Importance of C3=C4 double bond of plant alkaloid Harmine on its binding interaction with Hemoglobin: Multi-spectroscopic and molecular modelling studies

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Abstract

Harmine and harmaline are two structurally similar β-carboline plant alkaloids with various therapeutic properties, having difference in C3 = C4 double bond only. Nature of the interaction between hemoglobin (Hb) and harmine and harmaline was investigated in this study by employing different spectroscopic methods and molecular modeling. The spectrum of Hb showed hypochromic changes in the presence of both harmine and harmaline but with different efficiencies. The fluorescence quenching of harmine and harmaline occurred in the presence of Hb indicating stronger interaction of harmine with Hb. Stoichiometry study revealed that both harmine and harmaline interact with Hb in around 1:1 ratio. The circular dichroism study demonstrate no conformational change of Hb in the presence of alkaloids. From the melting study, it was found that both harmine and harmaline do not affect the stability of Hb. Molecular modeling study supported the above findings that harmine containing the C3 = C4 double bond in its structure, interacts strongly with Hb on the surface of α2 chain compared to harmaline. This study helps us to understand that slight structural differences in harmine and harmaline can alter interaction properties significantly and these key informations may help in the drug discovery processes.

Introduction

Hemoglobin (Hb) is an important component of the human body. It carries oxygen from the lung to different tissue. It consists of two parts, globin, protein part and heme, iron containing pyrol ring. In adult Hb, the globin part consists of four amino acid chains, two α chains and two β chains\(^1\). Each α chain is made up of 141 amino acids and β chain is made up of 146 amino acids\(^2\). Hb consists of four heme groups. The heme part is made up of an iron atom inside a porphyrin ring\(^3\). Normal Hb concentration in an adult man and woman is 13.2–16.6 and 11.6–15 g/dl respectively. The iron inside the heme generally carried oxygen and carbon dioxide\(^4\). Generally, it is found inside erythrocytes and in free form in the blood. Therefore, Hb molecules can reversibly bind indegenous or exogeneous molecules, such as flavonoids\(^5\), alkaloids\(^6\), and food colorants\(^7\) etc.

Harmalor Syrianrue is a trivial name of *Peganum harmala* (L) plant\(^8\). The plant is native to dry areas. Hence, it is found in north India, eastern Iranian, North Africa, Middle East China and some regions of the western USA\(^9\). *P. harmala* seeds contain different types of alkaloids such as harmine, harmane, harmalol, harmaline and harmalidine etc. Such β-carboline alkaloids are used for different therapeutic purposes\(^10\). Among these alkaloids, harmine and harmaline are one of the most important representatives. Harmine shows anti-inflammatory\(^11\), anti-osteoporesis\(^12\), anti-periodontitis\(^13\), anti-oxidant\(^14\) neuroprotective\(^15\), antitumor\(^16\), nephroprotective\(^17\) and antidiabetic\(^18\) effects. Harmaline shows anti-oxidant\(^14\), vasorelaxant\(^19\), hypothermic\(^20\), antimicrobial\(^21\), and antitumor activity\(^22\).

Both plant alkaloids harmine and harmaline (Figure. 1), have a methoxyl group at C-7 position and a methyl group at C-1. The only structural difference between the β-carbolines studied is the presence of
full aromaticity in harmine and partial aromaticity in harmaline. The interaction between β-carboline alkaloids and bovine serum albumin is well established.\textsuperscript{23}

Hb is one of the most abundant protein in our body. Hence, free Hb can expose to various small molecules which may interact with different binding efficiencies. The general structure and functional relationship of Hb is well studied.\textsuperscript{19} The predominance of glycosylated Hb in diabetes is another major aspects. As the glycosylation process is an oxidative reaction, the anti-oxidative property of alkaloids can hinder this glycosylation reaction\textsuperscript{23}. Therefore the binding characteristics of therapeutically important alkaloids with Hb will give us a more clear understanding in terms of drug actions. In addition to carrying oxygen from the lungs to the tissues and permitting the return of carbon dioxide, Hb also functions as a two-way respiratory carrier. Hb's strong affinity for oxygen and lower affinity for carbon dioxide, hydrogen ions, chloride ions and organic phosphates in arterial circulation are characteristics that distinguish them from others. In this study, we have focused on the interaction of Hb with two structurally similar plant alkaloids, harmine and harmaline (Figure. 1) and how the little structural difference of these molecules (double bond between C3 and C4 for harmaline) impact the interaction. It was found that harmine possesses more stronger interaction with Hb compared to almost structurally similar harmaline.

Results

Absorbance spectral studies. Soret band at 405 nm is one of the major absorption peak of human Hb in comparison to the other absorption peaks found\textsuperscript{24}. The Soret band originates from the heme moiety embedded in the hydrophobic pocket of the protein\textsuperscript{25}. In the presence of increasing concentration of the alkaloids, a decrease in the intensity of Soret band is observed. The Soret band absorption intensity is reduced after harmine treatment, while the maximum absorption wavelengths remain unaltered (Figure. 2A). Hence, the heme is unexposed from the crevices. This result suggests the formation of a complex of harmine with Hb. Two isosbestic points were noticed at 380 nm and 430 nm. The appearance of isosbestic points denote equilibrium between protein-bound and free dye in the complex of harmine with Hb. Harmaline shows an absorption peak around 375 nm which is merged with the absorbance of Hb. Therefore, the absorbance of harmaline (Figure. S1B) was subtracted from the absorbance of Hb-harmaline complex to get the exact results. It was found that after the gradual addition of harmaline in Hb, the soret band intensity was gradually decreased (Figure. 2B), though no such isosbestic points are noticed. This too implies, the complex formed between Hb and harmaline but according to the results the interaction between Harmine and Hb possesses more stronger complex formation.

Steady state fluorescence spectroscopy. With increasing concentration of the Hb, quenching of the fluorescence of the alkaloids are observed, finally reaching saturation in each case (Figure. 3A and 4A). In the case of harmine a red shift of the emission maxima by 26 nm is observed while no such shift of the emission maxima is observed in the case of harmaline. The results clearly indicate that the interaction of harmine with Hb was stronger compared to that of harmaline.
The Benesi–Hildebrand equation gave linear plots with association binding constant value ($K_b$) as $38.23 \times 10^4$ M$^{-1}$ and $19.78 \times 10^4$ M$^{-1}$ in the case of harmine and harmaline respectively (Figure 3B and 4B), clearly depicting stronger binding of harmine with Hb than harmaline.

**Stoichiometric Analysis.** The stoichiometry of alkaloids–Hb binding is analyzed by the continuous variation method (Job’s plot analysis). This is a very suitable method to understand the nature of the complex formed by the interaction between two compounds. In the experiments, the total concentration ($C_{Hb} + C_{Alkoids}$) is maintained constant while the molar fractions of Hb and alkaloids are altered. Then, the changes in fluorescence intensity ($\Delta F = F_{Hb} - F_{Hb+Alkoids}$) are plotted versus the alkaloids molar fraction (Figure 5).

The Job’s plot for Hb–harmine and Hb-harmaline are performed considering fluorescence emission at 420 and 480 nm respectively upon excitation at 350 nm. It was found that the stoichiometry of the Hb:harmine and Hb:harmaline complex at room temperature are approximately 1:1 (Figure 5).

**CD.** CD spectra are used to understand the secondary structure of proteins in different environments and different protein-ligand complexes. In the far UV CD (260 to 190 nm) the Hb exhibits two negative peaks around 208 and 222 nm which characterizes \(\alpha\)-helixes within the protein. The peaks occurred for n→\(\pi^*\) transition for the peptide bonds present in the \(\alpha\)-helix$^{26,27}$. The calculated percentage of \(\alpha\)-helicity in Hb was 29.2%. After addition of harmine and harmaline at increasing concentration (3,6,12 \(\mu\)M), the percentage of \(\alpha\)-helicity slightly changes to 31.2% and 30.7% respectively. Hence, the alkaloids did not cause any conformational changes in Hb upon interacting with it.

**Melting.** The interaction between alkaloids, harmine and harmaline and Hb was studied by UV-melting experiments. In this experiment, Hb exhibits melting temperature ($T_m$) of around 62.06°C (Figure 7). In the presence of alkaloids the $T_m$ of Hb (Hb:alkaloids – 1:1) was changed to 61.38°C and 60.36°C for harmine and harmaline respectively. These are very small changes regarding stability of Hb. To nullify the effect of absorbance change of alkaloids at 280 nm, we have performed the melting profile of alkaloids alone with varying temperature (Figure. S2). Hence, it can be stated that a small changes of melting temperature of Hb has been observed upon interaction with harmine and harmaline. Therefore, both harmine and harmaline have no effects on Hb stability.

**Docking.** Molecular docking is also used to determine the binding of both the alkaloids (harmine and harmaline) with Hb at the atomic level. The binding free energy calculated for harmine with Hb was -6.77 kcal/mol, whereas the binding energy for harmaline with Hb was -6.72 kcal/mol. From this study, it is found that both harmine and harmaline interacts at the periphery with the \(\alpha_2\) chain of Hb. Their binding site in Hb are similar (Figure. 8A and 8B). It is also found that glutamic acid with the residue number of 30 of \(\alpha_2\) chain of Hb interact with both harmine and harmaline with the bond length of 1.7 and 1.9 Å respectively. This study implies that a small structural difference of a double bond between C3 and C4 of harmine can change its mode of binding with Hb. The absence of a double bond between C3 and C4 in harmaline allows the compound to interact with the Hb weakly. The molecular docking result strongly
supports the previously mentioned experimental evidence that the complex formed between Hb and alkaloids and harmine binds strongly with Hb compared to harmaline.

**Discussion**

Several recent investigations have been reported the molecular interaction between protein and ligand through advanced multi- spectroscopic methods. The plant alkaloids, harmine and harmaline have different health beneficial activities such as anti-oxidant, anti-inflammatory anti-microbial, and anti-tumor activities. Various small molecules have been reported which showed potential to interact with Hb. Hence, studying the interactions of two structurally similar anti-oxidative plant alkaloids harmine and harmaline with Hb will provide us a clear scenario in terms of drug design and actions. The spectrophotometric titrations of Hb showed hypochromic changes in the presence of both alkaloids, harmine and harmaline indicating that both of them form complexes with Hb. The hypochromicity of harmine with Hb is higher compared to harmaline depicting strong interaction for harmine. Regarding the fluorimetric titrations, the fluorescence of harmine quenched much more with large red shift than harmaline upon interactions with Hb. This result implies harmine form more stronger complex with Hb compared to harmaline. The binding affinity studies also corroborated this fact. From stoichiometry, it is found that both harmine and harmaline interact with Hb in 1:1 ratio. Furthermore, circular dichroism studies indicated that in the presence of harmine and harmaline there was no significant change in the conformation of Hb. The melting experiments showed that alkaloids do not affect Hb stability significantly. The molecular docking study revealed that harmine interacts with Hb more strongly compared to harmaline according to the free energy calculations. Overall, the results suggest that harmine binds stronger with Hb than harmaline. So, a structure–interaction relationship was observed: the polycyclic planar full N-heteroaromatic structure of harmine showed to be more reactive towards Hb than the partial aromatic one harmaline. This study provides useful information to understand that a little difference in the structure can significantly change the interaction process, which may be useful for drug design purposes. Hence, this study will help to interpret the structural basis for screening and designing appropriate natural molecules that will be important in the advancement of clinical and pharmacological research.

**Methods**

**Materials.** Lyophilized powder of human Hb, harmine, harmaline, and ethanol were purchased from Sigma Aldrich, USA. Harmine and harmaline were dissolved in ethanol. The Hb concentration was measured by spectrophotometric absorbance considering the molar extinction coefficient of Hb at 276 nm (120,808 M$^{-1}$ cm$^{-1}$). All experiments were performed using buffer composed of 50 mM KCl, 10 mM KH$_2$PO$_4$, and 1 mM K$_2$EDTA (pH 7.4) at room temperature (25° C). The concentration of ethanol was always kept < 1% (by volume) in all experiments.

**UV-Vis absorption.** UV-Vis absorption spectra of Hb in the absence and presence of harmine and harmaline, were measured from 220 to 550 nm with Hitachi UH5300 using quartz cuvette with 1 cm
pathlength. Spectrophotometric titrations were performed by maintaining the concentration of Hb constant to 2.5 µM and titrated by elevating the concentration of harmine and harmaline.

**Fluorescence spectroscopy.** The fluorescence emission spectra were recorded with Biobase BK-F96Pro using quartz cuvette (Helma) with 1 cm pathlength at room temperature. For the emission spectral measurements both the excitation and emission spectral slit widths were set to 10 nm and the excitation wavelength for harmine and harmaline were set to 350 nm. In the experiments, the concentration of harmine and harmaline was maintained constant to 5 µM and titrated by elevating concentrations of Hb.

The fluorescence emission data is analyzed by the Benesi–Hildebrand Eq. 3 to determine the association binding constant

$$\frac{1}{F - F_0} = \frac{1}{\Delta F_{\text{max}}} + \frac{1}{K_b(\Delta F_{\text{max}})} \times \frac{1}{[\text{Alkaloids}]}$$

Where, $K_b$ is association binding constant.

**Stoichiometry.** The stoichiometry between alkaloids harmine and harmaline with Hb binding was analyzed by the method of continuous variation (Job's plot analysis)\(^\text{34}\). This is one of the useful methods for characterizing complexes formed between two compounds. During the experiments, the total concentration ($C_{\text{Hb}} + C_F$) was kept constant while the molar fractions of Hb and alkaloids were varied. Then, the fluorescence intensity changes ($\Delta F = F_{\text{alkaloid}} - F_{\text{Hb+alkaloid}}$) were recorded and plotted against the Hb molar fraction. The Job's plot for Hb–harmine and Hb-harmaline was calculated using fluorescence emission at 420 nm and 480 nm respectively upon excitation at 350 nm.

**CD Spectroscopy.** The CD measurements of Hb in the presence or absence of harmine and harmaline were performed in the range of 190–260 nm with a scan speed 100 nm/min using Jasco J-1500 CD Spectrometer. A cuvette with 1 mm pathlength was used in this study. Hb concentration was kept at 3 µM while the molar ratios of harmine or harmaline to Hb concentration were 1:1, 2:1, and 4:1. The buffer solution was considered as the blank and was subtracted from each scanned spectrum. Each sample was scanned three times to average for a CD spectrum. The CD spectra of Hb in the presence of ethanol (1%) was also performed (Figure S2). BeStSel (Beta Structure Selection) is a novel method for the secondary structure determination and fold recognition from protein circular dichroism spectra (https://bestsel.elte.hu/index.php). By using this method, the percentage of changes in alpha helical structure after adding harmine and harmaline to Hb was determined in our study.

**Melting.** Melting profiles (absorbance change against temperature curves) of 5 µM Hb in the absence and presence of 5 µM alkaloids (harmine and harmaline) complexes in 1:1 ratios were performed on a Hitachi UH5300 equipped with Julabo F12 water circulator and 3J1-0104 water circulated cell holder devise. A single chambered quartz cuvette with 1 cm pathlength was used. The samples were heated at the rate of 1°C/min and the change in absorbance at 280 nm was monitored. The measurement of melting temperature ($T_m$) was performed by analyzing the melting curves. The melting profiles of harmine and
harmaline alone were also performed at 280 nm. The melting profile of harmine was substracted from the melting profile of Hb-harmine complex melting profile to minimize the absorbance of harmine. Similar method was also performed in case of harmaline.

**Docking.** Molecular modeling studies were performed to know the molecular level interaction between harmine and harmaline with Hb. The crystal structure of Hb (PDB entry 2D60) has been downloaded from Protein Data Bank. The harmine and harmaline structures were downloaded from PubChem with PubChem CID 5280953 and 3564.

Autodock 4.2\textsuperscript{35} was used to dock alkaloids with the receptor (Hb). All the hetero atoms were deleted and non-polar hydrogens were merged. Kollman's charge was applied to the protein structure. A grid box with 126×104×106 grid points in size and grid-points spacing of 0.553 Å was utilized for the docking study. For both harmine and harmaline rotatable bonds were assigned and partial atomic charges were measured considering the Gasteiger–Marsili method after merging non-polar hydrogens. Protein-ligand docking was performed using AutoDock 1.5.6 program, which considers the Lamarckian Genetic Algorithm. In a docking total, 250 runs were executed and in each run, a maximum of 500,000 GA operations were executed with a single population size of 500 individuals. The data was analysed using PyMOL software.

**Declarations**

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**Author contributions**

S.B. and M.B. desigined the whole study. M.B. and S.Bag performed all the experiments. S.B., M.B. and S.Bag wrote the manuscript. All authors reviewed and approved the manuscript.

**Competing interests**

The authors declare no competing interests.

**References**


**Figures**

Figure 1

Structure of alkaloids (A) Harmine, and (B) Harmaline.

Figure 2
UV-Vis absorption spectra of Hb (2.5 µM) in the absence and presence of (A) harmine (0-9.5 µM) and (B) harmaline (0-9.5 µM) (subtracted absorbance).

Figure 3

(A) Change in fluorescence spectrum of Harmine (5 µM) upon addition of increasing amount of Hemoglobin (0-12 µM), (B) Benesi-Hildebrand plot for binding Hb with Harmaine using spectrofluorometric data.

Figure 4
(A) Change in fluorescence spectrum of Harmaline (5 µM) upon addition of increasing amount of Hemoglobin (0-12 µM), (B) Benesi-Hildebrand plot for binding Hb with Harmaine using spectrofluorometric data.

**Figure 5**

Continuous variation plot (Job plot) using spectrofluorometric data for (A) harmine/Hb and (B) harmaline/Hb complex. $\lambda_{\text{excitation}} = 350$ nm, $\lambda_{\text{emission}} = 420$ nm for harmine and 480 nm for harmaline, at room temperature.

**Figure 6**

CD spectra change of Hb (3 µM) in absence and presence of (3, 6 and 12 µM) (A) harmine and (B) harmaline. Black line = Hb, Blue line = 3 µM, green line = 6 µM, and violet = 12 µM of harmine and
harmaline in presence of Hb.

Figure 7

Melting graph of hemoglobin in the absence and presence of alkaloids, harmine and harmaline.
Figure 8

Best docking interaction between Hb and alkaloid complexes (A) Hb- harmine complex, and (B) Hb- harmaline complex.

Supplementary Files

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- SupplimentaryFile.pdf