2-((E)-(6-fluorobenzo[d]thiazol-2-ylimino) methyl)-4-chlorophenol; Synthesis, characterization, crystal structure, Hirshfeld surface analysis and BSA binding studies.

Savithri K¹, B C Vasantha Kumar¹, H D Revanasiddappa^{1*}, H K Vivek², Manpreet Kaur³ and Jerry P Jasinski³

Abstract

A new imine-based molecule 2-((E)-(6-fluorobenzo[d]thiazol-2-ylimino) methyl)-4-chlorophenol (FBt) was synthesized by microwave and conventional method. It was structurally characterized by spectral techniques (NMR, FT-IR, LC-MS and electronic absorption), elemental analysis and single-crystal X-ray diffraction methods. Hirshfeld surface analysis was employed to ensure the existence of intermolecular interactions in FBt structure. A preliminary *in vitro* susceptibility test against two pathogenic fungi with respect to standard has shown that the ligand is proved to be a potent antifungal agent. Since the carrying of a drug by BSA may effect on its structure and action, the investigation on the interaction between model protein BSA and FBt was carried out by employing UV-Vis and fluorescence spectroscopy. The characteristics of the binding, i.e., binding constant, number of binding sites, and nature of binding were determined. Besides, the Förster's parameters associated with the binding process were calculated. Molecular docking was also carried on interaction study of the FBt with BSA.

Key points: Imine base; X-ray crystal structure; BSA binding; Molecular docking; Hirshfeld surface analysis; Antifungal assay;

1. Introduction

In recent years, Imine based compounds have received much attention in clinical, biological, pharmaceuticals and analytical fields due to the similar structure of naturally occurring biological substances [1,2] and synthetic flexibility [3]. 2-Amino benzothiazole derivatives have received considerable attention owing to their unique structures and exquisite pharmacophores observed in many diagnostic and therapeutic agents [4]. 6-Fluorobenzothiazole derivatives possess high potency and selectivity as antifungal agents with a broad spectrum of action [5]. Moreover, the presence of electronegative atoms in one of the rings introduces high polarity to the structure. FBT is one of benzothiazole-based compounds. It was found that benzothiazole conjugates show an ideal affinity to protein *in vitro* which figure out the pharmacokinetic of the drug. [6,7].

In general, proteins are considered to be the primary molecular targets on the action of drugs through binding to proteins, which determine the therapeutic effectiveness of potent drugs and hence, studies on synthesis and interactions of compounds with proteins have been an active field of research [8]. Knowledge on drug-serum albumin binding is very useful for therapeutic drug monitoring as the blood proteins play a crucial role in drug pharmacology and pharmacokinetics and significantly influences the efficacy, distribution to cells *in vivo* and *in vitro* and metabolic rate of typical drugs. Moreover, the therapeutic drug effect is related to the balance between the bound and unbound fraction of a drug [9]. Plasma proteins' binding of ligands is necessary to determine the binding site(s) and the possible interactions for each compound since proteins are flexible molecules and most drugs and ligands are transported as complexes with serum albumin. Moreover, some of them may alter the binding affinity of other endogenous and or exogenous ligands as a result of an overlap of binding sites and or conformational changes [10,11]. The binding affinity also affects the rate at which the drug is released to sites of catabolism or pharmacological action. For its medical importance and ready availability, bovine serum albumin (BSA) was usually selected for studying drug-protein interaction *in vitro*.

The present research paper describes the investigation of the antifungal activity and general affinity of BSA binding towards a particular molecule containing Schiff base as well as the changes in the concentration of free and bound fractions are necessary from a pharmacological point of view is focused on potential new, unique and versatile scaffold for systemic drug design.

2. Experimental

2.1 Analytical and physical measurements

The melting point of a compound was determined in the open capillary tube using a precision digital melting point apparatus and was uncorrected. Mass spectrum of synthesized compound was recorded using a 2010EV LCMS Shimadzu spectrometer. Elemental analysis was performed on a Perkin–Elmer elemental analyzer. Electronic spectra were recorded (200–800 nm) in DMSO using a UV-Vis spectrophotometer (DU 730 ‘Life Science’ M/S Beckman coulter, USA). IR spectrum was recorded on a Perkin–Elmer 783 spectrophotometer *via* attenuated total reflection (FT-ATR) in the 4000–400 cm^{-1} range. Bovine serum albumin (BSA) was purchased from Sigma Corp.

2.2 Synthetic protocol for FBt: 2-((E)-(6-fluorobenzo[d]thiazol-2-ylimino) methyl)-4-chlorophenol

Classical method

The ligand (FBt) was prepared as in Scheme (**Fig.1**) by the condensation of an equimolar solution of 2-amino-6-fluoro benzothiazole and 5-chlorosalicylaldehyde in ethanol with a catalytic amount of glacial acetic acid and refluxed at 75 °C for 6 h. TLC was used to monitor the completion of the reaction in an n-hexane: ethyl acetate (7:3) solvent system. On standing the reaction mixture overnight at room temperature (25 °C), the yellow precipitate obtained was filtered and washed with cold ethanol. The product was recrystallized from ethanol twice to give yellow lamellar crystals. **Yield**=78 %.

Microwave method

The title compound FBt was synthesized by placing an ethanolic mixture of 2-amino-6-fluoro benzothiazole (0.1 M) and 5-chlorosalicylaldehyde (0.1 M) in a flask and irradiated in a microwave oven for 6 min. TLC monitored the completion of the reaction progress, and the reaction mixture was allowed to attain room temperature (25 °C). The yellow crystals obtained after recrystallized from ethanol. **Yield**=86 %.

Fig. 1. Scheme for the synthesis of FBt

Mp= 170-172 °C; **R_f** 0.55, **Anal.** (%) for C₁₄H₈ClFN₂OS: **Found (calc.):** C 54.80 (54.82), H 2.68 (2.63), N 9.11 (9.13), O 5.21 (5.22); **m/z** 307.14 [M+1] 309.14 [M+3]; **FT IR** ($\nu_{\max}$./cm⁻¹): (ν_{OH}) 3253, (HC=N) azomethine 1662; **¹H NMR** (295 K/ δ in ppm/DMSO-*d*₆ 400 MHz): HC=N 9.776 (1H, s); phenolic OH 11.45 (1H, s); Ar-H 7.0-7.9 (6H, m); **¹³C NMR** (295 K/ δ (ppm)/DMSO-*d*₆ 400 MHz): 164.176, 123.880, 109.423, 158.848, 109.150, 148.414, 135.710, 161.261, 119.463, 159.63, 115.714, 135.323, 124.494, 129.215.

2.3 Structural elucidation of FBt by X-ray crystallography

Data for the structural study of FBt was carried out on a Rigaku-Oxford Diffraction single crystal X-ray diffractometer, with Cu K α (λ = 1.54184) radiation. Φ and ω scans were chosen to give a complete asymmetric unit. A suitable yellow crystal was kept at 173(2) K during data collection. Using Olex2 [12], the structure was solved with the ShelXT [13] structure solution program using intrinsic phasing and refined with the ShelXL [14] refinement package using least squares minimization. Cell refinement gave cell constants corresponding to a triclinic cell (P-1 Space Group) whose dimensions are given in **Table 1** along with other experimental parameters. All of the non-hydrogen atoms were treated anisotropically.

Table 1. Crystallographic data and details of the structure determination for FBt

Empirical formula	C ₁₄ H ₈ ClFN ₂ OS
Formula weight	306.73
Temperature/K	173(2)
Crystal system	triclinic
Space group	P-1
a/Å	5.6358(3)
b/Å	7.5483(7)
c/Å	29.299(2)
α /°	96.598(7)
β /°	91.425(5)
γ /°	93.079(6)

Volume/Å ³	1235.73(16)
Z	4
$\rho_{\text{calc}}/\text{cm}^3$	1.649
μ/mm^{-1}	4.409
F(000)	624.0
Crystal size/mm ³	0.48 × 0.08 × 0.06
Radiation (Å), Cu K α	$\lambda = 1.54184$
2 θ range for data collection/°	9.12 to 142.606
Index ranges	-6 ≤ h ≤ 5, -9 ≤ k ≤ 8, -35 ≤ l ≤ 35
Reflections collected	8353
Independent reflections	4663 [R _{int} = 0.0407, R _{sigma} = 0.0526]
Data/restraints/parameters	4663/0/363
Goodness-of-fit on F ²	1.070
Final R indexes [I ≥ 2 σ (I)]	R ₁ = 0.1046, wR ₂ = 0.2813
Final R indexes [all data]	R ₁ = 0.1365, wR ₂ = 0.3307
Largest diff. peak/hole / e Å ⁻³	2.53/-0.47

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2 2.4 Fungal strain assay and Minimum inhibitory concentration (MIC)

3 *In vitro* antifungal activity of the titled compound was determined by the agar diffusion
4 method [15] against pathogenic fungi such as *Candida albicans* (ATCC 10231) and *Aspergillus*
5 *niger* (ATCC 6275). A disc dipped in DMSO served as negative control, and standard
6 cycloheximide (25 mg) served as the positive control. The results were recorded as the average
7 diameter of inhibition zones of fungal growth. This is further subjected to MIC evaluation. The
8 microdilution susceptibility in Sabouraud Liquid Medium (Oxoid) was used for the
9 determination of antifungal activity. The stock solution of test compound was prepared in DMSO
10 and diluted in the media to concentrations ranging from 200 to 0.15 $\mu\text{g/mL}$ so that DMSO never
11 exceeded 1% v/v. The suspension of the microorganism ($10^3/\text{mL}$) at 10^6 CFU/mL (Colony
12 Forming U/mL) concentrations was inoculated into the wells. After incubated the plates at 36 °C
13 for 2 days, the minimal fungicidal concentrations were determined as the lowest concentration of
14 the compound inhibit the fungal growth to a maximum extent compared to that of the DMSO-
15 free control.

16 2.5 Study with spectroscopic methods on protein BSA interaction activity

17 The binding interaction between BSA and FBt has been deciphered by employing absorption
18 and fluorescence measurements. The solution of BSA and compound were prepared by

dissolving them in the buffered solution of 50 mM NaCl/Tris-HCl pH 7.24 to required concentrations, respectively. For UV-absorption study, a fixed concentration of BSA was titrated with varying concentrations of ligand solution. The absorbance was recorded in the range of 200-800 nm. Fluorescence quenching experiment was performed by keeping the concentration of the BSA constant while varying concentrations of the quencher, producing the solutions with a varied molar ratio of the quenchers to BSA [16].

Fluorescence measurements have been recorded at λ_{ex} 280 nm and emission wavelength ranges from 200-700 nm on RF-5301P spectrofluorophotometer (Shimadzu) equipped with a 150-W Xenon lamp, slit width of 5 nm, the response time (auto) and excitation voltage (medium) were kept constant for each data set. Fluorescence intensities were corrected for inner filter and dilution effects before analysis of the binding and quenching data. In order to minimize the inner filter effects of protein and ligands, absorbance measurements were performed at the excitation and emission wavelengths of the fluorescence measurements. The correction was done for the fluorescence intensities using the following equation [17].

$$F_{\text{cor}} = F_{\text{obs}} e^{(A_{\text{ex}} + A_{\text{em}})/2}$$

where, F_{cor} and F_{obs} are the corrected and observed fluorescence intensity, respectively. A_{ex} and A_{em} are the absorptions of the fluorescence determination system at excitation and emission wavelength respectively. The used Tris buffer was taken as a blank and subtracted from the experimental spectra to correct for the fluorescence background.

2.5.1 Molecular docking

Crystal structure of BSA (4F5S.pdb) was downloaded from the protein data bank (www.rcsb.org) and used for docking studies. FBt was drawn using Maestro 2D sketcher and energy minimize was computed by OPLS 2005 [18]. The protein was reduced to some extent after hydrogen was added. The crystal structure was imported and refined by a multistep process through the protein preparation wizard of Maestro 9.3, which includes energy minimization using OPLS-2005 force field. Using PROPKA, pH was fixed and optimized to 7.5. Non-hydrogen atoms were minimized by restrained minimization to default RMSD to 0.3Å. Finally, a grid with the dimension of 25 Å × 25 Å × 25 Å of BSA was prepared wherein Trp residues are mapped, while building glide-grid geometry. The dimension of the grid was in such a manner that the grid covers the whole target biomolecule. Using Extra-precision (XP) docking and

scoring each compound were docking into the receptor grid and the docking calculation were judged based on the Glide score [19].

3. Results and discussion

3.1. Chemistry

A new potential N and O-bidentate imine-based molecule (FBt) and a microanalysis checked the purity for C, H, O and N. Microwave assisted ligand synthesis is easier, convenient, short reaction time and yield (86 %) was better than the conventional method (78 %). The compound was very stable at atmospheric conditions, non-hygroscopic and soluble in all common organic solvents. Analytical data of the compound obtained in good consistent with the proposed formulae and are summarized above.

3.2. X-ray crystal structure studies of FBt

A perspective view of 2-((E)-(6-fluorobenzo[d]thiazol-2-ylimino) methyl)-4-chlorophenol, $\text{FC}_6\text{H}_3(\text{SCN})\text{N}=\text{CHC}_6\text{H}_4\text{OCl}$ (FBt) contains two crystallographically independent molecules (A and B) in the asymmetric unit, which are almost identical. It crystallizes in the monoclinic crystal system under the space group $P-1/n$ and is displayed in an ORTEP diagram (Fig. 2).

Fig. 2. ORTEP plot of $\text{C}_{14}\text{H}_8\text{ClFN}_2\text{OS}$ (FBt) showing the atom- numbering scheme and two independent molecules in the asymmetric unit. The non-hydrogen atoms are drawn with 30 % probability ellipsoids.

In $\text{FC}_6\text{H}_3(\text{SCN})\text{N}=\text{CHC}_6\text{H}_4\text{OCl}$, the average length of the $\text{N2}=\text{C8}$ bond is 1.267(6), and bond angle of $\text{N2}-\text{C8}-\text{C9}$ is 123.8(4) (**Table 2**), obtained as expected. The bond angles in benzothiazole ring $\text{C1}-\text{N1}-\text{C7}$ and $\text{C2}-\text{S2}-\text{C1}$ are 109.9(4)° and 88.5(2)°, respectively, (**Table 3**)

and this is very close to the value reported [20]. The asymmetric unit of the FBt moiety shows an intra-molecular hydrogen bond, O1A---H1A...N2A and O1B---H1B...N2B between the O atom of OCHC₆H₄OCl and N-amino group of FC₆H₃(SCN)NH₂ (**Fig. 3**). While no classical intermolecular hydrogen bonds are observed, weak C5A---H5A...F1A and C5B---H5B...N2B intramolecular interactions are present and contribute to crystal packing (**Fig. 3**, **Table 4**).

Fig. 3. Molecular packing of FBt, C₁₄H₈ClFN₂OS, viewed along the b axis. Dashed lines indicate intramolecular O---H...N hydrogen bonds. No classical intermolecular hydrogen bonds are observed. H atoms not involved in hydrogen bonding have been removed for clarity.

Table 2. Bond Lengths [Å] for FBt, FC₆H₃(SCN)N=CHC₆H₄OCl.

Atom	Atom	Length/Å	Atom	Atom	Length/Å
S1A	C2A	1.721(5)	S1B	C2B	1.731(5)
F1A	C4A	1.378(6)	F1B	C4B	1.358(5)
O1A	C10A	1.350(6)	O1B	C10B	1.352(6)
N1A	C1A	1.296(6)	N1B	C1B	1.283(6)
N1A	C7A	1.404(6)	N1B	C7B	1.390(6)
N2A	C1A	1.382(6)	N2B	C1B	1.400(6)
N2A	C8A	1.267(6)	N2B	C8B	1.296(6)
C2A	C3A	1.403(6)	C2B	C3B	1.376(6)
C2A	C7A	1.393(7)	C2B	C7B	1.409(7)
C3A	C4A	1.362(7)	C3B	C4B	1.384(7)
C4A	C5A	1.375(7)	C4B	C5B	1.402(7)
C5A	C6A	1.376(7)	C5B	C6B	1.377(7)
C6A	C7A	1.391(6)	C6B	C7B	1.403(7)
C8A	C9A	1.447(6)	C8B	C9B	1.453(6)
C9A	C10A	1.407(6)	C9B	C10B	1.412(7)
C9A	C14A	1.388(7)	C9B	C14B	1.410(7)

C10A	C11A	1.381(6)	C10B	C11B	1.392(6)
C11A	C12A	1.386(7)	C11B	C12B	1.392(7)
C12A	C13A	1.387(7)	C12B	C13B	1.390(7)
C13A	C14A	1.372(6)	C13B	C14B	1.358(7)

Table 3 Bond Angles [°]

Atom	Atom	Atom	Angle/°	Atom	Atom	Atom	Angle/°
C2A	S1A	C1A	88.5(2)	C2B	S1B	C1B	88.1(2)
C1A	N1A	C7A	109.9(4)	C1B	N1B	C7B	109.8(4)
C8A	N2A	C1A	119.3(4)	C8B	N2B	C1B	118.2(4)
N1A	C1A	S1A	115.9(4)	N1B	C1B	S1B	116.9(3)
N1A	C1A	N2A	122.3(5)	N1B	C1B	N2B	121.5(4)
N2A	C1A	S1A	121.8(4)	N2B	C1B	S1B	121.7(4)
C3A	C2A	S1A	127.7(4)	C3B	C2B	S1B	127.1(4)
C7A	C2A	S1A	110.5(3)	C3B	C2B	C7B	123.5(4)
C7A	C2A	C3A	121.8(4)	C7B	C2B	S1B	109.4(3)
C4A	C3A	C2A	115.0(5)	C2B	C3B	C4B	115.4(5)
C3A	C4A	F1A	116.9(5)	F1B	C4B	C3B	118.3(4)
C3A	C4A	C5A	125.3(5)	F1B	C4B	C5B	117.7(4)
C5A	C4A	F1A	117.8(4)	C3B	C4B	C5B	124.0(4)
C4A	C5A	C6A	118.9(4)	C6B	C5B	C4B	118.8(4)
C5A	C6A	C7A	119.0(5)	C5B	C6B	C7B	119.8(5)
C2A	C7A	N1A	115.2(4)	N1B	C7B	C2B	115.8(4)
C6A	C7A	N1A	124.8(4)	N1B	C7B	C6B	125.7(4)
C6A	C7A	C2A	120.0(4)	C6B	C7B	C2B	118.5(4)
N2A	C8A	C9A	123.8(4)	N2B	C8B	C9B	122.5(4)
C10A	C9A	C8A	122.8(4)	C10B	C9B	C8B	123.0(4)
C14A	C9A	C8A	118.0(4)	C14B	C9B	C8B	117.4(4)
C14A	C9A	C10A	119.2(4)	C14B	C9B	C10B	119.6(4)
O1A	C10A	C9A	121.6(4)	O1B	C10B	C9B	122.1(4)
O1A	C10A	C11A	118.9(4)	O1B	C10B	C11B	119.1(4)
C11A	C10A	C9A	119.4(4)	C11B	C10B	C9B	118.8(4)
C10A	C11A	C12A	120.6(4)	C12B	C11B	C10B	121.1(4)
C11A	C12A	C13A	119.8(4)	C13B	C12B	C11B	118.8(4)
C12A	C13A	C11A	119.5(4)	C12B	C13B	C11B	119.0(4)
C14A	C13A	C11A	120.4(4)	C14B	C13B	C11B	119.1(4)
C14A	C13A	C12A	120.1(5)	C14B	C13B	C12B	121.9(5)
C13A	C14A	C9A	120.8(5)	C13B	C14B	C9B	119.7(5)

Table 4. Intramolecular hydrogen bonds and Weak intermolecular interactions [$\text{\AA}$, $^\circ$] of $\text{FC}_6\text{H}_3(\text{SCN})\text{N}=\text{CHC}_6\text{H}_4\text{OCl}$

D---H...A	d(D---H)/ $\text{\AA}$	d(H---A)/ $\text{\AA}$	d(D...A)/ $\text{\AA}$	$\angle(\text{DHA})/^\circ$
O1A---H1A...N2A	0.84	1.98	2.692(5)	142.3
O1B---H1B...N2B	0.84	1.98	2.692(5)	142.0
C5A---H5A...F1A #1	0.95	2.67	3.422(5)	136.5.0
C5B---H5B...F1B #2	0.95	2.67	3.410(6)	136.5.0

#1 -x, -y, -z; #2 -x, 1-y, -z

3.3. Hirshfeld surface analysis of FBt

The electrostatic potential mapped on Hirshfeld surface with d_{norm} or shape index functions provides a unique signature of a molecule in a crystal lattice as it strongly depends on the surrounding/surface. So, the same molecule appears different in different crystal packing. It gives a 3D picture of intermolecular interactions in a crystal [21,22]. The 3D Hirshfeld surfaces and 2D fingerprint plots of the proton-transfer compounds (FBt) were prepared using Crystal Explorer ver. 3.1.[23] and are shown in **Fig. 4**. For FBt, O1A--H1A...N2A and O1B---H1B...N2B intra-molecular hydrogen bond appears as sharp symmetric spikes in the two-dimensional histogram (2D fingerprint plots) which summarizes all the contact distances to the Hirshfeld surface (d_i , d_e) and expressed in terms of a percentage share.

Fig.4. A view of the Hirshfeld surface mapped over **a)** curvedness for FBt. The flat regions highlight the involvement of aromatic rings in the π - π stacking interactions. **b)** Surface mapped with shape-index property showing complementary triangular red hollows and blue bumps that are characteristic of six-ring stacking interactions. **c)** 2D fingerprint map displays a significant percentage of close contacts.

d_e and d_i are the distances to the nearest atom exterior and interior to the surface.

3.4. Spectral studies

The ^1H NMR spectrum of the FBt (**Fig. 5.**) exhibits a singlet signal at 9.776 ppm which is attributed to the azomethine ($-\text{CH}=\text{N}-$) proton. Phenolic OH leads to a singlet of integration intensity equivalent to one hydrogen at 11.13 ppm and aromatic hydrogens in the range 7.0-7.92 ppm which resonates as multiplet. The ^{13}C NMR spectrum of FBt displays a characteristic signal at 161.261 ppm for the azomethine carbon (C10). The signals at 164.176 (C2), 159.63 (C12), 158.848 (C6), 148.414 (C8), 135.710 (C9) and other aromatic carbon lies in the range 129.215 - 109.423 [24]. The mass spectrum (**Fig. 6.**) of FBt shows the molecular ion peak corresponding to their formulation. The peak m/z 307 and 309 representing $[\text{M}+1]^+$ and $[\text{M}+3]^+$. The Fourier Transform Infrared Spectrum (**Fig. 7.**) of the ligand exhibits a sharp band at 1662 cm^{-1} due to azomethine group vibration. FBt has the characteristic bands at 1562 cm^{-1} for $\nu\text{C}=\text{N}$ of thiazole ring, 3253 cm^{-1} for νOH and 748 cm^{-1} for $\nu\text{C}-\text{S}-\text{C}$ [25].

Fig. 5. ^1H NMR spectrum of FBt

Fig. 6. Mass spectrum of FBt

Fig. 7. FT-IR spectrum of FBt

3.5. Evaluation of antifungal activity

The title compound displays varying degrees of inhibitory effects of FBt on the growth of tested fungal species chosen. FBt shows 55 % lower than the standard cycloheximide in an inhibitory growth of *C. albicans*, as revealed from their MIC values of 64 ± 0.25 $\mu\text{g/mL}$, while reactivity of compound was 25 % lower than the standard against *A. niger* (125 ± 0.12 $\mu\text{g/mL}$). The obtained result highly encourages and proved that the imine-based ligand incorporating benzothiazole served as a prominent antifungal agent [26].

3.6. BSA binding studies

3.6.1. UV absorption titration

UV absorption spectroscopy is a simple method to explore the conformational changes occurred in BSA. The absorption spectra of BSA (2×10^{-6} M) with varying concentrations of FBt are shown in **Fig. 8**. As can be seen, BSA solution has two absorption bands near 280 and 320 nm though the former one is comparably weak. The absorption peak at 280 nm raised due to the phenyl rings of aromatic amino acids Trp, Tyr and Phe [27]. The intensity of the BSA absorption changes with a redshift of ~ 9 nm on addition of varying concentrations of the compound. Observed hypochromism with slight shift indicates binding of the compound to the hydrophobic sites of the protein results in some changes in the polarity of the BSA environment surrounded the compound [28]. It is also observed that an isosbestic point at about 425 nm where FBt: BSA has the same absorption intensity is an indicative of equilibrium between the bound and free form of the compound. The absorption after 425 nm for free BSA increases with continuing addition of compound, suggesting the interaction strongly with BSA by the static quenching mechanism [29], it might be the aggregation of protein induces enhancement in absorption at high concentration of FBt in that region. The fluorescence studies also validate this view.

Fig. 8. Absorption spectra of the FBt -BSA system in Tris/NaCl buffer (p^H 7.2).
The arrow indicates the change upon increasing the concentration of FBt.

3.6.2. Fluorescence quenching studies

The fluorescence of the aromatic amino acids in proteins offers a specific sensitive probe in detecting molecular conformational changes [28]. The concentration of BSA solution was stabilized, and the concentrations of FBt were varied in the experiment [30]. Successive addition of different concentrations of FBt solution to BSA has led to its fluorescence intensity excited at 320 nm; however, the emission maximum of BSA was observed at 338 nm (**Fig. 9a**) corresponded to tryptophan residues located within the hydrophobic binding pocket of the protein. The addition of compound could alter the fluorophore environment in BSA by interacting with it, but not the local dielectric environment of BSA. The Stern-Volmer equation is employed to quantify the magnitude of the compound to quench the emission intensity of BSA:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \dots (1)$$

where, F and F₀ correspond to the fluorescence intensities of the protein in the presence and absence of quencher, respectively, and [Q] is the concentration of the quencher. K_{sv} is the Stern-Volmer quenching constant.

Hence, this equation was applied to determine K_{sv} values by linear regression of a plot of F₀/F against [Q] to elucidate quenching mechanism (inset plot of **Fig 9a**) and K_{sv} was calculated from

the slope. Resulting plots exhibit a good linear relationship is an indicative of the collisional quenching process or static quenching process. K_{sv} a direct measure of the quenching efficiency and is related to the lifetime of the system according to the equation $K_{sv} = K_q \cdot \tau_0$ (2), where K_q is the bimolecular quenching constant which measures the exposure rate of tryptophan residues to the drug and τ_0 is the time frame of protein (order of 10^{-8} s) [31]. The high magnitude of K_q ($10^{12} \text{ M}^{-1}\text{S}^{-1}$) obtained can be attributed to a specific long-range interaction between FBt molecule and tryptophan residues on protein BSA. Thus, the process of energy transfer occurs through intermolecular interaction forces between tryptophan and FBt [32].

Binding parameters

On binding of small molecules independently to a set of equivalent sites on a macromolecule, the association between the fluorescence quenching intensity and the concentration of quencher can be calculated by employing a double-logarithmic equation (3), i.e., Scatchard equation [33,34]

$$\log \left[\frac{F_0 - F}{F} \right] = \log K_b + n \log [Q] \dots (3)$$

where, K_b and n are the binding constant and the number of binding sites, respectively.

The K_b value is significant to understand the distribution of the drug in plasma since the weak binding can lead to a short lifetime or poor distribution, while strong binding can decrease the concentration of free drug in plasma. The corresponding value of K_b and n (from Eqn 3) are listed in **Tables 5** and is graphed in **Fig 9b**. A linear logarithmic plot exhibited for non-cooperative binding mode [35] as the Hill coefficient $n=1$. At room temperature, i.e., 298 K, the value of n is found to be 1.02 (~ 1) implying the presence of a single class of binding site for FBt in BSA. The molar ratio of the FBt-BSA system at 320 nm was 1:1. FBt binds near to just one of the fluorophore present in the BSA structure (Trp-134 or Trp-213 residue). The molecular docking suggests that FBt can make better interactions with Trp-134 than Trp-213 site.

The binding affinity is the strength of the interaction between two (or more than two) molecules that bind reversibly which is translated into physico-chemical terms in the dissociation constant (k_d), free, unbound drug concentrations in plasma decrease as the degree of binding (k_b) to these compounds increases. Whenever a system is studied *in vivo*, the number of experimental variables becomes too high, to the point that it may be difficult to obtain unambiguous results.

a)

b)

Fig. 9. a) Fluorescence emission spectra of BSA in the presence of FBt in Tris/NaCl buffer (pH 7.2) The arrow indicates the emission intensity changes of BSA upon increasing the concentration of the FBt (0-30 μ L at the increment of 5), slit width = 5 nm. Inset picture of modified Stern-Volmer plot for quenching of BSA by FBt.

b) Plot of $\log[(F_0-F)/F]$ versus $\log[Q]$ for BSA- FBt complex at 298 K

Table 5. BSA interaction parameters obtained by fluorescence method

Compound	K_{sv} ($\times 10^4 \text{ Lmol}^{-1}$)	K_q ($\times 10^{12} \text{ Lmol}^{-1}$)	K_b ($\times 10^5 \text{ Lmol}^{-1}$)	n	R^2 (correlation coefficient for value K_b)
FBt	1.03 ± 0.12	1.03 ± 0.14	1.51 ± 0.21	1.02	0.985

1 FBT-BSA complex interactions involve hydrophobic and weak interactive forces lead to a
2 moderate affinity of K_b ($1.51 \pm 0.21 \times 10^5 \text{ Lmol}^{-1}$). Moreover, low-affinity binding may be
3 associated with conformational changes in the protein molecule. The specific delivery of ligands
4 by protein albumins results from the existence of two major and structurally selective sites
5 located in hydrophobic cavities in subdomains IIA and IIIA. Most ligands bind to these two sites
6 reversibly with an affinity constant of 10^4 – 10^8 LM^{-1} [34,36]. A K_b value larger than 10^4 indicates
7 a significant interaction between ligand and protein [37].

8 Observations confirm clear distinct values for obtained K_{SV} from Stern-Volmer plot
9 approved for the nature of static interaction occurred between the protein and FBT in the order of
10 10^4 LM^{-1} depends on the stability of complex formed, while for the static quenching interaction,
11 binding constant K_b determined from the Scatchard plot for the evidence of the existence of
12 binding sites in a molecule whose occupation depends on the affinity of the FBT to the receptor
13 such a binding behavior is very specific and can be interpreted as a particular characteristic of
14 FBT: BSA complex in the order 10^5 LM^{-1} [38].

16 3.6.3. Energy transfer from protein to compound

17 Foster's non-radiative energy transfer theory (FRET) depends on the distance between
18 the molecules in their excited states. The extent of energy transfer between the BSA (an
19 amphiphilic protein) and the compound FBT (hydrophobic fluorophore) is determined by the
20 degree of spectral overlap between emission spectrum of the donor and absorption spectra of the
21 acceptor (**Fig. 10**) [39].

Fig.10. Spectral overlap of UV-Vis absorption spectrum of FBt with the emission spectrum of BSA. $c(\text{BSA}) = c(\text{FBt}) = 2 \times 10^{-6} \text{ molL}^{-1}$, $T = 298 \text{ K}$

From Foster's theory, the efficiency of energy transfer, E is calculated by

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^2} \dots (4)$$

where F and F_0 are fluorescence intensity of BSA in the presence and absence of the acceptor. r is the binding distance between the acceptor and donor; R_0 is the critical distance for 50 % energy transfer which can be calculated using $R_0^6 = 8.79k^2N^{-4}J\phi N^{-4} \dots (5)$

where, the spatial orientation factor of the dipole $K^2 = 2/3$, and the refractive index of medium $= 1.336$, and the fluorescence quantum yield of the donor $\phi = 0.15$ [38, 39]. Overlap of the UV absorption spectra of the acceptor with the fluorescence emission spectra of donor is given by

$$J = \frac{\sum F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda}{\sum F(\lambda)d\lambda} \dots (6)$$

The calculated values of E , R_0 , and J are listed in **Table 6**. It is found that the r value obtained is 3.61 nm, which is less than 8 nm between a donor and acceptor, indicates that the energy transfer occurred between BSA and the complexes with a high probability and which also indicates a static quenching interaction as $r < R$.

Table 6. Distance parameters of BSA with compound FBt

Compound	J (cm ³ L mol ⁻¹)	E (%)	R _o (nm)	r (nm)
FBt	2.1±0.15 ×10 ⁻¹⁴	0.08	2.93	3.61

3.7. Molecular docking

Crystal structure provides accurate structural and functional information of biomolecules and chemical compounds. The determined 3D structure of FBt plays a significant role in exploring its structural consequences and conformational behavior during interactions with BSA [42]. FBt bind with protein principally through hydrophobic cavities located in sub-domains IIA and IIIA, which exhibit similar chemical properties [43]. The principal binding sites where quenching of fluorescence is brought about by Trp134 and Trp213 at the proximity of BSA. Our results show similar interaction as reported by D. Lu *et al.*, [44]. Henceforth to authenticate the experimental results regarding FBt interaction, possible docked conformations of FBt - BSA adduct the best pose is represented with the highest negative value has been considered to be the best-ranked results. The docking score of FBt for the most stable docked pose (**Fig. 11**) is evaluated as - 5.361 kcal/mol. Lys136 formed π - π interaction with FBt and forms hydrogen bond with Tyr139 side chain of protein backbone whereas it forms hydrogen bond chain of Glu140 and with Arg143 it forms bond *via* a salt bridge (**Fig. 11A and B**). The molecular docking studies show that the FBt is in proximity to the hydrophobic residues at sub-domain IB (**Fig. 11C**) surrounded by majorly by hydrophobic amino acids which is represented in green color.

Apart from π - π stacking, salt bridge and hydrogen bonding interaction between FBt and BSA, other force of interactions computed from docking studies are Van der Waals energy of -39.949 kcal/mol (Glide evdw), Coulomb energy of -2.347 kcal/mol (glide ecoul). Glide energy of -42.296 kcal/mol is a modified Coulomb-van der Waals interaction energy as stated by the developer of Schrödinger. Despite these interactions, it was also observed that the best pose obtained in Trp-134 site, part of the FBt structure remains exposed to the surface media rather than be accommodated inside the binding site. This result can justify the experimental K_b values which indicate only a moderate affinity of FBt for BSA. The results of docking study of BSA are consistent with the spectroscopic results.

Fig.11. (A) Molecular interaction of FBt with amino acids within the receptor of BSA (B) showing hydrogen bond, π - π stacking and the salt bridge with the backbone of BSA (C) Secondary structure of BSA wherein the FBt binds with the best-docked pose.

4. Conclusions

The facile synthesis of a new, titled imine-based molecule (FBt) structure was analyzed by X-ray crystallography, Hirshfeld surfaces analysis and spectral methods. FBt was screened *in vitro* for antifungal activity against selected fungal strains and binding activity towards BSA has been done using spectroscopic techniques. BSA interaction studies with biologically active FBt molecule revealed that the compound could strongly quench the intrinsic fluorescence of protein through a form predominantly static in nature with the ratio of FBt to BSA being 1: 1. A FRET study for the distance between the BSA and FBt suggests that the energy transfer occurs with high possibility. Molecular docking studies on the interaction of compound with BSA confirmed the experimental results of binding energy involving Coulomb-van der Waals interaction in addition to hydrophobic forces. The K_b and n values indicate that FBt moderately bind to the albumin and there is just one main binding site for this association.

Appendix

CCDC 1537326 contains supplementary crystallographic data for FBt. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44-1223-336033. E-Mail: deposit@ccdc.cam.ac.uk or at <http://www.ccdc.cam.ac.uk>.

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