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# Simultaneous binding of anti-tuberculosis and anti-thrombosis drugs to a human transporter protein: A FRET study

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ABSTRACT

Although rifampicin (Rf) is one of the most effective antibiotics against infection caused by *Mycobacterium tuberculosis*, interaction of the drug with universal carrier protein in human blood plasma is not fully understood. Reduction of medicinal efficacy of other drugs, including anti-thrombosis drug warfarin (Wf), to the patients on Rf therapy also needs molecular understanding. In the present work we have studied interaction of Rf with one of the model carrier protein (human serum albumin). By using circular dichroism (CD) spectroscopy we have characterized the change in the secondary structure of the protein. The consequence of the simultaneous binding of the two drugs, Rf and Wf, on the structure of the protein has also been explored. Picosecond resolved Förster resonance energy transfer (FRET) from Wf to Rf explores possible binding sites of the anti-tuberculosis drug on the protein. In this report, we have discussed the potential problem of using the single tryptophan of the protein (Trp 214) as energy donor in FRET experiment for the characterization of the binding site of the drug Rf on the protein.



## 1. Introduction

Efficacy of a drug essentially depends on the effective concentration of the drug in blood plasma. In mammals, serum albumins are the most abundant proteins in blood plasma and they are responsible for binding and transport of various drugs [1,2]. The albumins are the carrier for many endogenous substances like fatty acids, bilirubins, hormones and numerous small ligands [3,4]. Increasing the concentration of water-soluble drugs in blood plasma and hence interaction with the transporter albumins is relatively straightforward. However, the complexation of sparingly water-soluble drugs with the transporter proteins, in the blood plasma, could be heavily affected. Unfortunately, some of the sparingly soluble vital drugs for a few of the very detrimental diseases are still unavoidable. Rifampicin (Rf) (Scheme 1), one of such sparingly soluble drugs, is a first line anti-tuberculosis drug which is active against *Mycobacterium tuberculosis* as well as few other Mycobacterial species [5]. It can be used alone or in combination with other drugs like, isoniazid and pyrazinamide for the treatment of tuberculosis (TB). It is a potent inhibitor of DNA dependent RNA synthesis from bacteria [6]. TB, a contagious disease, caused by *M. tuberculosis*, is one of the diseases of major concern today as it afflicts nearly 1/3rd of human population [7] and there is a high occurrence of TB in HIV infected subjects as well.

Low solubility of Rf is a major setback while deciding the dose of the drug to be administered to a patient and, due to severe side effects of Rf, high oral dose is a problem as well. Reduction of medicinal efficacy of other drugs is one of the major side effects of the administered higher doses of Rf to the TB patients. It has been clinically observed that effective dose of an anti-thrombosis drug, warfarin (Wf) (Scheme 2) is relatively higher to the TB patients on Rf therapy [8,9]. In this regard it is of utmost significance to understand simultaneous binding of the two important life saving drugs (Rf and Wf) with the human transporter proteins. Rf has UV–visible absorption and circular dichroism (CD) bands at long wavelengths [10], which makes it an excellent spectroscopic probe. It is known that Rf quenches the tryptophan fluorescence through energy transfer and hence, has been used as an energy acceptor [11,12]. Wf, on the other hand, is known to bind preferentially to the drug binding site I of human serum albumin (HSA) [13–15]. A high-resolution X-ray crystal structure of the drug Wf with HSA [4,16,17] and the spectroscopic characterization of the HSA–Wf complex [18,19] are available in the recent literature. However, till date a detailed study on the binding interactions of Rf with the human transporter proteins using NMR or X-ray crystallographic studies is rare in the literature. In this study the characterization of the binding of Rf with one of the model human transporter proteins, HSA using CD and picosecond resolved Förster resonance energy transfer (FRET) is one of the motives. We have also explored the simultaneous binding of Rf and Wf with the model protein HSA. Our studies may be relevant for the understanding of the molecular basis of simultaneous binding of two vital drugs to human transporter protein.

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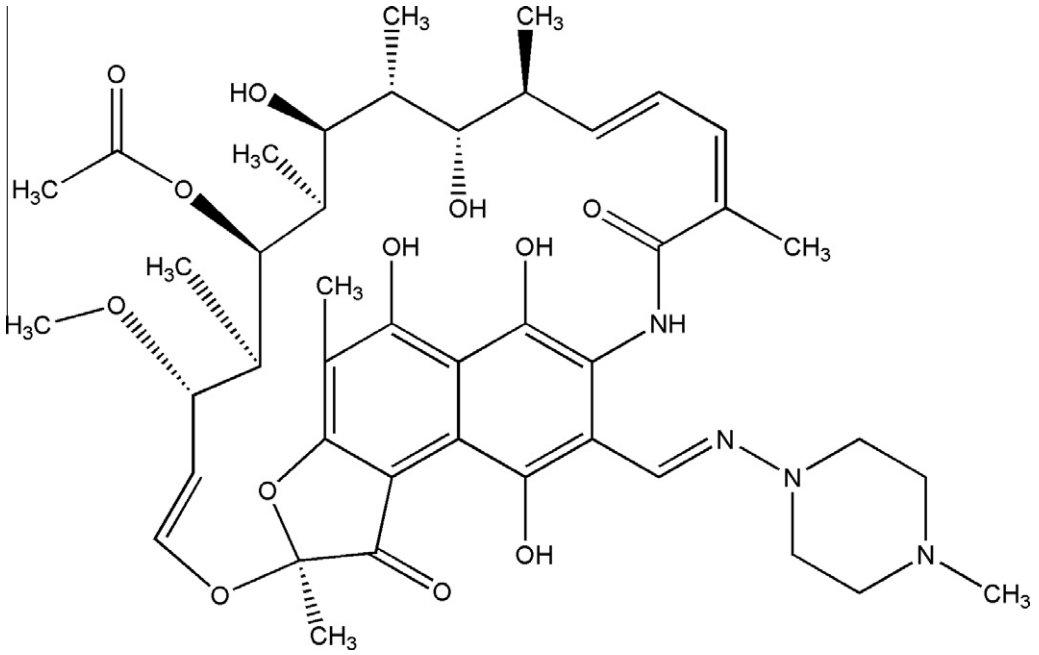
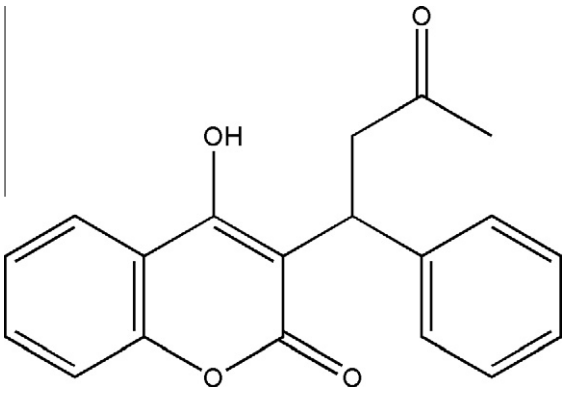


**Scheme 1.** Structure of rifampicin (Rf).



**Scheme 2.** Structure of warfarin (Wf).

## 2. Materials and methods

### 2.1. Materials

Human serum albumin (99% purity), Wf, Rf, Sodium dihydrogen phosphate and di-sodium hydrogen phosphate were all purchased from Sigma and used without any further purification. 100 mM phosphate buffer (50 mM Sodium dihydrogen phosphate and 50 mM di-sodium hydrogen phosphate, pH 7) was prepared by using Millipore water (Milli Q) and the sample solutions were prepared in the same.

### 2.2. CD spectroscopy

CD measurements were carried out on a JASCO 815 spectropolarimeter. A temperature controller was attached with it and all the CD measurements were done at 20 ± 0.1 °C. The scan speed of the measurements was 50 nm/min and each spectrum was the average of five scans. The spectral data were acquired over the range of 500–200 nm using a 1 cm path length cuvette. Buffer solutions containing the corresponding concentration of the probe, i.e. Rf, were subtracted from all the measurements. The difference spectra are presented for all the concentrations used. The concentration of the protein for the CD experiments was $1 \times 10^{-7}$ M HSA, whereas, the concentration of the drugs Rf and Wf were varied from $1 \times 10^{-6}$ M to $10 \times 10^{-6}$ M. It has to be noted that the reported binding affinity of Wf is much higher (more than 94%) than that of Rf (65–75%) with the model protein HSA [20,21]. The numbers in the parentheses indicate the "fraction bound" of the drugs with the protein HSA. For example, the "fraction bound" of Wf is more than 94% means that of the amount Wf in the blood (attached to the plasma protein). The remaining less than 6% (the fraction unbound) drug is the fraction that is actually active and may be excreted. The results are expressed in $\theta$, the optical rotation obtained from the instrument in milli degrees. The secondary structural data of the CD spectra were analyzed using CDNN deconvolution program [22]. CDNN is a method based on neural network theory to analyze and quantify the information content of far UV circular dichroism spectra. Using this CDNN software, it is possible to deduce five different secondary structure fractions (helix; parallel and anti-parallel beta-sheet; beta-turn and random coil) with satisfactory correlations between calculated and measured secondary structure data.

### 2.3. Steady state spectroscopy

All absorbance measurements were performed in a Shimadzu UV-2450 spectrophotometer. All fluorescence measurements were performed in a Jobin Yvon Fluoromax-3 fluorimeter. For all the steady state measurement, a quartz cuvette of 1 cm path length was used. In order to minimize the contribution from tyrosine, HSA was excited at 295 nm and the respective emission was collected from 300 to 550 nm. For the experiments, $1.6 \times 10^{-5}$ M HSA ($OD_{295}$ = 0.1) and $2.5 \times 10^{-5}$ M Wf was used. Rf concentration was maintained at $1.7 \times 10^{-4}$ M ($\lambda$ = 473 nm).

### 2.4. Time resolved spectroscopy

Details of the time-resolved spectroscopic data have been measured with a time correlated single photon counting (TCSPC) setup from Edinburgh Instruments, in which the sample was excited by the third harmonic laser beam (300 nm) of the 900 nm (0.5 nJ

per pulse) using a mode-locked Ti-sapphire laser with an 80 MHz repetition rate (Tsunami, Spectra Physics), pumped by a 10 W Millennia (Spectra Physics) followed by a pulse-peaker (rate 8 MHz) and a third harmonic generator (Spectra-Physics, model 3980). The third harmonic beam was used for excitation of the sample inside the TCSPC instrument (instrument response function, IRF = 50 ps) and the second harmonic beam was collected as for the start pulse. The picosecond-resolved fluorescence transients have been fitted with tri-exponential function, $\sum_{i=1}^{3} A_i \exp\left(-\frac{t}{\tau_i}\right)$, where, $A_i$'s are weight percentages of the decay components with time constants of $\tau_i$. The relative change in the overall excited state lifetime is expressed by the equation $\tau = \sum_{i=1}^{3} A_i \tau_i$, when $\sum_{i=1}^{3} A_i = 1$. For the energy transfer experiments, the concentration of the protein was $1.25 \times 10^{-6}$ M whereas, the same for Wf and Rf was $3.125 \times 10^{-6}$ M and $15 \times 10^{-6}$ M respectively.

The Förster distances of donor–acceptor pairs were calculated using the equation [23–25],

$$R_0 = 0.211(\kappa^2 n^{-4} Q_D J(\lambda))^{1/6} (\text{in Å}) \quad (1)$$

where $R_0$ is the distance between the donor and the acceptor at which the energy transfer efficiency is 50%, $\kappa^2$ is a factor describing the relative orientation in space of the transition dipoles of the donor and acceptor. The magnitude of $\kappa^2$ is assumed to be 2/3 for donor and acceptors that randomize by rotational diffusion prior to energy transfer and in the present study also, the same assumption has been made. The refractive index ($n$) of the medium was assumed to be 1.4 (aqueous medium). It was essential to compute the fluorescence quantum yield ($Q_D$) of donor chromophores for the estimation of the distance between a donor and acceptor pairs using the Förster's theory of dipole–dipole energy transfer. The relative quantum yields of HSA and Wf were determined with respect to tryptophan solution in water (0.14), which was used as a standard [26]. The fluorescence quantum yield of intrinsic Trp of HSA was calculated to be 0.11 and the quantum yield of bound Wf was calculated to be 0.08. $J(\lambda)$, the overlap integral, which expresses the degree of spectral overlap between the donor emission and the acceptor absorption, is given by,

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \quad (2)$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor in the wavelength range of $\lambda$ to $\lambda + d\lambda$ and is dimensionless. $\varepsilon_A(\lambda)$ is the extinction coefficient (in $M^{-1}$ $cm^{-1}$) of the acceptor at $\lambda$. If $\lambda$ is in nm, then $J$ is in units of $M^{-1}$ $cm^{-1}$ $nm^4$. Once the value of $R_0$ is known, the donor–acceptor distance ($r$) can easily be calculated using the formula

$$r^6 = \left[R_0^6(1-E)\right]/E \quad (3)$$

Here, $E$ is the efficiency of energy transfer. The transfer efficiency can be measured using the relative fluorescence intensity of the donor in the absence ($F_D$) and presence ($F_{DA}$) of the acceptor (Eq. (4a)). The efficiency, E can also be calculated from the lifetimes ($\tau_D$ and $\tau_{DA}$) using the Eq. (4b), where $\tau_D$ and $\tau_{DA}$ are lifetimes of the donor in absence and in presence of the acceptor:

$$E = 1 - (F_{DA}/F_D) \quad (4a)$$

$$E = 1 - (\tau_{DA}/\tau_D) \quad (4b)$$

The distances measured using Eqs. (4a) and (4b) are revealed as $r_S$ (steady state measurement) and $r_{TR}$ (time-resolved measurement), respectively. However, the potential danger of using Eq. (4a) for the estimation of donor–acceptor distance had been discussed in previous studies [27].

## 3. Results and discussion

The model transporter protein (HSA) used in our study has a distinct CD characteristic [28,29] owing to its alpha helical content in its secondary structure which makes CD spectroscopy a suitable tool to study the effect on the protein as a function of its structural changes. CD studies have been done on the protein and protein–Rf complexes in order to investigate the possibility of any structural change of the protein upon complexation with the drug. Fig. 1 shows the CD spectra of HSA with various drug concentrations in phosphate buffer at pH 7. On complexation with Rf, there was no peak shift of the CD bands (208 nm and 222 nm, characteristic of alpha helix) of HSA but there was definitely a decrease in the band intensity. As shown in the inset of Fig. 1, the quantitative analysis using CDNN software [22], indicates that the complexation of Rf to HSA has induced significant decrease in the α-helix content of the protein. For native protein, at 20 °C, we obtained 65% of α-helix which is in close agreement to the previously reported values [28,29]. However, in presence of 10 μM Rf (Fig. 1), the amount of α-helix decreased to 48.1%. The association constant $K_b$ between Rf and HSA is estimated utilizing the CD spectroscopy and found to be $3.46 \times 10^5$ $M^{-1}$ (details of the calculation is given in Supporting information). Wf, on the other hand, itself does not possess a distinct CD characteristic and, also, it has no significant effect on the protein structure upon binding (data not shown). We have also investigated from CD spectroscopy that the structure of HSA-Rf complex remains unaffected in the presence of the anti-thrombosis drug Wf (10 μM).

In order to estimate the binding site of the Rf on the model transporter protein HSA we have explored the possibility of using FRET. In Fig. 2a the emission from the single tryptophan (Trp214) of HSA and the absorption spectrum of Rf are shown. A significant spectral overlap, as indicated by yellow shaded region, is evident from the figure. As, is apparent from the spectral overlap, Trp214 and Rf could, unambiguously, form a FRET pair and same is depicted in the inset of Fig. 2a, which shows the steady state Trp214 fluorescence emission quenching in presence of Rf. Utilizing Eq. (4a), the efficiency of energy transfer, between Trp214 and the HSA-bound Rf, is calculated to be 75%. From the steady state measurements, the Förster's distance $R_0$ of the donor (Trp214)–acceptor (Rf) pair has been estimated to be 24.3 Å and the donor–acceptor distance in the protein is found to be 20.26 Å. However, we have recognized that the quenching of the Trp214 residue in the protein upon complexation with the drug

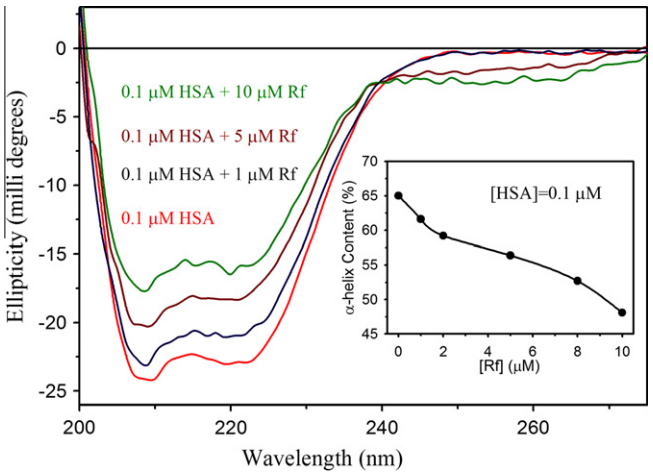



**Fig. 1.** CD spectra of HSA ($1 \times 10^{-7}$ M) in various concentrations of the drug Rf in aqueous buffer (pH = 7). The inset of the graph shows the decrease of α-helical content of the protein upon complexation with the drug.

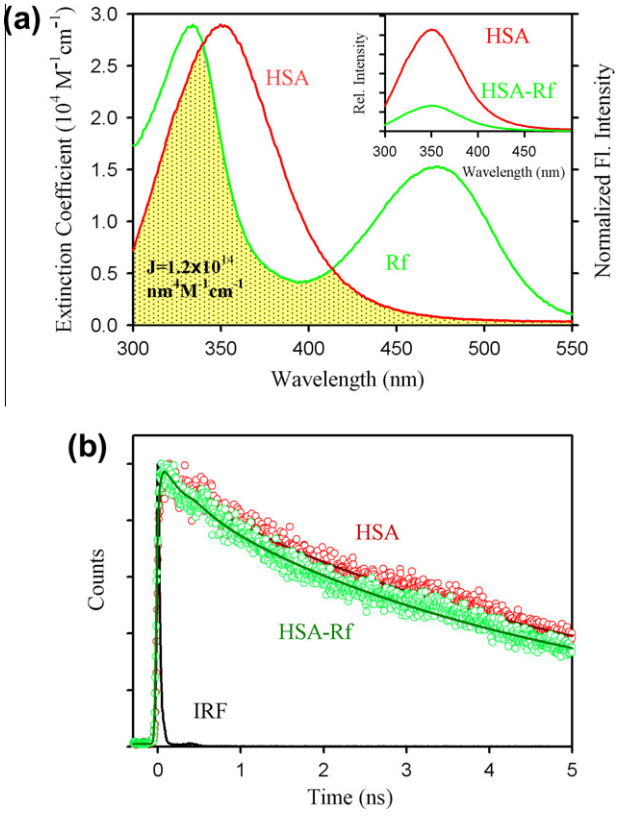



**Fig. 2.** (a) Normalized fluorescence of HSA and absorption extinction coefficient of Rf. Inset shows the steady state fluorescence emission of HSA (in red) and quenching of emission of HSA in presence of Rf (in green). (b) Picosecond-resolved fluorescence decay of HSA ($1.6 \times 10^{-5}$ M) and of HSA in presence of Rf ($1.7 \times 10^{-4}$ M). Instrument response is shown in black. Excitation wavelength of 299 nm has been used for both the experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

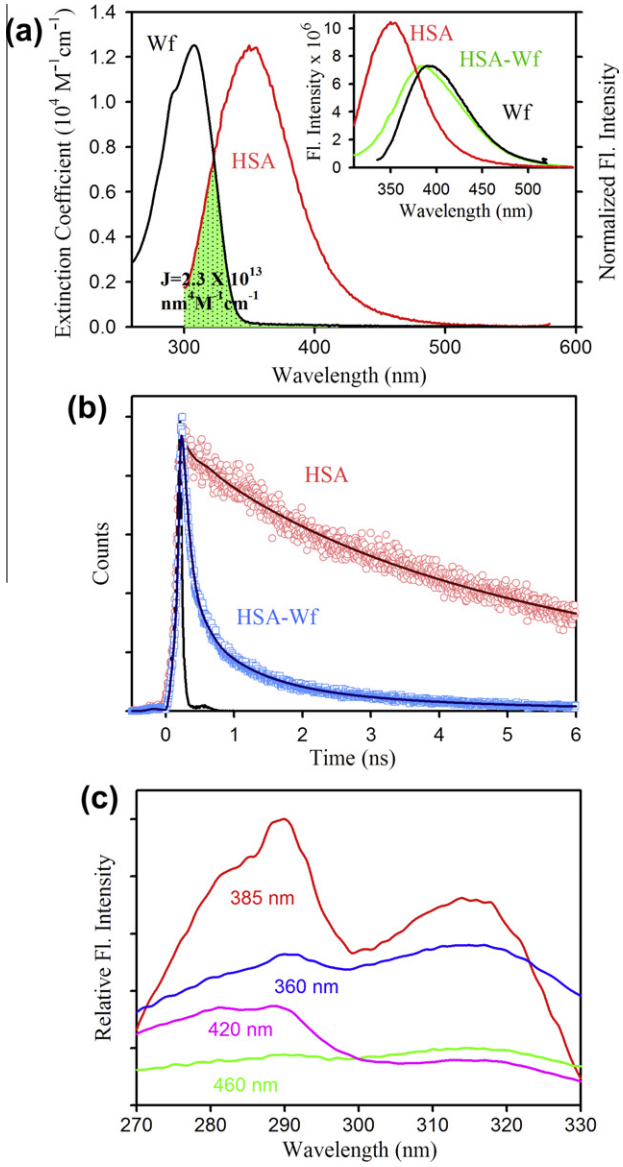



**Fig. 3.** (a) Normalized fluorescence intensity of HSA (in red) and absorption extinction coefficient of Wf (in black). The emission spectra of HSA (in red), Wf (in black) and Wf bound HSA (in green) are shown in the inset. (b) Picosecond-resolved fluorescence decay at 360 nm of HSA (in red) and of HSA in presence of Wf (in blue). Instrument response is shown in black. Excitation wavelength of 299 nm has been used for both the experiments. (c) Excitation spectra of HSA–Wf complex with detection wavelengths at 360 nm, 385 nm, 420 nm and 460 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Rf is not dynamic in nature. It has also been reported in the literature that the quenching of the tryptophan residue of HSA, in presence of a different derivative of Rf, is not dynamic in nature [30]. Negligibly small dipolar interaction of Trp214 with Rf is clearly evident from the Fig. 2b. The fluorescence transients of Trp214 in absence and presence of the drug Rf reveals similar fluorescence dynamics indicating the energy transfer from Trp214 to Rf to be radiative; *not* non-radiative resonance type. Thus application of FRET in order to estimate the distance between Trp214 and Rf is misleading. Our observation clearly justifies the exploring of an alternative FRET donor for the characterization of Rf binding to the protein.

In order to study the binding of Rf to the protein we have explored the possibility of using Wf as potential energy donor. The anti-thrombosis drug Wf binds to the protein at the site I [16,17]. There are several studies [18,19] on the FRET from Trp214 of HSA to the protein-bound Wf, because of a large spectral overlap of the emission of Trp214 with the absorption spectrum of Wf, as also shown in Fig. 3a. As a consequence, steady-state emission of Trp214 in the presence of Wf shows significant quenching as shown in inset of Fig. 3a. Apparent "strong" dipolar coupling of Trp214 with the Wf is evident from Fig. 3b. Faster fluorescence transient of HSA–Wf complex at 360 nm (excitation 299 nm), compared to that of the protein (Trp214 alone), apparently is indicative of the FRET from Trp214 to the Wf moiety. For further confirmation, we have studied fluorescence transient of Trp214 residue of HSA with varying concentration of Wf. Insignificant change in the fluorescence lifetime of tryptophan beyond a certain concentration of Wf, suggests that the quenching of tryptophan residue in the protein upon complexation with Wf is due to FRET (data not shown), which is consistent with the conclusion of the reported literature [31]. The fluorescent decay of Wf, which is very fast and is not incorporated in the Fig. 3b [18]. However, it should be noted that Wf has emission peak at 385 nm, close to the emission peak of Trp214 (350 nm) of the HSA protein. Thus the contribution of the Wf at 360 nm cannot be ruled out. The excitation spectra of the HSA–Wf complex with various detection wavelengths are shown in Fig. 3c. It is evident from the figure that contribution of the Wf (UV–visible absorption peak at 310 nm) at the detection wavelength of 360 nm is significant, but at the detection wavelength of 420 nm and longer, there is minimum contribution arising from the Trp214. Therefore, in order to measure the excited state lifetime of the donor Wf bound to HSA ($\tau_D$), the detection wavelength of 420 nm would be optimum, because at the

detection wavelength greater than 420 nm the fluorescence intensity of Wf decreases significantly. As evident from Fig. 3b, HSA, in presence of bound Wf, shows 92.5% energy transfer efficiency. The efficiency of energy transfer was calculated using Eq. (4b) and $R_0$ was estimated to be 18.4 Å. The distance between Trp214 and Wf was calculated utilizing Eq. (3) and found to be 12.1 Å. Utilizing the crystal structure data of HSA complexed with Wf [4,16,17], we have estimated the distance, between Trp214 and the Wf binding site using the Web viewer software, to be 13.1 Å. The estimated distance is quite close to the experimentally obtained value.

The spectral overlap of the HSA–Wf emission with the absorption of the HSA-bound Rf is shown in Fig. 4a. The inset shows the steady-state emission quenching of the Wf in HSA upon complexation with Rf. The dipolar interaction of the Wf with the Rf is confirmed from the faster fluorescence transient (Fig. 4b) of HSA–Wf complex upon binding with Rf. The estimated $R_0$ is 25.8 Å and the distance between the Wf and the Rf is found to be 28.1 Å. Our observations clearly indicate that simultaneous binding of Rf and Wf to the transporter protein is possible.

Using the Web viewer software and utilizing the available crystal structure data of HSA complexed with different drugs [16,17] we have estimated the Rf binding site with respect to the Wf binding site in the protein. Rf is hydrophobic in nature and the sites, on the transporter protein, where the probability of Rf binding is high at the sites on domain IIIA and IB of the protein [17]. The estimated average distance between the Wf binding site (located at domain IIA of the protein) and domain IIIA, as well as IB is 27.2 ± 0.1 Å. The calculated FRET distance is 28.1 Å which is quite close to the value corresponding to the distance from domain IIA to both the hydrophobic drug binding domains, IB and IIIA. So, it could be concluded that Rf binds at the site located at either of the domains IIIA and IB of the transporter protein. The affinity of other hydrophobic drugs (Diazepam, Diflunisal, Indomethacin) to both the domains has been reported from X-ray crystallographic study [17]. Our observation of the perturbation of protein structure (Fig. 1) upon binding of Rf in the domains IB and IIIA can also be correlated with the binding of fatty acids in the domains [17]. A significant structural perturbation of the protein upon fatty acid binding to the domains IB and IIA has been observed from X-ray crystallographic study [16,17].

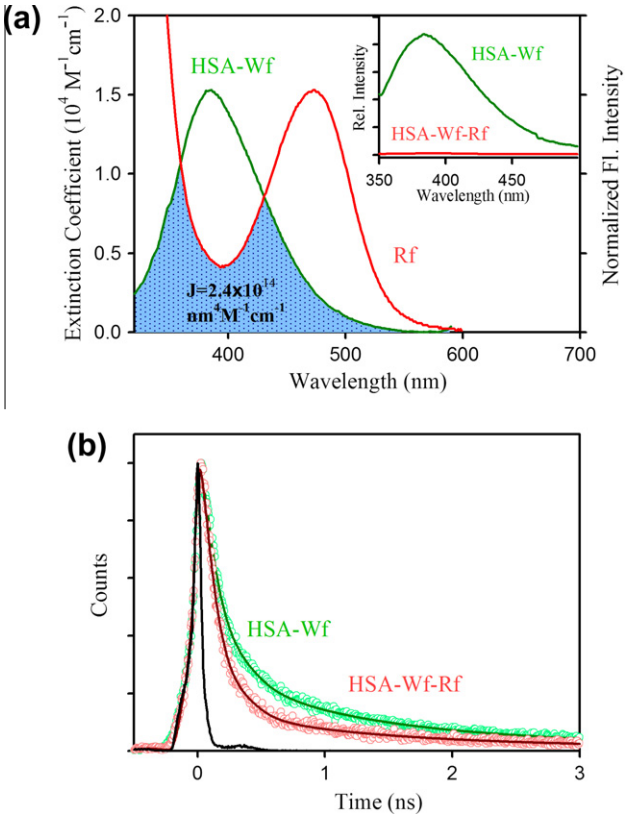



**Fig. 4.** (a) Spectral overlap between Wf emission complexed with HSA and Rf absorbance. Inset shows the fluorescence emission intensity of Wf complexed with HSA (in green) and of the same in presence of Rf (in red). (b) Picosecond-resolved fluorescence decay at 420 nm of HSA in presence of Wf (in green) and that of HSA–Wf complex in presence of Rf (in red). Instrument response is shown in black. Excitation wavelength of 299 nm has been used for both the steady state and time resolved experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 4. Conclusion

In this report, we have investigated the interaction of one of the important anti-tuberculosis drugs, Rf with one of the model transporter protein, HSA. The binding interaction in presence of another drug, an anti-thrombosis drug, namely Wf has also been characterized. From the far UV–CD, perturbed secondary structure of the transporter protein upon inclusion of the antibiotic Rf, has been confirmed. However, no perturbation is evident in the protein upon binding of the anti-thrombosis drug Wf. As there is no report on the binding site of the drug Rf in the protein, attempt has been made to characterize the drug binding site by using FRET. Careful studies on the energy transfer from the single tryptophan, Trp214 of the protein, to the drug Rf shows that the nature of the energy transfer is not resonance type, rather a radiative in nature, which rules out the possibility of using FRET formulism to estimate the distance from Trp214 to the bound Rf. Our studies also reveal that lack of careful analysis of steady state or time resolved Trp214 fluorescence may lead to estimate incorrect binding site of the drug Rf. In an alternative approach we have used well-characterized, protein bound drug Wf complex, as energy donor in order to apply FRET to the protein-bound Rf drug. We have recognized that adaptation of FRET from Wf to Rf is very crucial and depends on the detection wavelength due to the potential interference of Trp214 emission on the donor (Wf) fluorescence. Our studies are the first attempt to characterize the probable binding site of the important antibiotic to a model transporter protein in human blood. The effect of the drug binding in presence of other drug, namely Wf has also been addressed and it has been found from the FRET studies that simultaneous binding of the two drugs at two different sites in the protein is feasible. Simultaneous binding of the two drugs evidently rules out the possibility of competitive binding of the drugs. Thus the molecular basis of the reduced effectiveness of the drug Wf on the patients on Rf therapy could be due to the structural perturbation of the host transporter protein.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jphotobiol.2011.02.023.

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