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Differential interactions of imatinib mesylate with the membrane skeletal protein, spectrin and hemoglobin.

Debashree Das[1], Ushasi Pramanik[2], Malay Patra[3], Mousumi Banerjee[3], Abhijit Chakrabarti[1,*]

[1]Crystallography & Molecular Biology Division, Saha Institute of Nuclear Physics, HBNI, Kolkata 700064, [2]Biophysics, Molecular Biology and Bioinformatics and [3]Chemistry Department, University of Calcutta, Kolkata 700009, India.

Corresponding author: Abhijit Chakrabarti PhD

E-mail: abhijit.chakrabarti@saha.ac.in

abhijit1960@gmail.com

Abstract

Anti leukaemia drug, imatinib mesylate has been shown to bind to the membrane skeletal protein, spectrin and to the most abundant erythroid protein, HbA in its oxy-form with binding dissociation constants of 48 μM and 63 μM at 25°C respectively. Such bindings are detected by monitoring the imatinib induced quenching of the tryptophan fluorescence of the proteins with increasing concentrations. The thermodynamic parameters associated with such binding revealed the binding to be favoured by positive changes in entropy. Circular dichroism studies showed significant changes in the secondary structure of dimeric spectrin and in the tertiary structure of HbA, α-globin and the β-globin chains indicating marked difference in the molecular mechanism of binding of imatinib with the two different of proteins of erythrocytes. We have also found imatinib to bind to both the globin chains with higher affinity compared to the HbA tetramer. Molecular docking studies revealed imatinib to show preference for the β-globin chain, however, the binding parameters were comparable between the globin chains. This is the first study showing favourable interactions of this anticancer agent with the two major erythroid proteins which could throw light in understanding the mechanism of action and toxicity of this drug those are to be optimized for cancer treatment.

Keywords: Imatinib mesylate, HbA, α-globin, β-globin, spectrin dimer.

Abbreviations

HbA: Haemoglobin A

HSA: Human serum albumin

DTT: Dithiothreitol

EDTA: Ethylene diamine tetraacetic acid

PMSF: Phenyl methylsulfonyl fluoride

CD: Circular dichroism

PMB: p-hydroxymercuribenzoic acid sodium salt

DEAE cellulose: Diethylaminoethyl cellulose

CM cellulose: Carboxymethyl cellulose

CML: Chronic myelogenous leukaemia

Introduction

Imatinib mesylate is a tyrosine kinase inhibitor, binds to the catalytic cleft of tyrosine kinases inhibiting its activity successfully.[1,2] Imatinib mesylate is used for the treatment of chronic myelogenous leukaemia (CML) and gastrointestinal stromal tumors.[3,4] The drug partly occupies the ATP-binding pocket and stabilizes an inactive form of the Bcr-Abl oncogenic fusion protein.[4] Besides gene amplification and mutation, binding of imatinib to acute phase protein, α1-acid glycoprotein (AGP) also could inhibit its activity.[5,6] Binding studies of imatinib in plasma and with the two main plasma proteins, AGP and human serum albumin (HSA) proved that the stronger binding can be referred to the AGP component with association binding constant (K_a) values of 4.9×10^6 M^{-1} and 2.3×10^5 M^{-1} for AGP and HSA, respectively.[7,8] Little is known about the interaction of imatinib with other cellular components, e.g. membranes and other blood proteins, however, these anticancer agents can exert toxic effects when used for prolonged period. Moreover, a huge body of literature is available on the interactions of anticancer drugs with cytoskeletal proteins e.g. Estramustine alone or in combination with other anticancer agents could affect microtubule dynamics associated with tubulin acetylation, spindle abnormalities, and mitotic arrest to exert their anticancer properties.[8] We have also shown previously that Chromomycin and Mithramycin could favourably interact with spectrin from erythroid membrane skeleton.[9]

Spectrins are flexible rods 200 nm in length with binding sites for F-actin at each end.[10] It is composed of the two largest known, evolutionarily related polypeptide chains, α-subunit (280 kDa) and β-subunit (246 kDa), both of which are non-covalently associated in an antiparallel 'side-to-side' manner to form a two-stranded worm-like heterodimer.[11,12] To establish the planar network, spectrin interacts with a large number of proteins such asactin, adducin, ankyrin and protein 4.1. In addition to those proteins spectrin binds to a variety of hydrophobic ligands, tertiary amine local anaesthetics and Haeme and its derivative.[13-16] Tryptophan residues in spectrin are spread over the entire length of the dimer making them convenient intrinsic fluorescence reporter groups for monitoring conformational changes or binding of ligands.[16] Spectrin also contains a unique binding site for hydrophobic molecules such as Prodan and pyrene and large number of binding sites for fatty acids and phospholipids along the length of the protein molecule.[13,14,17-19]

Human haemoglobin A (HbA), the major protein in erythrocyte, exists as tetramer of globin chains, which is composed of two α and two β subunits with 141 and 146 amino acid residues respectively. The polypeptide chain of each subunit is attached with a prosthetic group, heme. Each haeme group contains an iron ion, encapsulated inside heterocyclic porphyrin ring, which can bind one O_2 molecule. HbA is an important functional protein as reversible oxygen carrier and storage.[20] The structure function relationship of HbA is well established.[21-25] Tetrameric HbA contains six tryptophan residues as each α subunit contains one tryptophan (α14) while each β subunit contains two tryptophans (β15, β37).[26] Additionally HbA contains five tyrosine residues in each αβ heterodimer.[27]

Some clinical studies showed that prolonged use of imatinib mesylate causes its partitioning to red blood cell.[28] A recent report also shows that long term use of this anticancer agent may have some role in regulation of reduced lifespan and rigidity of erythrocyte, associated with haemolytic anaemia.[29] Binding of imatinib to serum proteins e.g. human α1-acid glycoprotein and HSA have been already implicated in the context of its activities as these proteins could act as potential carriers of the drug molecules and show reversible binding due to its abundance in blood plasma. [5-7, 30] The present work is aimed towards studying the binding potentials of other abundant proteins in the blood, the erythroid membrane skeletal protein, spectrin and the most abundant HbA along with its subunits using fluorescence spectroscopy. Such binding studies could be important for better understanding of the drug induced toxicity, since it is highly probable that imatinib comes in direct contact with the most abundant proteins of the blood cells, particularly for the patients taking them for a longer period of time. We have seen moderate and comparable binding affinity of imatinib mesylate with spectrin and HbA with dissociation constants of 48 μM and 63 μM respectively. The binding affinities of both the globin chains were stronger with K_d of around 10 μM. The thermodynamic parameters indicated such binding to be largely entropy-driven. Circular dichroism (CD) spectroscopy was employed to find out the structural basis of recognition and indicated binding induced conformational changes, in both spectrin, HbA and the globin chains. It is also evident that imatinib induces conformational changes in these proteins which might contribute to the drug induced toxicity in patients.

Materials and Methods

Materials

Imatinib mesylate (4-(4-methyl-piperazin-1-yl-methyl)-N-[4-methyl-3-(4-pyridin-ylpyrimidin-2-yl-amino)-phenyl]-benzamidemethanesulfonate), p-hydroxymercuribenzoic acid sodium salt (PMB), Tris, PMSF, EDTA, DTT, Sephadex G100were obtained from Sigma(St. Louis, MO, USA). Deionised water, triple distilled on quartz, was used for preparing solutions and buffers. Stock solution of Imatinib mesylate prepared in distilled water.[30]

Methods

Isolation and purification of Spectrin, Haemoglobin A and its subunits

Dimeric spectrin was purified from erythrocyte ghosts prepared by hypotonic lysis in 5 mM phosphate, 1mM EDTA containing 20 μg/ml PMSF at pH 8.0 following published protocols.[31] Spectrin extraction was done by resuspending the erythrocyte ghosts in 20 vol of spectrin removal buffer (0.2 mM sodium phosphate, 0.1mM EDTA, 0.2 mM DTT, 20 μg/ml PMSF, pH 8.0), and incubating at 37°C for 30 min. The crude spectrin was purified after concentration by 30% ammonium sulfate precipitation elaborated earlier.[32] Spectrin was stored in the buffer containing 5 mM phosphate, 20 mMKCl, 1mM EDTA, pH 8.0 containing 0.2 mM DTT and was dialyzed extensively against the buffer containing 10 mM Tris-HCl, 20 mM NaCl, pH 7.4 to remove DTT before all fluorescence experiments. Spectrin concentrations were determined spectrophotometrically from an absorbance of 10.7 at 280 nm for 1% spectrin solution.

HbA was obtained from blood samples collected from normal volunteers and purified according to published protocol.[25,33] After removal of buffy coat and plasma red cells were washed extensively with phosphate buffer saline (5 mM phosphate, 0.15 M NaCl, pH 7.4). HbA was isolated from packed erythrocytes by osmotic lysis using three volumes of 1mM Tris, pH 8.0 at 4°C for 1 hr. HbA was further purified by gel filtration on Sephadex G100 column (30×1 cm) in buffer containing 5mM Tris, 50mM KCl, pH 8.0. Purified HbA was stored in oxy format at -70°C for less than 7 days and characterized by the measurements of absorpation at 415 nm and 541 nm respectively. The purity of the HbA was checked by 15% SDS-PAGE after staining with coomassie blue. The protein concentration was determined

spectrophotometrically in a Cary UV-Visible spectrophotometer using a molar extinction coefficient of HbA 125,000 M^{-1} cm^{-1} at 415 nm and 13,500 M^{-1} cm^{-1} at 541 nm respectively.

The PMB (p-hydroxymercuribenzoic acid) derivatives of HbA were prepared according to the published method.[34-36] The α-PMB and β-PMB chains were isolated by specific ionic exchange chromatography as described earlier. Hb-PMB mixture was equilibrated with 0.01 M phosphate buffer, pH 8.0 followed by passing through a DEAE-cellulose column, equilibrated with the same buffer. Under this condition α-PMB was eluted from the column. Now to get the β-PMB chain, Hb-PMB solution was applied on a CM-cellulose column, equilibrated with 0.01 M phosphate buffer, pH 6.6. The PMB was removed individually from the isolated subunits by the addition of 50mM β–mercaptoethanol in 0.1 M phosphate buffer, pH 7.4. PMB free globin chains were further purified by gel filtration on a Biogel P2 column. The intact subunits were dialyzed extensively against 0.1 M phosphate buffer, pH 7.4.[37] The concentration of globin subunits were measured spectrophotometrically at 280 nm as $E^{1\%}_{1cm}$ =8.5.[36] The globin subunits were not stored for more than 48 hrs at 4°C and characterized from their spectral characteristics to confirm their oxidative states.

Fluorescence measurement of ligand binding of proteins:

Steady-state fluorescence was measured in a Cary Eclipse spectrofluorometer using a1cm pathlength quartz cuvette. The buffer used in the study contained 10 mM Tris, 20 mM KCl, pH 7.4. Small aliquots of the stock solution of imatinibwere added to 0.2 μMspectrin for fluorescence measurements using excitation at 295 nm and slits with band passes of 5 nm for both excitation and emission channels while Trp residues of HbA and its subunits were excited at 280 nm. The concentration of HbA was kept at 4.0 μM and the concentration of imatinib was varied from 0 to 37 μM in each binding experiments. Protein-free buffer containing different concentrations of imatinib was used as reference blanks in all fluorescence measurements. Fluorescence intensities were corrected for the inner filter effect due to absorption of the spectrin and imatinib, when absorbance, at both excitation and emission wavelengths, of the samples exceeded 0.05.[38,39] All measurements were performed at 25ºC, unless otherwise mentioned, with multiple sets (three to five) of samples.

Analysis of binding parameters

Binding parameters for the interaction between imatiniband both the proteins spectrin and HbA were estimated from the quenching of protein fluorescence. The quenching data were

then analyzed to determine the dissociation constant (K_d) using the following equation, also elaborated earlier:[15,38,40]

$$1/\Delta F = 1/\Delta F_{max} + 1/ (K_{app} .\Delta F_{max}. (C_p - C_0)) \quad (1)$$

where ΔF is the change in fluorescence emission intensity and ΔF_{max} is the change in fluorescence intensity, when the protein is completely bound to the ligand. C_P is the concentration of ligand and C_0 is the initial concentration of protein. The linear double reciprocal plot of $1/\Delta F$ against $1/ (C_p - C_0)$ was extrapolated to the ordinate to obtain the value of ΔF_{max} from the intercept. The stoichiometry of binding of imatinib to the proteins could not be estimated accurately from the fluorescence data. Due to inner-filter effect of imatinib mesylate we could not reach near the saturation point. However, the stoichiometry of imatinib binding to both proteins were obtained from the intersection of two straight lines obtained from the least-square fit plot of normalized increase in fluorescence intensity against the ratio of input concentrations of imatinib (not shown).

Evaluation of thermodynamic parameters

Thermodynamic parameters were evaluated from the following equations:

$$\ln K_{app} = - (\Delta H/ RT) + \Delta S/ R \quad (2)$$

$$\Delta G = \Delta H - T \Delta S \quad (3)$$

where R and T are the universal gas constant and absolute temperature, respectively and ΔG is the Gibbs free energy change upon binding of the imatinib to the proteins, ΔH is the corresponding average change in enthalpy and ΔS is the same in entropy.[14,15, 41,42] Assuming no significant temperature dependence of ΔH in the temperature range we used, the values of ΔG, ΔH and ΔS were determined. The binding affinity constant K_a was measured at three different temperatures to evaluate ΔH and ΔS from the slope and the intercept in a plot of $\ln K_a$ against $1/T$.

Circular dichroism:

The far UV CD spectra of the proteins and the protein–ligand complexes were recorded using a CD spectrometer from Biologic, in the range of 200-250 nm using a quartz cell of 0.1 mm pathlength while the near UV spectra of HbA and globin chains were recorded in the range of 250-350 nm using a quartz cuvette of 10 mm pathlength. The concentrations of spectrin and HbA were kept at 0.5μM and 8.0 μM respectively for far UV experiments. All the spectra

were the average of five runs. Each spectrum was subtracted from the buffer base line and undergone smoothing within the permissible limits using the instrument's inbuilt software and was expressed in unit of millidegrees.

Molecular docking studies:

The specificity of imatinib interactions with HbA and the respective globin chains was studied using a computer docking study. The crystal structure of HbA (PDB entry 2DN1) has been downloaded from the Protein Data Bank and the structure of imatinib was obtained from their respective PDB structural database. Docking studies were performed with Auto Dock 4.2 software package to calculate the interaction between ligand and HbA. Auto dock uses the Lamarckian genetic algorithm to calculate possible conformer of the hydrophobic ligand that binds different domains of HbA.[14, 15, 43] To recognize the binding site in HbA, blind docking was carried out, the size of the grid box was set to 80, 75 & 60 along X-, Y- and Z-axes, with 1.00 Å grid spacing. During docking process, a maximum number of 10 conformers were considered for ligand binding to protein, and the lowest binding energy molecule undergoes further analysis. The lowest energy binding domain was visualized using PyMOL molecular graphics system.[44]

Results and Discussion

Binding of Imatinib to dimeric spectrin, HbA and globin chains

The emission intensities of the proteins, dimeric spectrin, tetrameric HbA and monomeric globin chains decreased upon successive addition of small aliquots of imatinib (data not shown). In spectrin, the fluorescence maximum appears at 337 nm upon excitation at 295 nm while in HbA at 330 nm clearly indicating the Trp residues to be localized in HbA in a more hydrophobic environment compared to spectrin. **Figure 1(A-C)** shows the non linear fit and the representative double reciprocal plotsin the inset, of binding of imatinib to spectrin (A), HbA in both oxy- and deoxy-form (B) and the globin chains (C) at 25°C. No spectral shift was observed upon addition of increasing concentrations of imatinib suggesting that the microenvironment around the fluorophore remained unaltered upon addition of the ligands. The nature of the binding isotherm and double reciprocal plot suggest non-cooperative reaction mechanism. The K_d values for the protein-imatinib complexes were estimated and summarized in **Table 1**. It is evident that both spectrinand HbA have comparable binding affinities towards imatinib with K_d values of 48 μM and 63 μM for spectrin and oxy-HbA at 25°C respectively. The binding affinity was 6 fold higher in case of both the globin chains

compared to intact HbA with K_d values for α- and β-globin chainsto be 10.8 μM and 9.2 μM respectively. An estimate of the stoichiometry of binding showed about 100 imatinib molecules to be associated with a spectrin dimer and about 4-5 imatinib molecules per HbA while the stoichiometry for the association of both the globin chain chains with imatinib were found to be 1 (data not shown).

Imatinib demonstrated high levels of efficacy at all stages of CML from the outset of clinical testing in phase I and phase II trials.[45,46] The phase I studies were dose-escalating trials in which the daily doses of imatinib ranged from 25–1,000 mg. Based on recently reported findings of the phase III International Randomized IFN versus STI571 (IRIS) trial, imatinib is now the gold standard for first-line pharmacotherapy of CML.[47] Though recent clinical reports raised the concern of prolong usage of imatinib because of haemolytic anaemia as its adverse side effect. In case of haemolytic anaemia, loss of heme occurs and hence level of free heme increases inside erythrocyte.[48] Earlier we have shown that spectrin could act as potential heme acceptor.[15] A recent report also showed that imatinib alters the heme binding ability of HSA.[30] To check if imatinib could also modify the association of heme with spectrin we performed binding experiments of imatinib with heme bound spectrin and found comparable imatinib binding with that of heme free spectrin, indicating a probable lowering of oxidative stress inside red cell occurring due to loss of heme caused by binding of imatinib to HbA.

Determination of thermodynamic parameters

Thermodynamic parameters give us the idea of molecular basis of any interaction and the molecular forces, like hydrophobic force, hydrogen bonding, van der Waal's force and electrostatic forces, involved in the protein-ligand association.[41] Changes in free energy, enthalpy and entropy,associated with such binding were determined in spectrin, oxy-HbA and oxy-globin chains by studying the binding at different temperatures and **Figure 2** shows the representative Van't Hoff plots for the associations of the proteins with imatinib mesylate. The negative values of the free energy of formation of protein-drug complexes account for the spontaneity of the interactions. **Table 2** summarizes the thermodynamic parameters. The difference in the magnitude of the change in enthalpy and entropy for spectrin and HbA is due to the enthalpy-entropy compensation mechanism associated with solvent re-organization.[14,15,49,50] It is evident from the data that imatinib bind the proteins with positive changes in both entropy and enthalpy suggesting such interactions to be endothermic in

nature. So these associations are favoured by large positive change in entropy which arises due to the hydrophobic effect of removal of water molecules from the binding surface, as well as conformational restriction due to complex formation.[51] The large positive ΔS are the major driving force for the imatinib-spectrin, imatinib-HbA and imatinib-globin chain associations. The linear nature of the Van't Hoff's plots also suggests that the association of imatinib with the proteins do not result in any major reshuffling of the subunits.

Far and near UV CD studies of Imatinib binding of proteins

Figure 3 shows representative CD spectra of spectrin, HbA and its subunits in the presence and absence of imatinib mesylate in the far UV region (190-250nm). **Figure 3 (A-E)** shows the representative backbone structure of spectrin, HbA and its subunits with appreciable change in the backbone conformation and the extent of helicity of the worm-like protein, spectrin was observed upon binding to imatinib. In case of intact HbA such a change was insignificant in presence of the drug. However, we observed structural alterations in the globin chains reflected in the spectral changes of both the globin chains due to complex formation with imatinib.

The near UV CD spectra provide information about the contributions of aromatic side chains and disulfide bonds. **Figure 4 (A-D)** shows the representative near UV CD spectra of HbA and globin chains showing significant alterations in the tertiary structures of the tetrameric HbA and its subunits in presence of imatinib. The spectra showed characteristic positive peak at 260 nm which is strictly affected by the addition of the drug.The intensities of the peak at 260 nm for both the oxy and deoxy-HbA and oxy globin chains decreased with increasing concentrations of imatinib.

Identification of the binding sites in HbA by molecular docking studies:

To obtain insight into the imatinib interaction with haemoglobin, molecular modelling simulations were applied to examine the binding of imatinib at the active site of HbA as described by our own and other research groups.[15,16,52] The best binding energy resulted from the docking study was ΔG°= - 3.97 kcal/ mol, shown in **Figure 5**. It can be seen from **Table 3** summarizing the binding energies that imatinib was situated within the domain of both α- and β-globin chains. The specific interactions between imatinib β-globin are shown in **Figure 5**. An extended network of hydrophobic contacts was found with the amino acids Lys-17, Asn-19, Glu-22, Gly-25, Glu-26, Met-55, His-117, and His-127. HbA has six Trp residues,

two Trp-α14, two Trp-β15, and two Trp-β37. In the present study, among the six Trp residues, only Trp-β15 is at the closest proximity and interacts with imatinib. Other Trp residues are present in the hydrophobic core of the protein; therefore, those Trps are not accessible for the imatinib molecule. Imatinib molecule preferably binds to the surface of the HbA, therefore, do not go into the hydrophobic cavity of HbA causing quenching of Trp fluorescence. In addition, the docking studies show that imatinib interacts not only with the Trp residues alone but also with other amino acids. **Table 3** also shows few of these amino acids involved in imatinib binding. Along with Trp β15, Trp β37 and Trp α14 residues also interact closely with imatinib leading to the apparent discrepancy in the binding energies obtained here from those obtained experimentally (**Table 2**).

Conclusion

This study for the first time showed the interactions of Imatinib mesylate, the antileaukemic drug with erythroid proteins, spectrin, HbA and the globin chains and compared the nature of binding of this anticancer agent with these proteins using fluorescence spectroscopy, CD, and molecular docking techniques. Results from this study raised possibilities of alternative binding sites of imatinib and related small molecule kinase inhibitors to erythroid spectrin and haemoglobin variants to their hydrophobic cavities, in addition to serum proteins, HSA and α1-acid glycoprotein, sequestering large amount of imatinib from the pool of the dose administered, hence contributing to the drug induced toxicity in patients.

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References

1. R. H. Gunby, E. Sala, C. J. Tartari, M. Puttini, C. Gambacorti-Passerini and L. Mologni, *Anticancer Agents Med.Chem.*, 2007, 7, 594-611.
2. P. W. Manley, S. W. Cowan-Jacob, E. Buchdunger, D. Fabbro, G. Fendrich, P. Furet, T. Meyer and J. Zimmermann, *Eur. J. Cancer.*2002, 38, (Suppl.5), S19–S27.
3. C. M. de Silva and R. Reid, *Pathol. Oncol. Res.*, 2003, 9, 13–19.

4. B. J. Druker and N. B. Lydon, *J. Clin. Invest.*, 2000, 105, 3-7.
5. C. Gambacorti-Passerini, R. Barni, P. le Coutre, M. Zucchetti, G. Cabrita, L. Cleris, F. Rossi, E. Gianazza, J. Brueggen, R. Cozens, P. Pioltelli, E. Pogliani, G. Corneo, F. Formelli and M. D'Incalci, 2000. *J. Natl. Cancer. Inst.*, 92, 1641–1650.
6. J. M. Kremer, J. Wilting and L. H. Janssen, *Pharmacol Rev.,* 1988, 40, 1-47.
7. I. Fitos, J. Visy, F. Zsila, G. Mády and M. Simonyi. *Biochim. Biophys. Acta.*, 2006, 1760, 1704-1712.
8. R. Mohan, D. Panda, *Cancer Res.*, 2008, 68, 6181-6189.
9. S. Majee, D. Dasgupta and A. Chakrabarti, *Eur. J. Biochem,* 1999, 260, 619-626.
10. V. Bennett and A. J.Baines, *Physiol. Rev.,* 2001, 81, 1353–1392.
11. A. Chakrabarti, D. A. Kelkar and A. Chattopadhyay, *BioSci. Rep.*,2006, 26, 369-386.
12. J. H. Hartwig, *Protein Profile.,* 1995, 2, 703-800.
13. A. Chakrabarti, 1996, *Biochem. Biophys. Res. Commun*., 226, 495-497.
14. M. Patra, M. Mitra, A. Chakrabarti and C. Mukhopadhyay, *J. Biomol. Struct. Dyn.,* 2014, 32, 852-865.
15. D. Das, M. Patra and A. Chakrabarti, *Eur. Biophys. J.*, 2015, 44, 171-182.
16. A. Chakrabarti and M. Patra, *Biochim. Biophys. Acta.*, 2015, 1848, 821-832.
17. W. Diakowski, A. Prychidny, M. Swistak, M. Nietubye, K. Bialkowska, J. Szopa and A. F. Sikorski, *Biochem. J.*, 1999, 338, 83-90.
18. S. Ray and A. Chakrabarti, *Mol. Membr. Biol.*, 2004, 21, 93-100.
19. M. Mitra, M. Patra and A. Chakrabarti, *J Fluorescence*, 2015, 44, 635-645.
20. X. C. Shen, X. Y. Liou, L. P. Ye, H. Liang and Z. Y. Wang, 2007, *J. Coll. Inter. Sci.*, 311, 400-406.
21. K. Imai, A. Tsuneshige, T. Harano and K. Harano, *J. Biol. Chem,* 1989, 264, 11174-11180.
22. J. A. Lukin and C. Ho, *Chem. Rev.*, 2004, 104, 1219-1230.
23. A. Marengo-Rowe, *J. Proc. Bayl. Univ. Med. Cent.*, 2006, 19, 239-245.
24. W. L. Nichols, B. H. Zimm and L. F. Ten Eyck. *J. Mol. Biol.,* 1997, 270, 598-615.
25. M. Banerjee and A. Chakrabarti and S. Basu, *J. Phys. Chem. B,* 2012, 116, 6150-6157.
26. S. Venkateshrao and P. T. Manoharan, *Spectrochim. Acta. Part A,* 2004, 60, 2523-2526.
27. T. C. Mueser, P. H. Rogers and A. Arnone, *Biochemistry*, 2000, 39, 15353-15364.

28. H. Prenen, G. Guetens, G. D. Boeck, M. Highley, A. T. van Osterom, E. A. de Brujin, *J. Pharm. Pharmacol.*, 2006, 58, 1063-1066.
29. M. C. Z. Novaretti, G. H. H. Fonseca, M. Conchon, P. E. Dorlhiac-Llacer and D. A. F. Chamone, 2003, *Eur. J. Haematol.*, 71, 455-458.
30. E. D. Muzio, F. Polticelli, V. Trezza, G. Fanali, M. Fasano and P. Ascenzi, *Arch. Biochem. Biophys.,* 2014, 560, 100-112.
31. W. B. Gratzer *Methods Enzymol.,* 1985, 85, 475-480.
32. M. Patra, C. Mukhopadhyay and A. Chakrabarti, *PLoS ONE,* 2015, 10, e0116991.
33. P. Datta, S. Chakrabarty, A. Chakrabarty and A. Chakrabarti, *Biochim. Biophys. Acta.,* 2008, 1778, 1-9.
34. C. Bucci and C. A. Fronticelli, *J. Biol. Chem.*, 1965, 240, 551-552.
35. G. Geraci, L. J. Parkhurst and Q. H. Gibson, *J. Biol. Chem.*,1969, 244, 4664-4667.
36. P. Datta, S.Chakrabarty, A.Chakrabarty and A.Chakrabarti, *J Biosci.,* 2007, 32, 1147-1151.
37. E. Bucci, 1981, *Methods Enzymol.*, 76, 97-106.
38. A. Chakrabarti, S. Bhattacharyya, S. Ray and M. Bhattacharyya, *Biochem. Biophys. Res. Commun,.*2001, 282, 1189-1193.
39. J. R. Lakowicz, *Priciple of fluorescence spectroscopy,* Springer, New York, 3rd Edn, 2006, pp. 63-606.
40. J. L. Wang and G. H. Edelman, *J. Biol. Chem.*,1971, 246, 1185-1192.
41. D. P. Ross and S. Subramanian, *Biochemistry.*, 1981, 20, 3096-3102.
42. G. W. Castellan, Addison Wesley/Narosa Publishing House, New Delhi, India, 3rd Edn, 1989, pp. 799-815.
43. G. M. Morris, R. Huey, W. Lndstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell and A. J. Olson, *J. Comp. Chem.*, 2009, 30, 2785–2791.
44. W. L. Delano, Pymol: An open source for molecular graphics tool. De Lano Scientific, San Carlos, CA, 2002.
45. B. J. Druker, M. Talpaz, D. J. Resta, B. Peng, E. Buchdunger, J. M. Ford, N. B. Lydon, H. Kantarjian, R. Capdeville, S. Ohno-Jones and C. L. Sawyers, *N. Engl. J. Med.*, 2001, 344, 1031-1037.
46. C. L. Sawyers, A. Hochhaus, E. Feldman, J. M. Goldman, C. B. Miller, O. G. Ottmann, C. A. Schiffer, M. Talpaz, F. Guilhot, M. W. N. Deininger, T. Fischer, S. G. O'Brien, R. M. Stone, C. B. Gambacorti-Passerini, N. H. Russell, J. J. Reiffers, T. C. Shea, B. Chapuis, S. Coutre, S. Tura, E. Morra, R. A. Larson, A. Saven, C. Peschel,

A. Gratwohl, F. Mandelli, M. Ben-Am, I. Gathmann, R. Capdeville, R. L. Paquette, B. J. Druker, 2002, Blood, 99, 3530-3539.

47. K. Peggs and S. Mackinnon, *N. Engl. J. Med.*, 2003, 348, 1048-1050.
48. S. Kumar and U. Bandyopadhyay, Toxicol. Lett*.,* 2005, 157, 175-188.
49. L. Das, S. Gupta, D. Dasgupta, A. Poddar, M. E. Janik and B. Bhattacharyya, *Biochemistry*, 2009, 48, 1628-1635.
50. M. Mondal and A. Chakrabarti, *FEBS Lett.,* 2002, 532, 396-400.
51. H. Torigoe, T. Nakayama, M. Imazato, I. Shimada, Y. Arata and A. Sarai, *J. Biol. Chem.*, 1995, 270, 22218-22222.
52. S. J. Campbell, N. D. Gold, R. M. Jackson and D. R. Westhead, *Curr. Opin. Struct. Biol.,* 2003, 13, 389-395.

Figure legends:

Figure 1: Binding isotherms for the interaction of proteins with Imatinib in 10 mM Tris, 20 mM KCl buffer, pH 8.0 at 25°C. Figure 1 (A-C) shows non linear fit for the association of (A) spectrin dimer; (B) Hb oxy (▼), Hb deoxy (▲); (C) α-globin (●), β- globin (■).

Figure 2: Thermodynamics of Imatinib- protein association in 10 mM Tris, 20 mM KCl, pH 8.0 showing Van't Hoff's plots of ln K_{app} against 1/T for the interaction of (A) spectrin dimer; (B) Hb oxy (▼), Hb deoxy (▲); (C) α-globin (●), β- globin (■).

Figure 3: CD spectra (195-240 nm) of Imatinib in buffer containing 10 mM Tris, 20 mM KCl, pH 8.0 at 25°C in presence (dashed line) and absence of (A) Hb oxy, (B) Hb deoxyand (C) spectrin dimer, (D) α- globin, (E) β- globin.

Figure 4: CD spectra (230-300 nm) of (A) Hb oxy, (B) Hb deoxy, (C) α- globin, (D) β- globin in presence of successive addition of Imatinib. The arrow down towards indicates the increasing concentration of Imatinib.

Figure 5: Theoretical model of Hb tetramer (A); Residues involved in binding of Imatinib with Hb tetramer (B): Energy minimized complexes of Hb tetramer with Imatinib (C).

Table 1: Binding constants of Imatinib to the proteins at 25°C, pH8.0

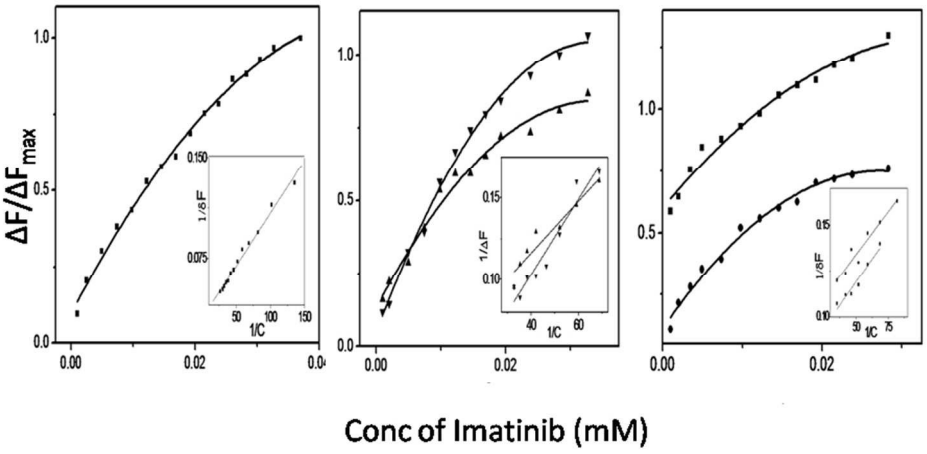
Proteins	**K_d (μM)**
Spectrin	48
Deoxy-HbA	129
Oxy-HbA	62.9
α-globin	10.8
β-globin	9.2

Table 2: Thermodynamic parameters of Imatinib binding to the proteins

Proteins	ΔG(kJ/mole)	ΔH(kJ/mole)	ΔS(eu)
Spectrin	-128.07	0.105	+103.47
Deoxy-HbA	-154.19	0.133	+124.59
Oxy-HbA	-161.59	0.138	+130.51
α- globin	-69.72	0.0357	+55.96
β- globin	-59.53	0.0249	+47.77

Table 3: Computed binding energies of Imatinib with HbA

	Binding energy(k-cal/mole)	**Binding site on subunits**	**Residue involve in the binding subset**
1	-3.97	beta	LYS-17, ASN-19, GLU-22, GLY-25, GLU-26, HIS-116, HIS-117.
2	-3.68	beta	GLU-22, GLU-26, MET-55, LYS-61, HIS-116, HIS-117, PHE-118, GLY-119, GLU-121.
3	-3.60	beta	LYS-17, ASN-19, GLU-22, PRO-114, HIS-116, HIS-117, PHE-118, GLU-121.
4	-3.34	beta	PR0-100, GLU-101, ASP-102, HIS-143, HIS-146, TYR-145.
5	-2.97	beta	ASP-73, THR-87, LEU-88, GLU-90,LEU-91, ASP-94, LYS-95.
6	-2.9	alpha+beta	LEU-109, HIS-112, LEU-113, PRO-114, HIS-116, HIS-117, PHE-118, LYS-120.
7	-2.61	alpha	LYS-61, ASP-64, ALA-65, ASN-68, ALA-71, HIS-72, LEU-80, ALA-82, LEU-86.
8	-2.53	alpha	PRO-36, TRP-37, LYS-40, GLN-39, GLU-43, ASP-47, LEU-48, SER-49.
9	-2.4	alpha	VAL-96, PRO-100, ARG-104, TRY-145, HIS-146.
10	-2.05	alpha	LYS-40, PHE-43, PRO-44, PHE-46, LEU-48, SER-49.



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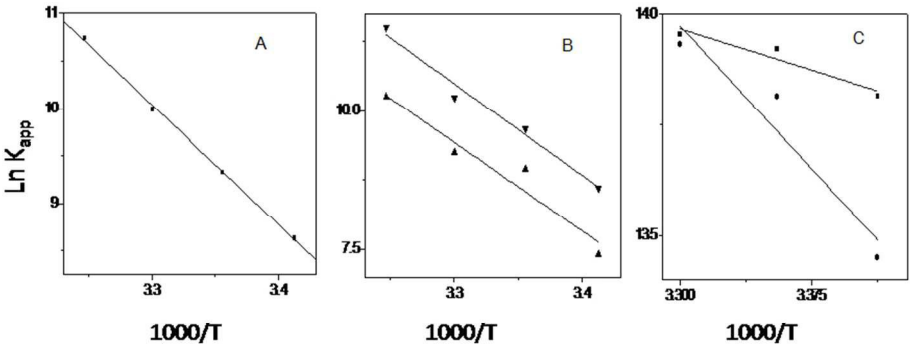

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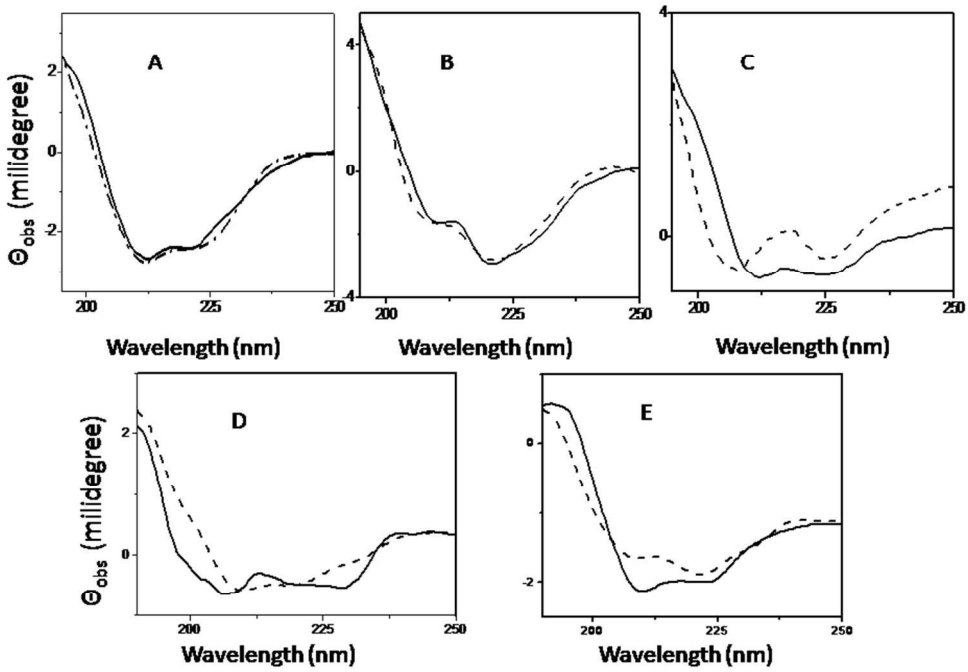

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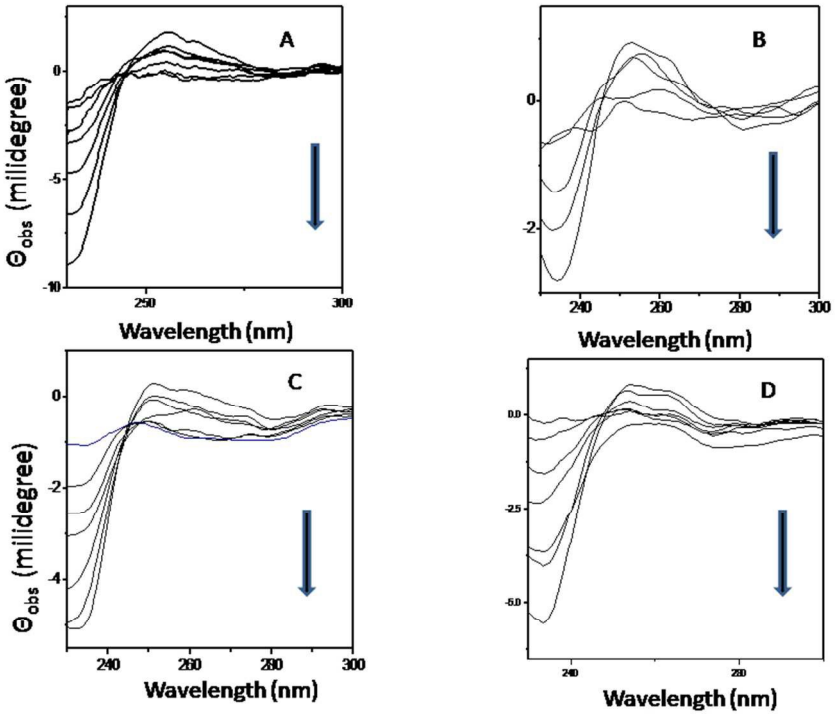

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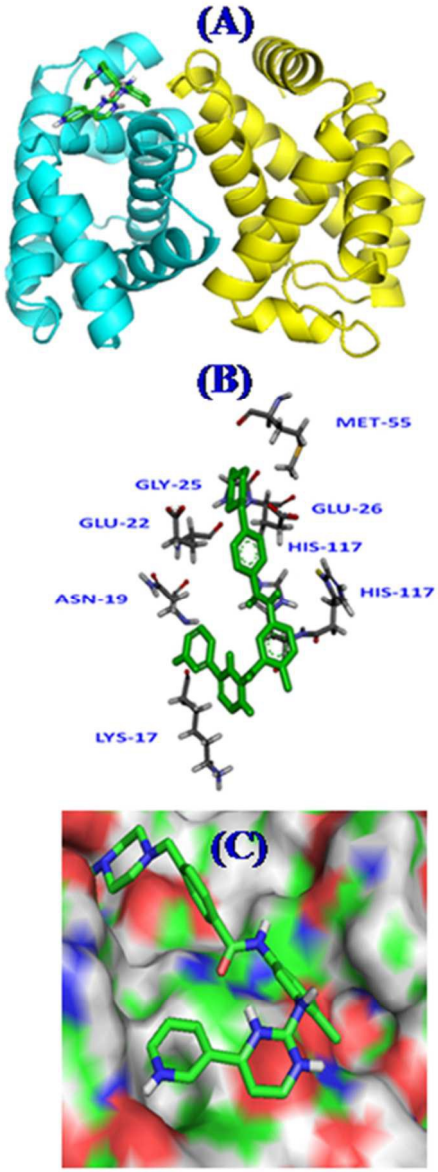

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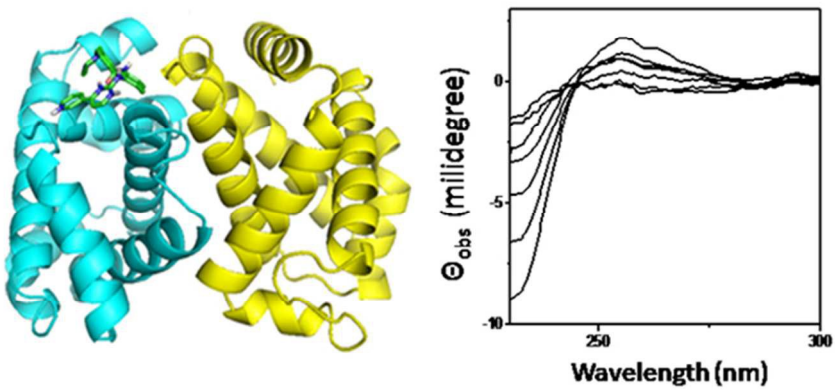
Graphical Abstract



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