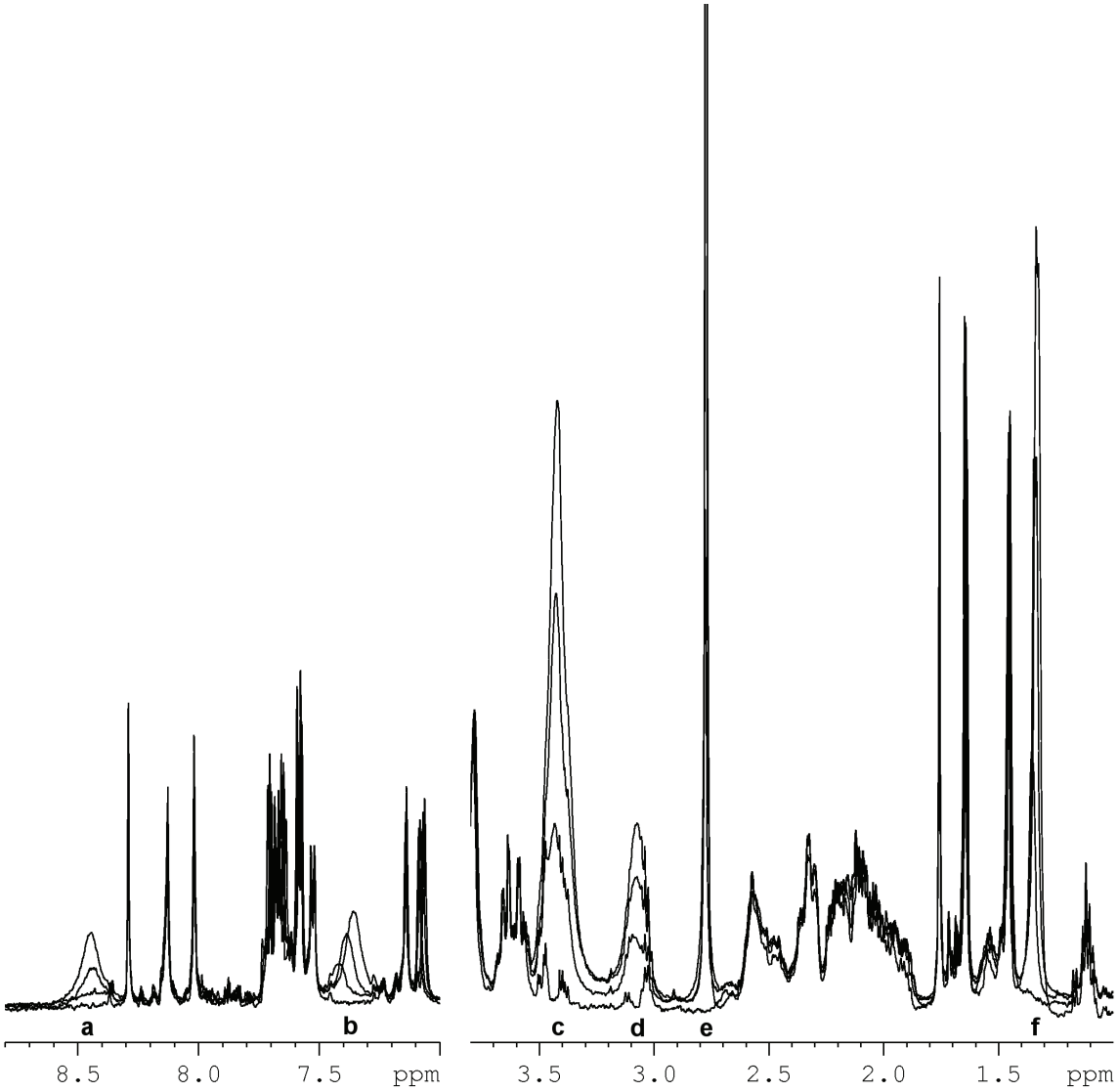

Figure S-2. Stacked plot of 1D ^{1}H NMR spectra of levo: **3**:Mg(II) at ratios 0.5:1:16, 1:1:16, 2:1:16 and 3:1:16. Spectra were recorded in 99.96 % D_2O at 281 K with 70 mM phosphate buffer, 45 mM NaCl, pD 7.64. Assignment of levo peaks: H2 (a), H5 (b), H2′/6′ (c), H3′/5′ (d), Me4′ (e) and Me3′′ (f).

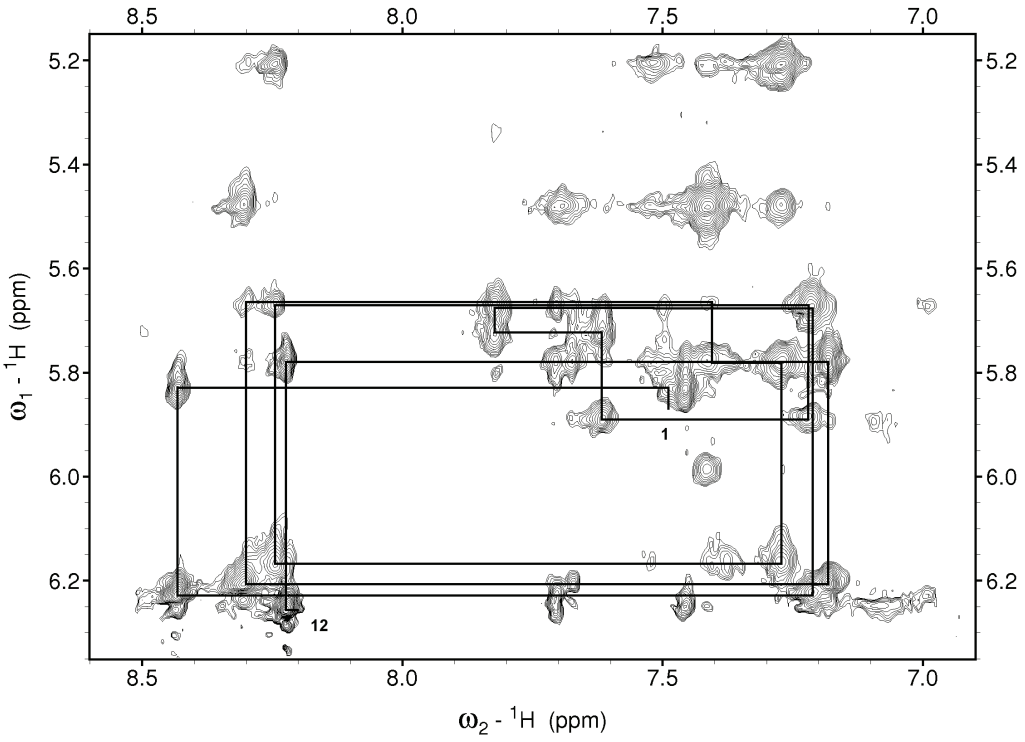

Figure S-3. The anomeric-aromatic regions of the 2D NOESY spectrum showing the sequential walk for **1** at ratio 1:1:4 (levo:DNA:Mg(II)). NOESY mixing time 250 ms, 20 mM NaCl, 10 mM Tris at pH 8.8 (pD 9.2), 301 K.

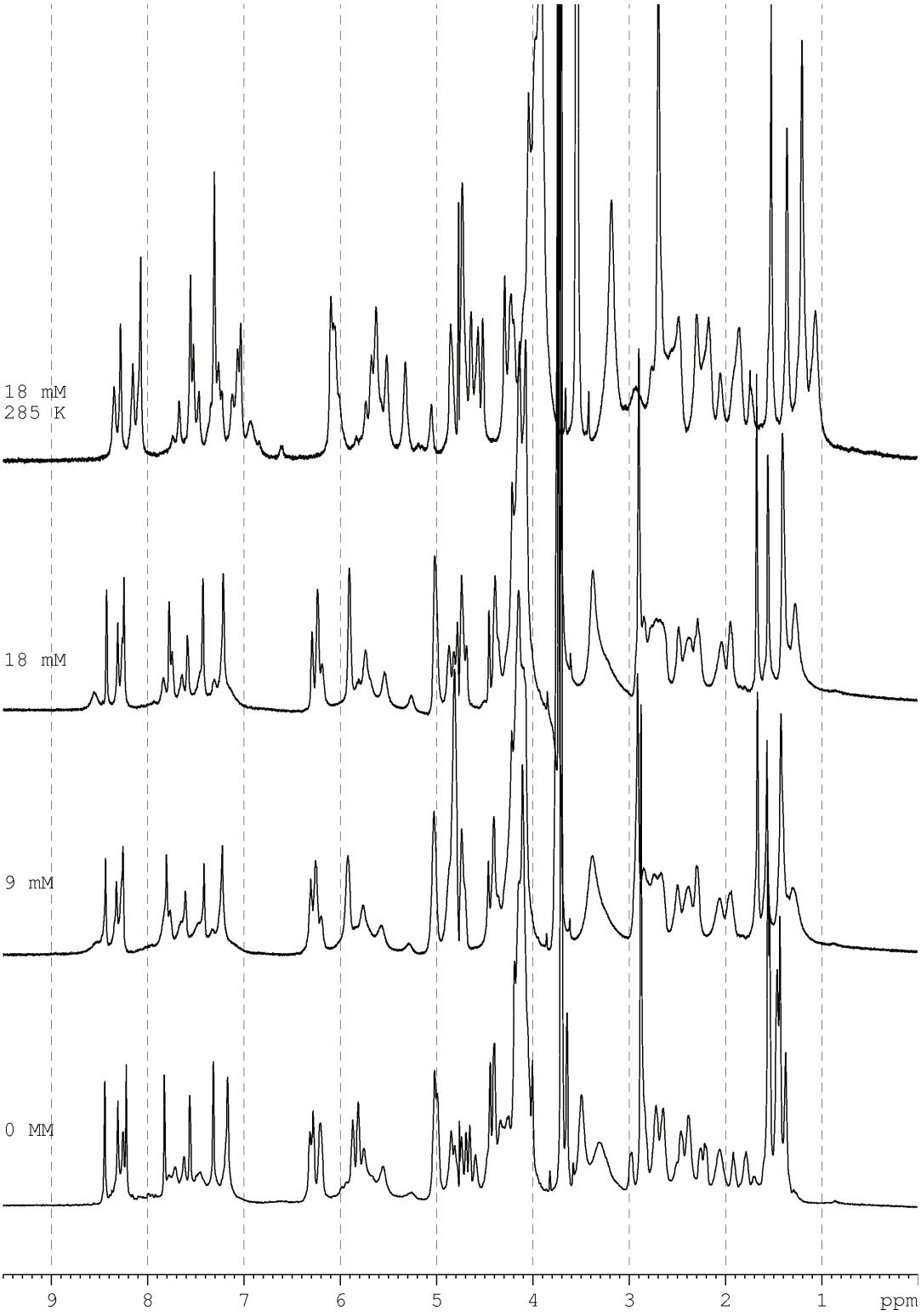

Figure S-4. Proton NMR spectra of 4.0 mM levofloxacin and 4.4 mM **1** with increasing Mg(II) concentrations. Conditions were 20 mM NaCl, 10 mM Tris buffer, pH 8.8 (pD 9.2) at 301 K.

Table S-4. Energies for the energy minimised structures I-IV as calculated by Amber 6.0. All energies in kcal/mol. The first 250 cycles were steepest descent, convergence criteria was rms < 0.05. All calculations converged between 2000 and 2500 cycles.

Structure	E_{Total}	E_{Bond}	E_{Angle}	$E_{Dihedral}$	E_{vdW}	$E_{1\text{-}4\ vdW}$	$E_{El.stat}$	$E_{1\text{-}4\ El.stat}$
I[a]	388.03	16.15	107.62	352.59	-474.46	193.44	620.70	-428.01
II[a]	391.07	16.29	109.34	357.24	-482.09	192.47	626.35	-428.52
III[b]	368.79	15.54	110.42	349.27	-481.85	191.91	609.83	-426.33
IV[b]	356.06	15.59	112.03	346.30	-498.96	192.58	612.65	-424.12

[a]minor groove conformation, [b]major groove conformation

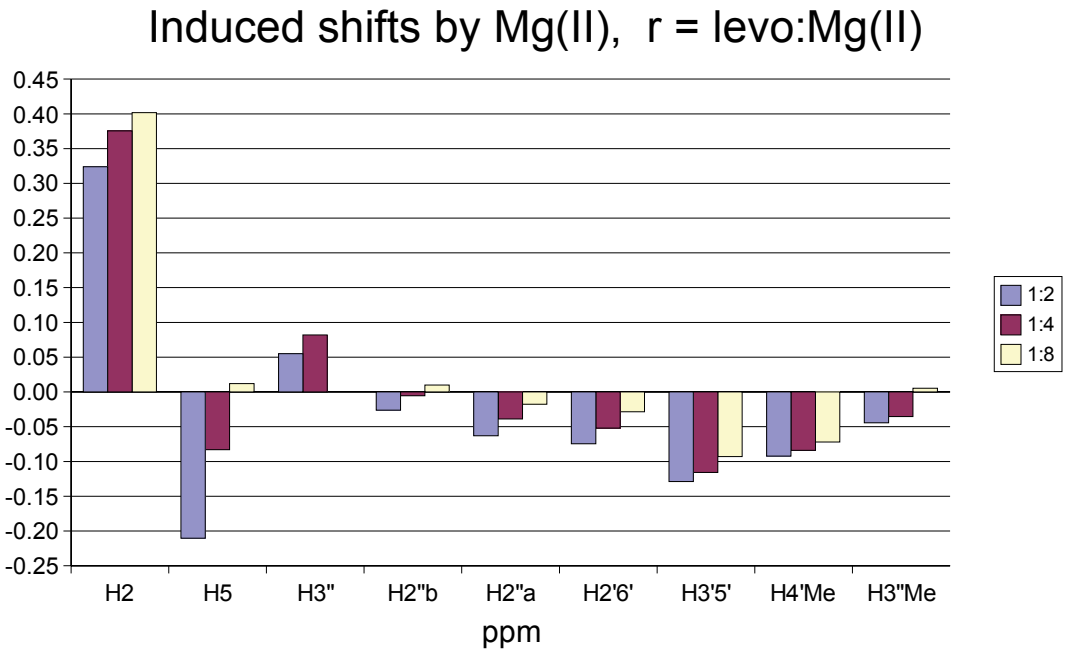

Figure S-5. Chemical shift differences of levofloxacin resonances induced by the titration of Mg(II) to a 4.4 mM levofloxacin solution at pH 9.2, 301 K.