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SPECTROFLUORIMETRIC STUDIES ON THE INTERACTION OF MOXIFLOXACIN WITH HERRING SPERM DNA

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ABSTRACT: Moxifloxacin (MOX) is a fourth-generation synthetic fluoroquinolone antibacterial agent with many important therapeutic properties. Fluorescence measurement was used to study the interaction of MOX with herring sperm DNA (Hs-DNA) in Tris-HCl, buffer of pH 7.4. The intercalative binding mode and a static quenching mechanism were confirmed by the Stern-Volmer quenching rate constant (*Kq*) of 5.611 x 10^{13} mol^{-1} s^{-1}(R^2 = 0.99876) at 298 K. The thermodynamic parameters (ΔH = -133.55 kJ mol^{-1} and ΔS = -349.98 J mol^{-1} K^{-1}) were calculated at different temperatures, and they indicate that the main forces between MOX and Hs-DNA are hydrogen bonding and Van der Waals force. We proved at the same time the presence of one single binding site on Hs-DNA, and the binding constant is 1.48 x 10^5 M^{-1} at physiological pH. The results may provide a basis for further studies and clinical application of antibacterial drugs.

INTRODUCTION: As the most common genetic material of various biosomes in nature, deoxyribonucleic acid (DNA) plays a key role in different kinds of vital processes including gene expression, mutagenesis, cell death, etc. An increasing number of studies have suggested that there is an essential linkage between environmental factors (chemical drugs, nuclear radiations, etc.) and genetic damage.[1] increasing attention has been paid to the study of molecular interactions between DNA and many drugs.[2-4] Studying the binding mechanism is of great importance in terms of life science, chemicals, pharmaceuticals, and clinical medicines.

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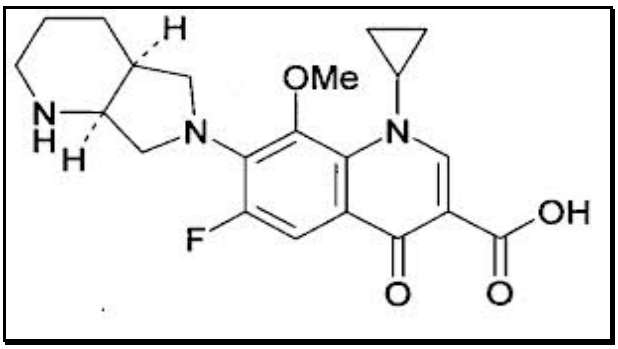
The fluoroquinolones are one of the most useful types of synthetic antibacterial agents due to their broad spectrum of activity against gram-positive and gram-negative bacteria and mycoplasma pneumoniae.[5-7] Because of this; fluoroquinolones are used in the treatment of bacterial infections, including respiratory, soft tissue, urinary tract, and joint infections. Moxifloxacin (MOX), with fewer side effects and extended half-life, is a novel fourth-generation quinolone antimicrobial drug. Its chemical structure is shown in **Scheme 1**.



SCHEME 1. STRUCTURE OF MOXIFLOXACIN

As far as we know, the interaction between MOX and Hs-DNA has not been investigated. MOX, however, is not limited to clinical applications. It is also widely used in the treatment and prevention of veterinary diseases in animals intended for human consumption and commercially farmed fish,[11] hence the interaction of MOX with Hs-DNA is worthy of further study.

Consequently, this study is expected to provide important insight into the essence and the potential toxicity between drugs and Hs-DNA in realistic situations and may also provide an available clinical reference for future combination and therapy guidelines for the development of new low toxicity and more efficient drugs. What is more, this study will contribute to our understanding of the interaction mechanism and the reason for the difference in the biological activity and clinical efficacy of MOX and its analogues.

MATERIALS AND METHODS:

Instrument: Fluorescence measurements were performed by using Hitachi F2700 spectrofluorimeter (JAPAN) equipped with a 150 W Xenon lamp and 1 cm quartz cell. The widths of excitation and emission slits were set at 5.0 and 10 nm respectively. The pH measurements were made with Scott Gerate pH meter CG 804. An electronic thermostat water-bath was used for controlling the temperature.

Reagents and samples: Commercially prepared herring sperm DNA (Hs-DNA, purity >99.0%), acridine orange (AO) and Hoechst 33258 were purchased from Sigma Aldrich. MOX (CAS#, 151096-09-2) was obtained from Sigma (purity grade inferior 99.9%). Ethidium bromide (EB, CAS#, 1239-45-8) was purchased from Sigma (purity grade superior 99%). All the other chemicals and solvents were of reagent grade and used without purification.

Sample preparation: Stock solutions of 2×10^{-3} M were prepared by dissolving the appropriate amount of the drug with double distilled water and then diluting to the mark of a 100 cm^3 volumetric flask. Exactly 11.6 mg of EB was dissolved with double distilled water and diluted to the mark of a 50 cm^3 volumetric flask. The concentration of solution was 5.89×10^{-4} M. The concentration of Hs-DNA solution was determined by UV absorbance at 260 nm using molar extinction coefficient $\varepsilon 260nm = 6600\ cm^{-1}\ M^{-1}$. Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, pH 7.4) was used as standard buffer solution, the concentrations of HCl and NaCl were 0.6 M and 0.2 M, respectively, and double distilled water was used for all solution preparation. All the solutions were kept at 277 K.

PROCEDURE:

Fluorescence Studies: Fluorescence emission spectra of MOX were performed by using Hitachi F2700 spectrofluorimeter (JAPAN) equipped with a 150 W Xenon lamp and 1 cm quartz cell. The widths of excitation and emission slits were set at 5.0 and 10 nm respectively. Excitation was fixed at 280 nm[8] and emission spectra were recorded from 400 nm to 850 nm after setting the widths of both the excitation and the emission slits at 10 nm. Appropriate blanks corresponding to the buffer were subtracted to correct the background fluorescence.

The fluorescence titration was carried out by keeping the concentration of MOX constant (50 mM) and varying Hs-DNA concentration (0 - 45 mM). In case of EB displacement assay, a solution containing 2 mM of EB and 20 mM of DNA was titrated with increasing concentration of MOX. EB-Hs-DNA complex was excited at 471 nm and emission spectra were recorded from 500–700 nm.

In another experiments, AO-DNA complex was excited at 490 nm while DNA-Hoechst 33258 complex was excited at 343 nm and emission spectra were recorded from 500 - 600 nm and 360–600 nm respectively. Iodide quenching experiments were performed in presence and absence of DNA. Emission spectra were recorded either in presence or absence of 50 mM Hs-DNA in 3 ml reaction mixture which included 50 mM MOX, 10 mM Tris- HCl (pH 7.4) and varying concentration of KI between 0–8 mM. Excitation was done at 280 nm and emission spectra were recorded from 400–850 nm. Effect of ionic strength was studied by varying the concentration of NaCl between 0–70 mM in total volume of 3 ml containing 50 mM MOX, 50 mM Hs-DNA and 10 mM Tris-HCl (pH 7.4). Excitation was done at 280

nm and emission spectra were recorded between 400–850 nm.

The effect of temperature on the fluorescence of MOX-Hs-DNA: This part was conducted by fixing the concentration of MOX and pH 7.4 Tris-HCl while varying the Hs-DNA concentration. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static for 25 min at 298 K, 308 K, and 313 K.

The effect of KI or NaCl on the fluorescence of MOX-Hs-DNA: The salt effect experiments were conducted by adding various amounts of NaCl to MOX-Hs-DNA mixture. Iodide quenching experiments were carried out by adding various concentrations of potassium iodide stock solution to MOX and MOX-Hs-DNA mixture, respectively. The experiments were conducted at 298 K.

The competitive effect of EB on the fluorescence of MOX-Hs-DNA: Fixed concentrations of EB and Hs-DNA and varied concentrations of MOX were added to colour comparison tubes. The samples were kept static for 25 min at 298 K.

RESULTS AND DISCUSSION:

Steady state fluorescence: To elucidate the interaction of MOX with Hs-DNA, steady state fluorescence was employed in our study. Since the endogenous fluorescence property of DNA is poor, we studied the fluorescence spectra of MOX in all subsequent studies. Emission spectra of MOX in 10 mM Tris-HCl, pH 7.4, showed a broad unstructured peak with maxima around 503 nm (**Figure 1A**).

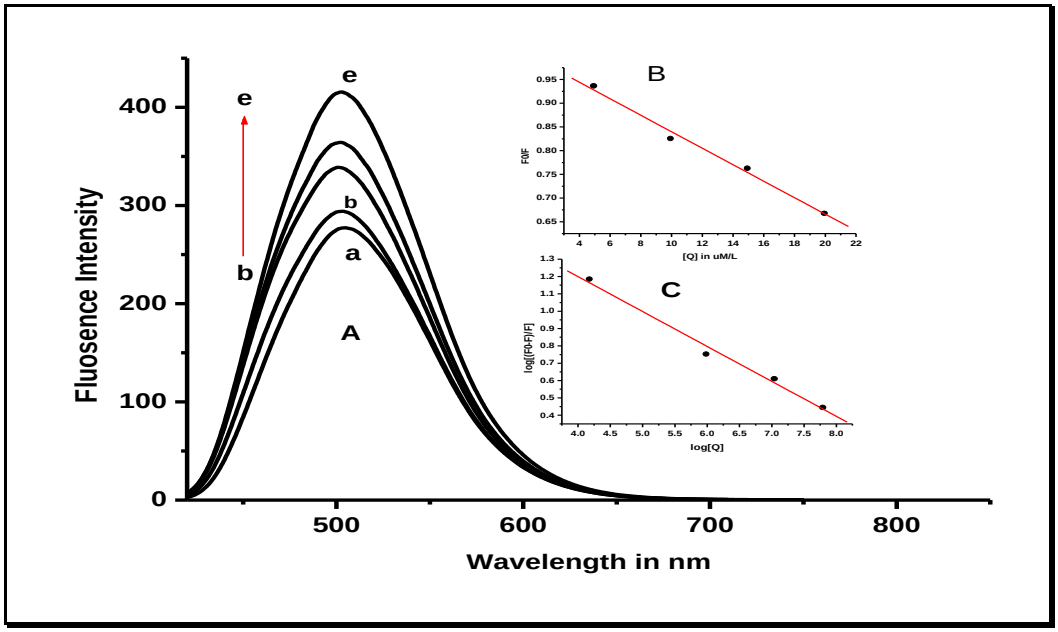


FIG. 1 A Fluorescence spectra of a) 1.5×10^{-4} M MXF in the absence of Hs-DNA and the presence of C_{DNA}= 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0 µM L^{-1} Hs-DNA (b to h) in Tris HCl buffer of pH-7.4, **(B)** Stern-volmer plot of (F / F_0) *vs.* [Q] for MXF-Hs-DNA system and **(C)** Plot of log [(F_0 - F)/F] *vs.* log [Q] for MXF-Hs-DNA system

On addition of Hs-DNA, enhancement in the fluorescence yield occurred with no detectable shift in the absorption peak position. This hyperchromism establishes the binding interaction of MOX with Hs-DNA. To further understand the interaction, ratio of peak fluorescence intensity in presence and in absence of Hs-DNA (F/F_0) was plotted as a function of DNA concentration (**Figure 1B**). The plot indicated that the fluorescent intensity is directly proportional to the Hs-DNA concentration. Further, Ksv (Stern-Volmer quenching constant) was calculated since it is considered as a measure for efficiency of fluorescence quenching by DNA. Ksv was obtained from the slope of **Figure 1B** and was calculated to be 5.611 x 10^5 mol^{-1}, which was much lower than the other classical intercalators [9, 10] and hence indicating less possibility of intercalation of MOX with Hs-DNA. Thus, MOX is suggested to interact with Hs-DNA via non-intercalative binding mode.

Competitive displacement assay: DNA binding dyes are extensively used to study the mode of drug-DNA interaction. Binding of such dyes to DNA are well studied and their binding mode is well established. Any small molecule that competitively replaces a bound dye from DNA helix is expected to bind the DNA in similar fashion as the bound dye[11–14]. Thus any change in fluorescence intensity of dye-DNA complex on addition of small molecule is easily interpreted. EB is a well known probe that binds to the DNA in intercalative fashion[11]. Since EB works as an excellent spectral probe to investigate the binding mode of drug with DNA, it was used to confirm the mode of binding of MOX to Hs-DNA. With continuous addition of MOX to the system, there was no significant change in the fluorescence intensity. This suggested that MOX does not replace EB from Hs-DNA helix as MOX binds to Hs-DNA in non-intercalative mode.

To further confirm the binding mode, we used AO in a similar competitive replacement assay. AO is a classical intercalating dye[12] and it was not replaced by MOX as expected. In another experiment, Hoechst 33258, which binds to the minor groove of double stranded Hs-DNA[13], was used to study competitive replacement by groove binders. Hoechst 33258 on binding with DNA showed enhancement in the fluorescence intensity[14]. Groove binding molecules are able to displace Hoechst 33258 from the minor groove of DNA helix, resulting in decreased fluorescence intensity of DNA-Hoechst system. On addition of MOX, the fluorescent intensity of Hoechst-DNA system was found to decrease as MOX could replace the groove bounded dye from the CT-DNA. This further suggested the binding mode of MOX to be groove binding rather than intercalation.

Iodide quenching studies: Iodide ion quenching experiments provide great help in deducing the binding interaction of drug with DNA[15–17]. Iodide ions, being negatively charged, can effectively quench the fluorescence of small molecules in an aqueous medium. However, in presence of DNA, iodide ions are repelled by negatively charged phosphates present in DNA backbone. Any small molecule intercalated into the DNA helix is well protected as the approach of anionic quenchers towards such molecule is restricted. However, this is not the case with electrostatic binding and groove binding molecules which are exposed to the external environment and are easily approachable for quenchers even in presence of DNA[15]. The relative accessibility of small molecules to anionic quencher in free medium and in presence of DNA is studied by calculating Ksv using Stern-Volmer equation

$$F_0 / F = 1 + K_{sv} [Q]$$

Where, F_0 and F are the highest fluorescence intensity in the absence and presence of the anionic quencher [Q]. Ksv is Stern-Volmer quenching constant calculated from the slope of $[F_0/F]$ vs [Q] plot. Ksv obtained in absence and presence of DNA environment signifies the binding mode of drug. Relative decrease in Ksv in presence of DNA occurs in case of intercalation, however it remains unchanged when interaction is electrostatic or groove binding. As seen in **Figure 2**, KI could effectively quench the fluorescence of MOX in a buffer solution and a Ksv value of 5.611 x 10^5 M^{-1} was obtained.

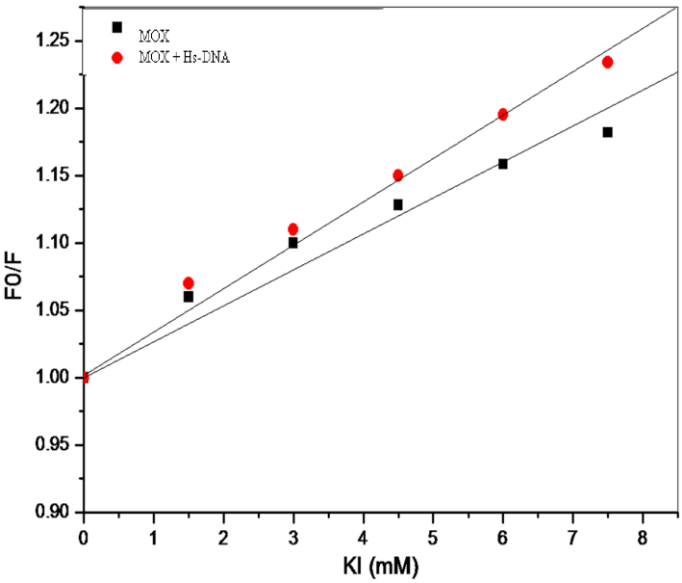


FIGURE 2. STERN-VOLMER PLOT FOR FLUORESCENCE QUENCHING OF MOX (50 mM) BY KI IN ABSENCE AND PRESENCE OF Hs-DNA (100 mM).

However, in presence of Hs-DNA, there was an increase in the K_{sv} value to 32.61 M^{-1}. Since, earlier experiments suggested for a groove binding mode of interaction between MOX and Hs-DNA, relatively similar Ksv value was expected in KI quenching studies. However, this unexpected increase in Ksv value, decrease in fluorescence

yield, can be explained by involving the role of ionic strength. Firstly, on addition of KI there is increase in the ionic strength in the medium resulting in the release of DNA bound MOX. Since the fluorescence intensity of free MOX is less than MOX-Hs-DNA complex, there is a decrease in fluorescence yield. Also, KI effectively quenches the fluorescence of free MOX in solution. Hence two factors operate together resulting in enhanced quenching of fluorescence intensity by KI in presence of DNA causing an increased Ksv value. Thus, it can be confirmed that groove binding mode of interaction occurs between MOX and Hs-DNA.

Effect of ionic strength: Studying the effect of ionic strength is also a resourceful method to differentiate the binding mode between small molecules and DNA. Generally, strong electrolyte such as NaCl is used where the addition of NaCl does not affect the fluorescence yield of drug alone. In presence of DNA, Na^+ partly neutralizes the negative charges of DNA phosphate backbone resulting in reduced electrostatic repulsion between them. The electrostatic attraction between small molecule and DNA surface is weakened by the addition of Na^+. In case of surface binding molecules, the electrostatic binding takes place out of the groove, addition of NaCl will weaken the interaction resulting in the weakening of quenched fluorescent intensity[18]. In our study, addition of NaCl to MOX-Hs-DNA complex (**Figure 3**) increased the fluorescence intensity.

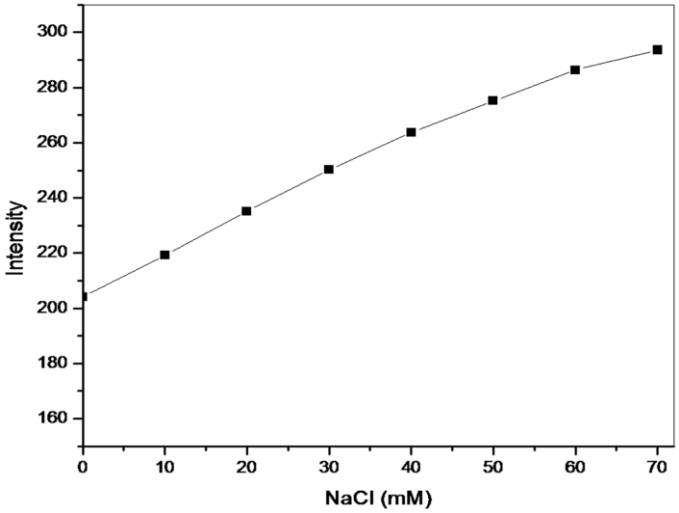


FIGURE 3. EFFECT OF IONIC STRENGTH: PLOT OF MAXIMUM INTENSITY OF MOX-Hs-DNA vs. INCREASING CONCENTRATION OF NaCl (0 - 70 mM).

This observation could be explained on the basis that the negative charge of DNA phosphate backbone is neutralised with the addition of NaCl causing negatively charged MOX to further interact with Hs-DNA resulting in enhanced fluorescence intensity. This enhancement may have masked the probable decrease in the electrostatic interaction on increasing the ionic strength. Thus, electrostatic interaction between MOX and Hs-DNA cannot be ruled out but such data indicated that the interaction between MOX and Hs-DNA was not surface-binding mode.

The composition of the binary complex: In terms of the mechanism, it is generally accepted that small molecules are bound to large biological molecules by 3 binding modes: intercalative binding, electrostatic binding, and groove binding.[19] The process of electro-static binding involves cationic species reacting with the negatively charged Hs-DNA phosphate backbone in the external Hs-DNA double helix, and this kind of binding mode has low selectivity. The intercalation binding was first proposed by Lerman in 1961.[20] Typically, intercalative small molecules with a planar aromatic system can insert into between two adjacent base pairs in a helix. In groove binding, direct hydrogen bonding or Van der Waals force is always formed between the two grooves of the Hs-DNA double helix generally.[21] If the static binding reaction exists, and if the binding capability of Hs-DNA is equal at each binding site, then the composition of the binary complex can be deduced from Eqs. (2) and (3): [23, 22]

$$Q + nL = QLn \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$\text{Log } (F_0 - F)/F = \log Ka + n \log (C_{DNA}) \ldots\ldots (3)$$

Where, Q is the quencher, L is the pharmaceutical molecule with fluorescence, QLn is the binary complex of which the binding constant is Ka, and F_0 and F are the fluorescence of the pharmaceutical molecules without and with Hs-DNA, respectively. As shown in **Figure 1C**, the plot of log $[(F_0 - F)/F]$ *vs*. log $[Q]$ gives a straight line at different temperatures, and the binding stoichiometry (n) and log Ka are calculated from the slope and y-axis intercept, respectively. The fluorescence titration data are well filled to Eq. (3) and can infer both the binding constant (Ka) and the binding

stoichiometry (*n*) for the complex formation of MOX with Hs-DNA. The values of *Ka* and *n* are found to be 1.48 x 10^5 M^{-1} and 0.98 at 298 K, respectively. The result indicates that MOX can form a stable 1:1 complex with Hs-DNA. As we all know, EB is embedded in parallel into the Hs-DNA double helix typically and the *Ka* of MOX-Hs-DNA and that of EB-Hs-DNA (4.94 x 10^5 M^{-1}) belong to the same order of magnitude. Therefore, the binding mode of MOX with Hs-DNA should be intercalative.[24] Further, the *Ka* (2.71 x 10^4 M^{-1}) at 308 K and that (9.18 x 10^3 M^{-1}) at 313 K were less than that at 298 K, which proved further that the quenching mechanism of the MOX-Hs-DNA binding reaction is static.

Analysis of data and obtaining thermodynamic parameters : Given the temperature-dependent equilibrium constants (*Ka*) for the Hs-DNA quinolone complex formation, the entropy, enthalpy, and Gibb's free energy at 298 K can be easily obtained from the fundamental thermodynamic relationships coupled with the van't Hoff equation (4) and (5).[25]

$$R\ln Ka = \Delta S - \Delta H/T \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

$$\Delta G = -RT\ln Ka = \Delta H - T\Delta S \quad \ldots\ldots\ldots\ldots (5)$$

The thermodynamic parameters, namely the enthalpy change ΔH, the entropy change ΔS, and Gibb's free energy, can be calculated from the above equation; *R* denotes molar gas constant. ΔH and ΔS for the association of MOX with Hs-DNA can be obtained according to the basic thermodynamic relation. For the association of MOX with Hs-DNA, the plot of *RlnKa vs.* 1/T gives a straight line and the ΔH and ΔS are calculated from the slope and y-axis intercept, respectively. Then we found that ΔS was -349.98 J mol^{-1} K^{-1} and ΔH was -133.55 KJ mol^{-1}. Previous research[3] showed that ionic and hydrophobic interactions are characterized by positive ΔS and ΔH; the non bonded (Van der Waals) interactions and hydrogen-bond formation in low dielectric media and protonation accompanying association are characterized by predominantly negative ΔS and ΔH, and electrostatic interactions are characterized by positive ΔS, $\Delta H \approx 0$. Therefore, we can conclude that the main force between MOX and Hs-DNA is hydrogen bonding or Van der Waals force.

CONCLUSIONS: In conclusion, we have studied the mode of interactions between MOX and Hs-DNA using spectrofluorimetric studies and confirmed that there could be mixed type of binding interactions. Groove binding of MOX to Hs-DNA was confirmed while role of electrostatic interactions cannot be ruled out. However, MOX does not intercalate into the strands of Hs-DNA. This study could also be useful to classify drug as phototoxic or nonphototoxic and also provide a deep insight in determining the mechanism of action of various drugs.

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