N-Arylhydroxamic acids as a drug like molecule: A motif of binding mode with calf thymus DNA

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A drug-like molecule, which has a propensity of binding with DNA play a vital role in drug designing mechanism. In this paper, we tried to find out the DNA binding affinity of two derivatives of N-arylhydroxamic acids: (i) N-p-Chlorophenyl-2-methoxybenzohydroxamic Acid (Cl-2-MBHA) and (ii) N-p-Chlorophenyl-3-methoxybenzohydroxamic Acid (Cl-3-MBHA) with calf thymus DNA (ct-DNA) by applying techniques such as UV-visible spectroscopy, Fluorescence spectroscopy, and Viscometry measurements. The findings concluded with experimental techniques were verified with theoretical calculation using computer-based method, Molecular Docking. Absorption spectra revealed that both the hydroxamic acids derivatives bind to ct-DNA, among two, Cl-2-MBHA exhibits the higher value of binding affinity $K_b = (9.52 \pm 0.08 \times 10^3)$ M$^{-1}$. Fluorescence spectra showed that ct-DNA successfully quenches the emission spectra of N-arylhydroxamic acid. Ethidium bromide displacement method was used as a standard for analyzing the mode of binding. Both the hydroxamic acids were found to be groove binders. The Stern–Volmer Constant was found to be 2.05 $\times 10^{-2} \pm 0.001$ M$^{-1}$ and 3.35 $\times 10^{-2} \pm 0.002$ M$^{-1}$ for Cl-2-MBHA and Cl-3-MBHA respectively. Theoretical analysis molecular docking was done using Hex software for validating the experimental findings. Hence, it was observed that both experimental and computational method complimented the results and deduces groove binding as the mode of interaction.

Keywords: Calf thymus DNA, Ethidium Bromide, Fluorescence, Hydroxamic Acid, Viscosity

Hydroxamic acids for the present investigations are a drug-like molecules according to Lipinski rule of five$^1$. –NOH.C=O functionality shows both hydrogen bond donor and hydrogen bond acceptor capability$^{2,4}$, and this characteristic feature of the molecule is responsible for their medicinal properties. A number of derivatives of hydroxamic acids have been reported to have pharmacological and pathological activities toward many diseases like cancer, Alzheimer’s disease, tuberculosis, etc$^{5-10}$. Suberoylanilide hydroxamic acid (SAHA) was a promising agent for performing clinically useful chemotherapy against chondrosarcomas$^{11}$. N-hydroxy-4-(4-phenylbutyryl-amino) benzamide, a hydroxamate-tethered phenyl butyrate derivative with sub-micromolar potency in inhibiting HDAC activity and cancer cell proliferation$^{12}$. Benzohydroxamic acid was proved to be a potent inhibitor of $P. falciparum$ in the human system$^{13}$. N-arylhydroxamic acids analogous have been reported as anti-cancer drug-like molecule against MCF-7 breast cancer cell line$^{14}$. Nucleic acid (DNA/RNA) interactional study plays a vital role in drug design mechanism and the molecules possessing this unique feature proves to be very important for ruling out their actual biological pathway of action. Small molecules that bind to genomic DNA are screens as prime effective candidates as anticancer, antibiotic, and antiviral therapeutic agents. Designing new therapeutic agents will most probably affect the well being of millions of people worldwide. It is obviously and conceptually straight forward to target DNA, the genetic material for regulation of cell functions by altering gene expression or by interrupting replication. As DNA is core of the central dogma machinery of protein synthesis, small molecules with the potential of binding with DNA have been studied extensively$^{15}$. Small molecules interact with DNA mainly through non-covalent interactions namely three modes: intercalative binding, groove binding, and electrostatic binding$^{16}$. Intercalative binding and groove binding are related to the grooves in the DNA but electrostatic binding can be seen at the surface of the double helix. In groove binding, interactions with the grooves of the DNA double helix usually involve direct van der Waals interactions or hydrogen bonding with the bases of nucleic acid in the shallow major
groove or the wide narrow minor groove of the DNA helix. Electrostatic binding is the interaction between cationic species and the negatively charged DNA phosphate backbone that occurs along the DNA double helix and does not possess selectivity. Drugs which have the efficacy of DNA binding as anticancer agents were centrally placed in drug design mechanism. The characterization of small molecule interaction to DNA provides new insights into DNA–drug interactions and this, together with structural studies, may be used in rational drug designing. We aimed to shed some light on the interaction of N-arylhydroxamic acid with calf-thymus DNA. In this context, we determined the DNA binding interaction of following hydroxamic acids: N-p-Chlorophenyl-2-methoxybenzohydroxamic Acid (Cl-2-MBHA) and N-p-Chlorophenyl-3-methoxybenzohydroxamic Acid (Cl-3-MBHA).

Materials and Methods

Chemicals and materials

Cl-2-MBHA [C\textsubscript{14}H\textsubscript{12}NO\textsubscript{3}Cl; Mol wt = 277.70; melting point = 116°C; % yield 75; λ\textsubscript{max} = 270; IR, ν/cm\textsuperscript{-1}: 3185 (O-H), 1613 (C=O)] and Cl-3-MBHA [C\textsubscript{14}H\textsubscript{12}NO\textsubscript{3}Cl; Mol wt = 277.70; melting point = 104°C; % yield 85; λ\textsubscript{max} = 278; IR, ν/cm\textsuperscript{-1}: 3106 (O-H), 1625 (C=O)] (Fig. 1) were prepared according to the standard procedure. Their purity was confirmed by the determination of their melting points, and UV and IR spectral analyses. The data were then compared with the reported literature.

Hydroxamic acids are sparingly soluble in water, the stock solution (1 mM) of the experimental compounds was prepared in dimethyl sulfoxide (DMSO) for ct-DNA binding studies and stored in a cool and dark place. DMSO from Merck (dry, analytical grade) and Calf thymus DNA (Merck Chemical Industry, Osaka, Japan) was used without further purification. It was dissolved in double distilled deionised water for the stock preparation (1 mM) and stored at 4°C. The concentration was determined at 260 nm using UV molar absorption coefficient. The absorbance ratio (A\textsubscript{260}/A\textsubscript{280}) was determined for the purification of the DNA. A\textsubscript{260}/A\textsubscript{280} was found to be >1.8, which indicates the protein free nature of DNA. Ethidium bromide was purchased from Merck. For the determination of interaction between hydroxamic acid-DNA, tris-HCl buffer solution (100 mM) was used throughout. pH was maintained at 7.4 with 0.01 M HCl prepared by standard procedure. All the chemicals used were of spectroscopic grade and doubly distilled deionized water was used for the preparation of solutions.

Instrumentation

Absorption spectra were measured on Biospectrum BL-198 (Elico) using quartz cells of path width 1.0 cm. Fluorescence spectra were scanned on Cary Eclipse fluorescence spectrophotometer (Varian) equipped with xenon flash lamp using quartz cells of path width 1.0 cm. The viscosity measurements were carried out using thermo stated Ubbelohde viscometer. pH measurement was done using Cyber510 digital pH meter with a coupled glass-calomel electrode. For proper mixing, tarsons spin centrifuge (1.5 mL tube) of 6000 rpm was used.

UV spectroscopic titration

UV spectroscopic titration of hydroxamic acids and ct-DNA complex were measured in 200–400 nm wavelength. The binding interaction studies were carried out by keeping the amount of hydroxamic acids fixed (100 μM) and titrating with varying concentration of 1 mg/mL ct-DNA from 0 to 125 µL. The reference solution was the corresponding buffer solution. The intrinsic affinity constant of the hydroxamic acid-ct-DNA complex was calculated from equation (1),

$$A_0/ (A−A_0) = \varepsilon_f/(\varepsilon_b−\varepsilon_f) + \varepsilon_f/(\varepsilon_b−\varepsilon_f) \times 1/K_a[\text{DNA}]$$

where, [DNA] is the DNA base pair concentration, A\textsubscript{0} and A are the absorbance of the complex in the free and fully bound state and ε\textsubscript{b} and ε\textsubscript{f} are their respective extinction coefficients. K\textsubscript{a} was calculated from the ratio of intercept to that of the slope, obtained from a plot of A\textsubscript{0}/(A−A\textsubscript{0}) vs 1/[DNA].

Fluorescence emission spectra

Fluorescence emission spectra of hydroxamic acids were recorded at 200–580 nm upon excitation at 210 nm wavelength using slit width 5 nm each. The
fluorescence titration was performed by keeping the concentration of hydroxamic acids solution constant (100 μM) and varying the volume of 1 mg/mL ct-DNA from 0 to 125 μL. Stern–Volmer quenching constant $K_{sv}$ is used to evaluate the fluorescence quenching efficiency of hydroxamic acids. The binding stoichiometry of the DNA–hydroxamic acids complex was calculated by following equation (2)\(^{25}\). It signifies the number of bound DNA per hydroxamic acid molecule.

$$\log \left( \frac{F_0 - F}{F} \right) = \log K_b + n \log [DNA] \quad (2)$$

The value of $K_b$ was obtained from the graph plotted between $\log \left( \frac{F_0 - F}{F} \right)$ versus $\log [DNA]$. $F_0$ is the fluorescence intensity of compound alone, while $F$ is the fluorescence intensity of hydroxamic acid with the presence of DNA.

**Ethidium bromide displacement method**

Ethidium bromide displacement method was performed by adding 10 μL of 50 μM of EB to 0.033 M ct-DNA at λ\(_{\text{exit}}\) = 475 nm and λ\(_{\text{em}}\) = 610 nm and the fluorescence quenching analysis were implemented by varying amount of hydroxamic acids (0–250 μL) that can bind to ct-DNA may modify the binding of ethidium bromide to ct-DNA. The loss of ethidium bromide fluorescence as a function of hydroxamic acid has been used for assessing the stern-Volmer constants of hydroxamic acids and ct-DNA complex\(^{26}\). The Stern–Volmer quenching plots from the fluorescence titration data of ethidium bromide bound to DNA\(^{27-29}\) and the hydroxamic acids were investigated according to the Stern–Volmer equation (3),

$$\frac{I_0}{I} = 1 + K_{sv}[Q] \quad (3)$$

where, $I_0$ and $I$ are the fluorescence intensity of ethidium bromide bound to DNA in the absence and presence of the quencher. $[Q]$ is the ratio of the concentration of hydroxamic acid with DNA. The Stern–Volmer quenching constant, $K_{sv}$, for ethidium bromide bound to DNA has been obtained from the plot between $I_0/I$ and $[Q]$.

**Viscosity measurements**

The viscosity experiment was conducted at 298.15 K on thermostated Ubbelohde viscometer, 20 mL of Tris-HCl was transferred to the viscometer and flow time was noted. About 1 mL ct-DNA in 19 mL Tris-HCl buffer was then taken in viscometer and flow time was recorded. The concentration of ct-DNA was kept constant and an appropriate amount of hydroxamic acid (HA) was added at certain $r = [HA/DNA]$ and flow time was measured. The data were presented as $(\eta/\eta_0)^{1/3}$ versus $r$, where $\eta$ and $\eta_0$ are the viscosity of ct-DNA in presence and absence of hydroxamic acids\(^{30}\).

**Molecular Docking**

The molecular docking was performed by using HEX Cuda 6.1 software\(^{31}\). HEX is the molecular graphics software used for calculating and displaying feasible docking modes of DNA. It necessitates the ligand and DNA as an infant in PDB format. The parameters used for docking include correlation type-shape only, FFT made-3D grid dimension-0.6, receptor range 180, Ligand range 180, twist range-360, distance range-40.

**Results and Discussion**

**Absorption spectral studies**

UV-Vis absorption measurement is a simple and effective method for detection of complex formation. When a small molecule interacts with DNA and forms a new complex, usually, changes in absorption spectra were observed with a shifting of the band which means λ\(_{\text{max}}\) position will be differing as compared to compound alone\(^{32}\). Fig. 2 shows UV absorption spectra of Cl-2-MBHA and Cl-3-MBHA in absence and presence of ct-DNA at different $r$ values i.e. $r = [DNA]/[HA]$. Upon addition of increasing concentration of ct-DNA to derivatives leads to hyperchromic effect. Generally hyperchromism and hypochromism are the spectral features of DNA concerning with its double helix pose; hyperchromism reveals that the molecule interacts in such manner which leads to degradation or splitting of DNA and hypochromism correlated with DNA binds with molecule through intercalation mode resulting into stabilizing the DNA duplex structure\(^{33}\). Both hydroxamic acid derivatives show hyperchromic effect due to π-π* transition on the addition of increasing amount of DNA\(^{34-36}\). Both the hydroxamic

![Fig. 2—UV Absorption spectra of (A) Cl-2-MBHA and (B) Cl-3-MBHA (100 μM) without and with ct-DNA in DMSO at 298 K. Concentration of added DNA is 1.0 mg/mL.](image-url)
acid derivatives show a blue-shift, indicating both are binding to ct-DNA. This observed shifting effect, either in the peak intensity (hyperchromic or hypochromic) or \( \lambda_{\text{max}} \) value (hypsochromic or bathochromic shift) show interactions between ct-DNA and the hydroxamic acid derivatives. The observed hyperchromic shifts in the absorption spectrum shown in Fig. 3 can be attributed to groove mode of interaction between small molecules and double helix of DNA. The value of intrinsic affinity constant, \( K_a \) for both the hydroxamic acids are 9.52 \( \times \) 10^3 \( \pm \) 0.08 M\(^{-1}\) and 4.36 \( \times \) 10^3 \( \pm \) 0.11 M\(^{-1}\) for Cl-2-MBHA and Cl-3-MBHA respectively. Higher \( K_a \) of Cl-2-MBHA indicates that it was a stronger ct-DNA binder as compared to Cl-3-MBHA.

**Viscosity measurements**

The DNA interactions of hydroxamic acids were further confirmed via viscometric studies. Viscosity experiments are proved as effective tools to determine the binding mode of small molecules and DNA. In present study slight changes in the relative viscosity of ct-DNA was observed pointing out surface binding i.e. electrostatic or minor/major groove may be the binding mode\(^{37}\). The relative viscosity of DNA solution increased slightly in a linear trend (Fig. 4) in both compound of hydroxamic acids may be due to the lengthening of DNA helix which may be due to adherence of molecule in the groove region.

**Fluorescence spectra**

Fluorescence spectra of the drugs provide information about their localization and interaction mode with DNA. Fluorescence emission spectrum of small molecules with increasing concentration of ct-DNA shows significant spectral changes\(^{38}\). Both of the compounds gave a fluorescence emission peak at 423.93 nm in Tris-HCl buffer solution at pH 7.4 at room temperature. The results shows (Fig. 5) that the fluorescence spectra of Cl-2-MBHA and Cl-3-MBHA could be quenched by ct-DNA resulting in a gradual decrease in emission intensity at 423.93 nm which is usually symbolises as groove mode of binding. Thus, it can be concluded that the molecules bind with the ct-DNA where they are protected from aqueous environment due to its hydrophobic nature. The binding constant, \( K_b \) and number of binding sites n of the compound at 298 K have been calculated (Fig. 6) as 1.56 \( \times \) 10^8 \( \pm \) 0.78 M\(^{-1}\) for Cl-2-MBHA with ‘n’ equals to 2.134\( \pm \)0.19 and 3.66 \( \times \) 10^3 \( \pm \) 0.21 M\(^{-1}\) for Cl-3-MBHA with the value of ‘n’ found as 1.091\( \pm \)0.05. (Table 1) The values obtained clearly marks the molecules as DNA binders with a probability of binding sites more than 1 which means that both the compound were able to associate closely with DNA in their curved i.e. groove region of DNA. The conformation of the molecule can be

<table>
<thead>
<tr>
<th>Hydroxamic acids</th>
<th>Binding constant ( K_b ) (M(^{-1}))</th>
<th>Number of binding sites n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-2-MBHA</td>
<td>1.56 ( \times ) 10^8 ( \pm ) 0.78</td>
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</tr>
<tr>
<td>Cl-3-MBHA</td>
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<td>1.091 ( \pm )0.05</td>
</tr>
</tbody>
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Fig. 5—The fluorescence emission spectra of (A) Cl-2-MBHA and (B) Cl-3-MBHA with DNA. A compound concentration (100 µM) in Tris-buffer, pH 7.4. Concentration of added DNA is 1.0 mg/mL

Fig. 6—Graph plotted between log (F0-F)/F versus log [DNA] for (A) Cl-2-MBHA and the graph (B) Cl-3-MBHA
characterized by the binding stoichiometry of the hydroxamic acid-DNA complexes. Ethidium Bromide Displacement Method

The displacement of ethidium bromide (EB) bound to ct-DNA by the drug molecules has been used to analyze ct-DNA binding ability as it shows the intercalative mode of binding with ct-DNA owing to its planar structure. The emission spectra of EB-DNA system in the presence and absence of Cl-2-MBHA and Cl-3-MBHA are shown in Fig. 7. Both hydroxamic acids were unable to compete with EB and fail to quench the emission spectra. Hence, it can be deduced that both the molecules show potency of groove binder. The quenching plots of $I_0/I$ vs $[Q]$ (Fig. 8) are in good agreement with the linear Stern–Volmer equation with $K_{sv}$ values of $2.05 \times 10^{-2} \pm 0.001$ M$^{-1}$ and $3.35 \times 10^{-2} \pm 0.002$ M$^{-1}$ for Cl-2-MBHA and Cl-3-MBHA respectively.

Molecular Docking

Molecular docking emerges as a helping tool for DNA binding studies which is useful in solving the puzzle of DNA binding; tentative sights were identified as a key zone of interaction. Structure of DNA dodecamer d(CGCGAATTCGCG)$_2$ was retrieved from protein data bank using (PDB ID:1BNA) while hydroxamic acid structure was drawn using ChemSketch and ChemDraw and then files are converted into PDB formate for docking. Both the ligands Cl-2-MBHA, Cl-3-MBHA and DNA files were prepared proceeded by docking. The results of analysis observed as groove binding. The prediction on most probable binding sites of ct-DNA helps in knowing the basic interactional mode. Fig. 9, represent the surface view of Hydroxamic docked into groove region of DNA. The $E_{total}$ energy obtained is $-214.67$ eV and $-221.89$ eV for Cl-2-MBHA and Cl-3-MBHA, respectively. The negative values indicate that the molecules bind well to the DNA within close proximity. Higher the negative value higher will be the binding affinity of drugs with DNA. The stability of complexes drawn by the comparison of the total empirical energies which shows that both the complexes are stable as it possesses minute energy.

Conclusion

In the present work, we have explored the binding interaction of Cl-2-MBHA and Cl-3-MBHA with ct-DNA in the physiological buffer by spectroscopic, viscometric techniques, and molecular docking method. Both the compound possesses the affinity of binding with ct-DNA. Out of two compounds, Cl-2-
MBHA has shown the higher value of the binding constant as compared to Cl-3-MBHA in both the spectroscopic techniques, which clearly mark its potency in binding with ct-DNA. In addition, at various concentrations of DNA, a hyperchromic shift was observed in absorption studies for both the compounds. Ethidium bromide competitive binding assay of both the hydroxamic acids illustrates that the compound is unable to compete with ethidium bromide. Viscometric studies revealed that there is a slight increase in the relative viscosity of both the compounds, indicating the lengthening of DNA helix predicting groove as the mode of binding. Thus, Cl-2-MBHA and Cl-3-MBHA show strong interaction with ct-DNA through groove mode of binding. Computer analysis using molecular docking complemented the results. Hence, the present study is helpful in the screening of other hydroxamic acids DNA binders, and prepare a novel complex with specific, effective and more potent drug to target DNA which may indirectly help in cancer study.

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